# Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 35, Issue of December 15, pp. 20827-20830, 1989


# SecB Protein Stabilizes a Translocation-competent State of Purified prePhoE Protein*

(Received for publication, August 10, 1989)

**Ron Kusters‡, Truus de Vrije‡§, Eefjan Breukink‡, and Ben de Kruijff‡§**

*From the ‡Centre for Biomembranes and Lipid Enzymology and the §Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

**Efficient translocation of pure precursor of PhoE protein (prePhoE) could be accomplished in an *in vitro* system consisting of only inverted *Escherichia coli* inner membrane vesicles, ATP, and SecA and SecB protein. In this *in vitro* system SecB and not trigger factor could stabilize a translocation-competent state of prePhoE. In contrast, translocation competency of proOmpA could be induced by both trigger factor and SecB protein, suggesting specificity in interactions between cytosolic factors and precursors in outer membrane protein translocation.**

Over the last years genetic and biochemical studies have rapidly led to the identification of several protein factors that are necessary for bacterial protein export (see Ref. 1 for review). However, the precise function of most of these factors is still largely unknown. Recently, a strong indication on the function of a soluble factor, SecB protein, has been obtained. It was suggested that this protein, which affects the translocation of only a subset of precursor proteins, interacts with the mature region of maltose-binding protein and in doing so retards its folding into a translocation-incompetent state (2). Purified SecB protein, isolated as multimer, was shown to promote translocation of *in vitro*-synthesized precursor proteins (3–5), probably by retardation of the folding into a conformation which is incompatible with translocation. Photochemical cross-linking studies identified the 20 S heat shock GroEL protein as a factor which, like SecB protein, interacts with the unfolded state of a precursor of a periplasmic protein (6).

*In vitro* translocation systems using purified precursor proteins offer the advantage to identify and characterize individual factors required for membrane translocation. With such a system it was demonstrated that translocation of purified (diluted from urea) proOmpA protein across inverted *Escherichia coli* inner membrane vesicles was solely dependent on ATP (7). The precursor could be stabilized for translocation by stoichiometric interaction with a soluble protein called trigger factor (8). By means of the same reconstituted system it was demonstrated that SecA protein, which *in vivo* as well as *in vitro* has been shown to be essential for bacterial protein export (9, 10), is active in a membrane-bound form and couples ATP hydrolysis to OmpA protein translocation (11). However, it is not clear whether the proposed involvement of trigger factor in proOmpA translocation (12) applies for other outer membrane proteins as well.

In earlier studies we characterized the translocation of the *in vitro*-synthesized precursor of the *E. coli* outer membrane pore protein PhoE (prePhoE) across inner membrane vesicles in the presence of cell lysate. ATP was essential for membrane translocation, and both the proton motive force (13) and the presence of the negatively charged membrane phospholipid phosphatidylglycerol (14) were found to be required for an optimal process. We now report a very rapid and efficient purification of prePhoE which can be effectively translocated into inverted membrane vesicles in the presence of only ATP and SecA and SecB protein. Trigger factor is much less active than SecB in this system, suggesting specificity in the functionality of cytosolic proteins in protein translocation.

## EXPERIMENTAL PROCEDURES

*Materials*—An S-135 cell extract and inverted inner membrane vesicles were prepared from *E. coli* strain MRE600 (15) according to De Vrije *et al.* (13). *E coli* strain CE1150 (16), which is deficient in OmpA protein, was used for the preparation of inner membrane vesicles to study proOmpA translocation. *E coli* strain MM52 (9) was used for the purification of prePhoE. ATP and proteinase K were obtained from Boehringer (Federal Republic of Germany), PMSF[1] and dithiothreitol from Merck (Federal Republic of Germany). [$^{35}$S]Methionine (1000 Ci/mmol) was purchased from Amersham (United Kingdom). Urea was from Baker (United Kingdom). Purified proOmpA (8), trigger factor (8), SecA (17), and SecB proteins were a generous gift of Dr. W. Wickner (Molecular Biology Institute and Dept. of Biological Chemistry, UCLA).

