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A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*

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The trimethylamine *N*-oxide (TMAO) reductase of *Escherichia coli* is a soluble periplasmic molybdoenzyme. The precursor of this enzyme possesses a cleavable N-terminal signal sequence which contains a twin-arginine motif. By using various *moa*, *mob* and *mod* mutants defective in different steps of molybdocofactor biosynthesis, we demonstrate that acquisition of the molybdocofactor in the cytoplasm is a prerequisite for the translocation of the TMAO reductase. The activation and translocation of the TMAO reductase precursor are post-translational processes, and activation is dissociable from translocation. The export of the TMAO reductase is driven mainly by the proton motive force, whereas sodium azide exhibits a limited effect on the export. The most intriguing observation is that translocation of the TMAO reductase across the cytoplasmic membrane is independent of the SecY, SecE, SecA and SecB proteins. Depletion of Ffh, a core component of the signal recognition particle of *E.coli*, appears to have a slight effect on the export of the TMAO reductase. These results strongly suggest that the translocation of the molybdoenzyme TMAO reductase into the periplasm uses a mechanism fundamentally different from general protein translocation.

Keywords: metalloenzyme/periplasm/Sec system/signal recognition particle/twin-arginine motif

Introduction

Proteins localized to the periplasmic space or the outer membrane of *Escherichia coli* are translocated through the inner membrane via the translocase complex of the general export Sec pathway (Pugsley, 1993; Murphy and Beckwith, 1996). The minimal set of proteins constituting a functional translocase complex comprises SecA, SecY, SecE and SecG, which mediate the ATP- and proton motive force-dependent translocation across the membrane (Schatz and Dobberstein, 1996). Genetic and biochemical investigations have shown that the SecYEG complex is the integral part of the general protein translocation apparatus and that this complex is required for all proteins to be translocated into the periplasm of *E.coli*. However, both Sec-dependent and Sec-independent protein insertion into the *E.coli* cytoplasmic membrane have been reported. While the membrane assembly of leader peptidase (Wolfe *et al*., 1985) and mannitol permease (Werner *et al*., 1992) requires SecY, that of M13 procoat and melibiose permease (Bassilana and Gwizdek, 1996) appears to be Sec independent.

In *E.coli*, synthesis of secretory proteins and translocation through the cytoplasmic membrane are not coupled. Consequently, it is necessary to maintain precursor proteins in an export-competent conformation prior to the interaction with the translocase. It was first reported that precursor maltose-binding protein is competent for transport into the periplasm only before it has achieved its final, stably folded, conformation. Once in this folded state, it can no longer be translocated (Randall and Hardy, 1986). Some small proteins (such as the coat protein of phage M13) can spontaneously maintain a loose conformation in the cytosol and travel from the ribosome to the target membrane unassisted. However, large proteins, particularly those with hydrophobic internal domains or hydrophobic signal sequences, require the assistance of cytoplasmic chaperones or similar factors to prevent premature folding into a conformation that cannot be translocated (Pugsley, 1993; Schatz and Dobberstein, 1996).

Translocation of metalloenzymes is a more complicated process since formation of the metallocenter must be coordinated with translocation. Furthermore, many bacterial metalloenzymes are synthesized as precursors with an unusually long and distinctly structured signal sequence (see Discussion). It is thus conceivable that there is a particular pathway required for translocation of this subset of proteins. To address this question, we studied translocation of a molybdoenzyme, trimethylamine *N*-oxide (TMAO) reductase of *E.coli*. The TMAO reductase catalyzes reduction of TMAO to trimethylamine and functions as a component of anaerobic respiratory chain, which provides energy for the bacterial growth (Barrett and Kwan, 1985). It is a periplasmic enzyme composed of two identical subunits of 90 kDa (Silvestro *et al*., 1989). The precursor of the TMAO reductase possesses an N-terminal signal sequence (MNNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATA↓AQAA) which is cleaved at the position indicated by the arrow after the precursor has been translocated into the periplasm (Méjean *et al*., 1994). The TMAO reductase is encoded by the *torA* gene of the *torCAD* operon, and it is thus also called TorA. Expression of the *torCAD* operon is induced anaerobically solely if TMAO or related compounds are available in the growth medium (Iobbi-Nivol *et al*., 1996; Jourlin *et al*., 1997).