*Purification of prePhoE*—*E. coli* strain MM52 (*secA*51(Ts)) (9), which is defective in protein transport, carrying plasmid pJP29 encoding PhoE protein and chloramphenicol acetyltransferase (18) was induced for PhoE synthesis by growth at 30 °C in a low phosphate medium (19) containing 25 μg/ml chloramphenicol in the presence or absence of [$^{35}$S]methionine (0.5 mCi/50 ml of culture). Cells were harvested 6 h after induction and washed with 10 mM Tris/HCl, pH 8.0. An osmotic shock was carried out by suspending the cells in 30 mM Tris/HCl, pH 8.1, 20% sucrose, 1 mM EDTA and stirring for 15 min at 0 °C. After centrifugation (15,000 × *g*, 15 min) the pellet was resuspended in distilled water, and the suspension was stirred again for 15 min at 0 °C. The pellet obtained by centrifugation (15,000 × *g*) was resuspended in 10 mM Tris/HCl, pH 8.0, 3 mM EDTA, and 0.1 mM PMSF. Cells were disrupted by ultrasonication at 0 °C with a B12 sonifier (Branson) equipped with a microtip 8 times for 15 s at maximal power. Aggregates of overproduced prePhoE were separated from most contaminants, including membranes, by low speed centrifugation (1,500 × *g*), and the pellet, containing most of the protein, was dissolved in 8 M urea, 10 mM Tris/HCl, pH 8.0. The suspension was centrifuged at 235,000 × *g* for 60 min, and the supernatant was further purified by FPLC anion exchange chromatography using a monoQ column (Pharmacia, Sweden) in 8 M urea, 10 mM Tris/HCl, pH 9.0. The purification of prePhoE at each step was analyzed by SDS-PAGE (Fig. 1*A*). Fig. 1*B* shows the elution profiles from the monoQ column. After rechromatography of the indicated pooled fractions, prePhoE was recovered as a single peak. The overall purification was estimated to be 5-fold with a 53% yield with an estimated purity of >98%. The specific activity of the purified [$^{35}$S]prePhoE ranged from 1.1 to 2.3 Ci/mmol.

*In vitro translocation of prePhoE and proOmpA*—[$^{35}$S]prePhoE in

* This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid of the Netherlands Organization for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

8 M urea was diluted into 25 μl of translocation buffer (40 mM Tris acetate, pH 8.0, 10.8 mM $Mg^{2+}$ acetate, 28 mM $K^+$ acetate, 2 mM dithiothreitol) containing 4 mM ATP, inner membrane vesicles (0.43 mg of protein/ml), S-135 cell extract, or purified cytosolic factors as indicated, whereafter the mixture was incubated for 20 min at 37 °C. Translocation was demonstrated by protection against externally added proteinase K (200 μg/ml). After addition of the protease the samples were incubated at room temperature for 30 min. Protease activity was stopped by the addition of PMSF (2 mM), and the samples were analyzed by SDS-PAGE and autoradiography. Translocation of unlabeled proOmpA (0.4 μg/25 μl of reaction mixture) was studied using the same assay. ProOmpA and OmpA proteins were detected by Western blotting using a rabbit antiserum directed against OmpA (20). The precursor and mature forms of PhoE and OmpA were quantified from autoradiographs and Western blots using a LKB Ultroscan XL laser densitometer and liquid scintillation counting of the rehydrated excised protein bands of dried gels. Twenty percent of a translocation mixture, depleted of ATP, was taken as a quantitative standard for prePhoE translocation. Translocation activity is defined as the percentage of precursor and mature protein protected against proteinase K.

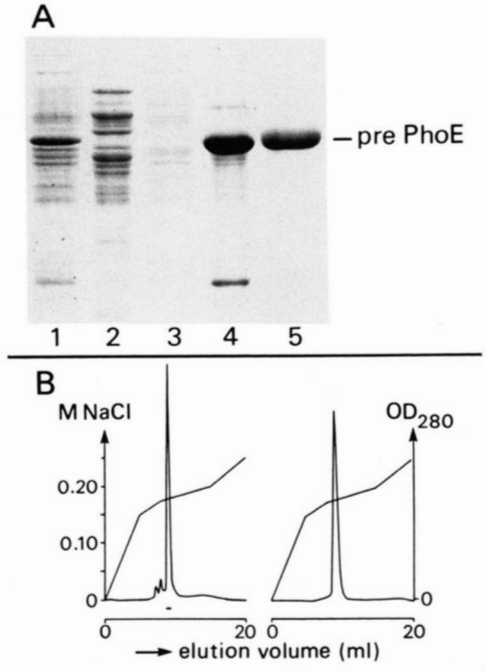


FIG. 1. **Purification of prePhoE.** *A*, samples were analyzed by SDS-PAGE and Coomassie Blue staining at different purification steps. *Lane 1*, protein pattern of *E. coli* strain MM52 carrying plasmid pJP29 after induction for PhoE synthesis; *lane 2*, osmotic shock fluid containing periplasmic and outer membrane proteins but virtually no prePhoE; *lane 3*, supernatant and *lane 4*, pellet fraction after ultrasonic disruption and low speed centrifugation; *lane 5*, protein after final run on monoQ column. *B*, FPLC elution profiles of the first and second run on a monoQ column. The *bar* underneath the main peak in the first elution profile indicates the fractions used for the second run.

RESULTS AND DISCUSSION

[$^{35}$S]prePhoE was isolated and purified after overproduction in a strain defective in protein translocation due to the presence of the *secA*51(Ts) allele. First, the translocation competency of the purified prePhoE was tested in an *in vitro* translocation assay. [$^{35}$S]prePhoE in 8 M urea was diluted into translocation mixtures that contained inverted inner membrane vesicles. In the presence of only ATP virtually no prePhoE was translocated (Fig. 2, *lane 3*). In agreement with Crooke *et al.* (8) we found under similar conditions a substantial amount (approximately 10%) of purified proOmpA to be translocated (data not shown). Addition of an S-135 cell extract resulted in a concentration-dependent increase of the translocation activity of prePhoE (*lanes 4–7*). At the optimal concentration of the cell extract (*lane 6*) translocation was approximately 15%; a higher concentration inhibited the translocation activity (*lane 7*). When ATP was omitted from the reaction mixture, translocation of prePhoE was completely inhibited (*lane 2*). Translocated prePhoE was digested by proteinase K when the membranes were disrupted by Triton X-100 (*lane 8*). As the translocation of both prePhoE and proOmpA (data not shown) was stimulated by the cytosolic fraction, the presence of one or more factors in the cell extract might be necessary for efficient translocation of these precursor proteins. To test this, purified SecA protein, trigger factor, and SecB protein were added to the translocation mixtures in the absence of cell extract and assayed for their ability to stimulate translocation (Fig. 3). prePhoE translocation was increased upon preincubation of the vesicles with SecA protein (*lanes 2* and *3*). Apparently the amount of functional SecA protein, peripherally membrane-bound or soluble in the translocation mixture, was suboptimal. The presence of trigger factor in the reaction mixture did not significantly stimulate prePhoE translocation (*lane 4*). Addition of SecB protein resulted in a large increase in translocation of prePhoE (*lane 5*). The combined addition of SecA protein and trigger factor very slightly stimulated prePhoE translocation compared with the addition of SecA protein alone (*lanes 3* and *6*). However, the combined addition of SecA and SecB protein gave rise to a dramatic increase in translocation activity. In this case approximately 35% of the total amount of prePhoE in the mixture became translocated (*lane 7*). This result is consistent with recent observations[2] that prePhoE processing was retarded in a $SecB^-$ strain.

Optimal concentrations of SecA and SecB protein for prePhoE translocation were determined by titration of these purified factors into the reaction mixture (Fig. 4). Already at low concentrations both SecA and SecB gave rise to a substantial increase in prePhoE translocation, reaching an optimal activity around 0.5 μM, corresponding to an equimolar SecB/prePhoE ratio. Fig. 4 confirms that trigger factor also

[2] H. de Cock and J. Tommassen, personal communication.

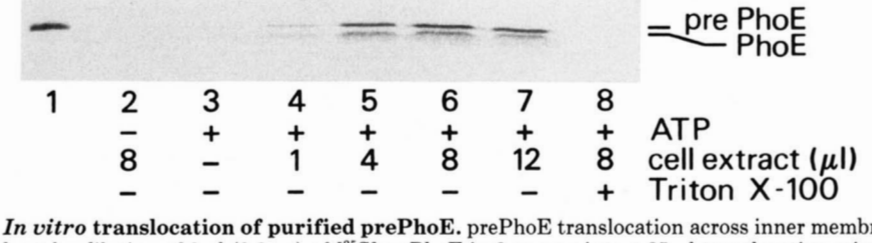


FIG. 2. ***In vitro* translocation of purified prePhoE.** prePhoE translocation across inner membrane vesicles was carried out by dilution of 2 μl (0.2 μg) of [$^{35}$S]prePhoE in 8 M urea into a 25-μl translocation mixture without (*lane 2*) or with added 4 mM ATP (*lanes 3–8*) and the indicated amounts of a S-135 cell extract. In *lane 8* 1% Triton X-100 (v/v) was added before protease treatment. *Lane 1* shows a prePhoE standard.

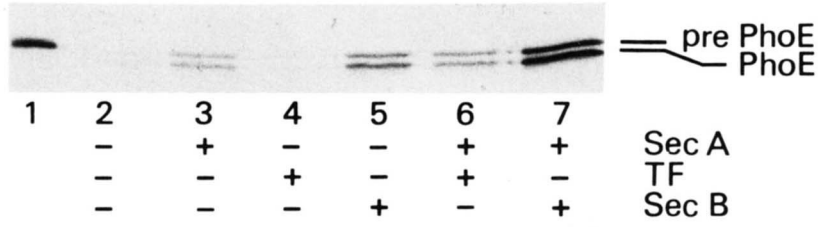


FIG. 3. ***In vitro* translocation of prePhoE is dependent on SecA and SecB protein.** Purified protein factors were added to translocation mixtures in the absence of a S-135 cell extract and the presence of ATP. Two $\mu$l (0.2 $\mu$g) of [$^{35}$S]prePhoE in 8 M urea was diluted into a 25-$\mu$l translocation mixture supplemented with trigger factor (0.8 $\mu$g; *lanes 4* and *6*) or SecB protein (0.16 $\mu$g; *lanes 5* and *7*). Inner membrane vesicles, used in samples 3, 6, and 7, were preincubated for 5 min at 37 °C in the presence of 1.3 $\mu$g of SecA protein before translocation was carried out. *Lane 1* shows a prePhoE standard.

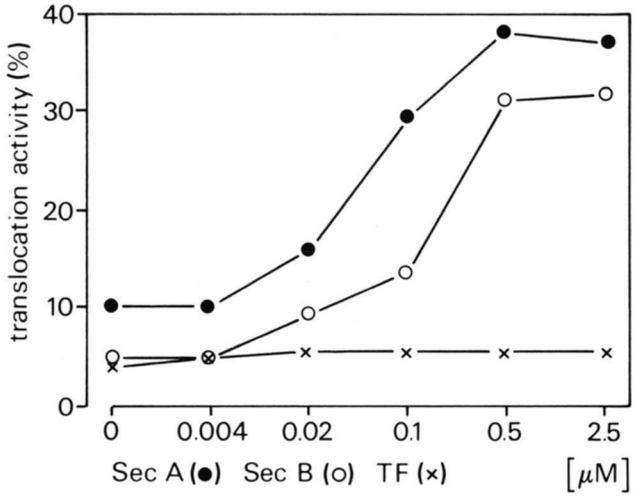


FIG. 4. **SecA and SecB protein concentration dependence of prePhoE translocation.** The translocation activity was determined by preincubation of vesicles with varying concentrations of SecA (●) at a constant SecB concentration of 0.4 $\mu$M (0.16 $\mu$g of protein/25 $\mu$l) and at varying concentrations of SecB (○) or trigger factor (×) at a constant SecA concentration of 0.5 $\mu$M (1.3 $\mu$g of protein/25 $\mu$l). The prePhoE concentration was 0.4 $\mu$M.

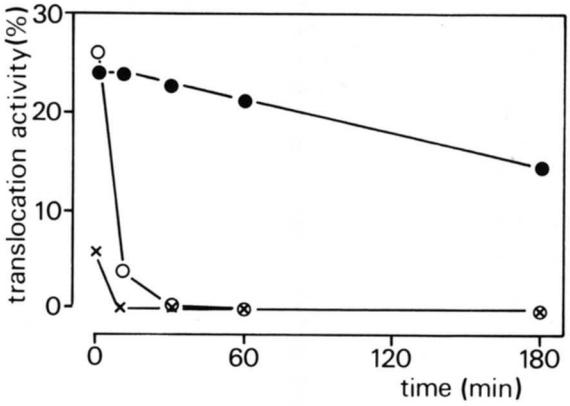


FIG. 5. **SecB keeps prePhoE in a translocation-competent state.** 0.4 $\mu$g of prePhoE in 8 M urea was diluted 14-fold into translocation buffer and incubated at room temperature. At different time points, samples were drawn from this preincubation mixture, and after addition of SecA preincubated vesicles (see legend to Fig. 3) and ATP in translocation buffer, translocation was assayed. Shown are the effects on translocation efficiency of prePhoE incubated in the presence (●, 0.16 $\mu$g/25 $\mu$l) or the absence (×) of SecB and the effect of SecB addition after prePhoE incubation (○).

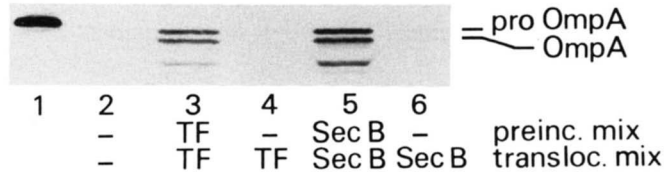


FIG. 6. **Trigger factor and SecB keep proOmpA in a translocation-competent state.** 0.4 $\mu$g of proOmpA in 8 M urea was diluted 7-fold into translocation buffer and incubated for 3 h at room temperature. After addition of SecA-preincubated vesicles and ATP in translocation buffer, translocation was assayed (*lane 2*). *Lanes 3* and *5* show the translocation activity when trigger factor (0.65 $\mu$g/25 $\mu$l) or SecB (0.3 $\mu$g/25 $\mu$l), respectively, was present during the preincubation of proOmpA. *Lanes 4* and *6* show the translocation activity when trigger factor or SecB, respectively, was added after preincubation of the precursor.

at higher concentrations is virtually inactive in promoting prePhoE translocation.

To test whether SecB protein influenced the translocation competency of prePhoE, the precursor was diluted in the absence of vesicles and incubated at room temperature. Samples of the incubation mixture were taken at different time points and assayed for translocation (Fig. 5). In the absence of SecB, prePhoE lost its translocation competency almost instantaneously. On the other hand, when SecB was present during the preincubation, the larger part of the prePhoE was still in a translocation-competent form, even after a 3-h preincubation. Addition of SecB protein after the preincubation did not restore the translocation competency, suggesting that SecB has no unfolding activity.

As might be expected from the low translocation activity accomplished by the addition of trigger factor to a translocation mixture (Figs. 3 and 4), preincubation of prePhoE in the presence of trigger factor did not result in maintenance of a translocation-competent conformation (data not shown). In contrast to the low activity of trigger factor in prePhoE translocation, proOmpA was retained translocation-competent for at least 3 h in the presence of trigger factor (Fig. 6, *lane 3*), as reported before (7). Also for this precursor protein, SecB turned out to be more potent in retarding the folding into a conformation incompetent for translocation (Fig. 6, *lanes 3* and *5*).

In accordance with the observed differences in activity in translocation of both cytosolic factors, SecB has recently been found to form isolable complexes with both precursors, whereas trigger factor is only able to form a (weaker) complex with proOmpA.[3]

Our data do not allow a detailed conclusion on the nature of the observed differences in the activity of cytosolic factors

[3] W. Wickner, personal communication.

in prePhoE and proOmpA translocation. However, they appear to relate to differences in kinetics of folding of the precursor into a translocation-incompetent form. Whereas prePhoE in the absence of SecB protein rapidly folds (or aggregates) into a translocation-incompetent form, proOmpA apparently folds (or aggregates) much more slowly (7).

*Acknowledgments*—We would like to thank H. de Cock and J. Tommassen for providing strain MM52 carrying plasmid pJP29 and strain CE1150. We also thank W. Wickner for generously supplying materials used in this study and for communicating data prior to publication.

REFERENCES

1. Baker, K., Mackman, N., and Holland, B. I. (1987) *Prog. Biophys. Mol. Biol.* **49,** 89–115
2. Collier, D., Bankaitis, V., Weiss, J., and Bassford, J., Jr. (1988) *Cell* **53,** 273–283
3. Kumamoto, C. A., Chen, L., Fandl, J., and Tai, P. C. (1989) *J. Biol. Chem.* **264,** 2242–2249
4. Weiss, J. B., Ray, P. H., and Bassford, P. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8978–8982
5. Watanabe, M., and Blobel, G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2728–2732
6. Bochkarera, E. S., Lissin, N. M., and Girshovich, A. S. (1988) *Nature* **336,** 254–257
7. Crooke, E., Brundage, L., Rice, M., and Wickner, W. (1988) *EMBO J.* **7,** 1831–1835
8. Crooke, E., Guthrie, B., Lecker, S., Lill, R., and Wickner, W. (1988) *Cell* **54,** 1003–1011
9. Oliver, D. B., and Beckwith, J. (1981) *Cell* **25,** 765–772
10. Cabelli, R. J., Chen, L., Tai, P. C., and Oliver, D. B. (1988) *Cell* **55,** 683–692
11. Lill, R., Cunningham, K., Brundage, L., Ito, K., Oliver, D., and Wickner, W. (1989) *EMBO J.* **8,** 961–966
12. Lill, R., Crooke, E., Guthrie, B., and Wickner, W. (1988) *Cell* **54,** 1013–1018
13. De Vrije, T., Tommassen, J., and De Kruijff, B. (1987) *Biochim. Biophys. Acta* **900,** 63–72
14. De Vrije, T., De Swart, R. L., Dowhan, W., Tommassen, J., and De Kruijff, B. (1988) *Nature* **334,** 173–175
15. Cammack, K. A., and Wade H. E. (1965) *Biochem. J.* **96,** 671–680
16. Van Alphen, L., Van Alphen, W., Verkleij, A. J., and Lugtenberg, B. (1979) *Biochim. Biophys. Acta* **556,** 233–243
17. Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W., and Oliver, D. (1989) *EMBO J.* **8,** 955–959
18. Bosch, D., Leunissen, J., Verbakel, J., De Jong, M., Van Erp, H., and Tommassen, J. (1986) *J. Mol. Biol.* **189,** 449–455
19. Tommassen, J., Leunissen, J., Van Damme-Jongsten, M., and Overduin, P. (1985) *EMBO J.* **4,** 1041–1047
20. Overbeeke, N., Van Scharrenburg, G., and Lugtenberg, B. (1980) *Eur. J. Biochem.* **110,** 247–254