Analysis of several molybdoenzymes in *E.coli* and other bacteria has shown that all of them contain molybdopterin

guanine dinucleotide (MGD) as their molybdocofactor (Rajagopalan, 1996). Crystallographic structures reveal that formate dehydrogenase H (FDH-H) of *E.coli* and dimethylsulfoxide (DMSO) reductases from *Rhodobacter sphaeroides* and from *R.capsulatus* all contain one molybdenum and two MGD per polypeptide chain (Schindelin *et al*., 1996; Schneider *et al*., 1996; Boyington *et al*., 1997). The molybdenum is coordinated by the dithiolene group of MGD and one amino acid side chain of the enzymes. The two MGD are ligated within the enzymes through an extensive network of hydrogen bonds, salt bridges and van der Waals interactions, which involve more than two dozen residues distributed throughout the entire sequences of these enzymes.

In *E.coli*, mutations in the five chlorate resistance loci (*moa*, *mob*, *mod*, *moe* and *mog*) lead to the pleiotropic loss of all molybdoenzyme activities (Rajagopalan, 1996). The *moa* and *moe* loci encode functions required for the biosynthesis of molybdopterin (MPT), which is the basic structure of the molybdocofactor, and the *mob* locus is required for addition of guanine dinucleotide to MPT. The *mod* locus encodes the high affinity specific molybdate transport system. The *mog* gene product may function as a molybdochelatase and it is responsible for the assembly of molybdocofactor from MPT in the presence of low concentrations of molybdate (Joshi *et al*., 1996).

By using the *moa*, *mob* and *mod* mutants, we demonstrate that acquisition of the molybdocofactor in the cytoplasm is a prerequisite for the translocation of the TMAO reductase. The most intriguing observation is that the translocation of the TMAO reductase across the cytoplasmic membrane was found to be independent of the Sec system. Depletion of Ffh slightly reduced the TMAO reductase export. The results suggest the existence of a particular translocation machinery for the TMAO reductase.

Results

Translocation of TMAO reductase depends on intracellular molybdate availability in the mod mutant

In *E.coli*, molybdenum is taken up in the form of molybdate mainly through a periplasmic binding protein-dependent ABC transport system encoded by the *mod* operon (Maupin-Furlow *et al*., 1995). Deficiency of the Mod transporter reduces the intracellular molybdenum concentration to <10% of wild-type level (Scott and Amy, 1989). The poor intracellular molybdenum availability results in a pleiotropic effect on activities of molybdoenzymes. This defect, however, can be phenotypically suppressed by addition of molybdate in the growth medium (Campbell *et al*., 1985). In this case, molybdate is taken up through the sulfate transport system (Rosentel *et al*., 1995), and the intracellular molybdenum concentration in the *mod* mutants is restored to the level of a wild-type strain. We exploited the particular phenotype of the *mod* mutant to study the influence of the intracellular molybdate availability on translocation of TMAO reductase.

Periplasmic and cytoplasmic proteins from wild-type strain MC4100 and the *mod* mutant RK5209 were resolved on native gels, and TMAO reductase activity was revealed by activity staining (Figure 1A1). The crude extract of the mutant LCB620 was used as a negative control (Figure 1, lane 1). This strain contains a Mu insertion at the amino acid position 6 of *torA* and is thus deficient in the TMAO reductase. As expected, neither the periplasm nor the cytoplasm of the *mod* mutant contained TMAO reductase activity (Figure 1A1, lanes 5 and 9). Addition of molybdate in the growth medium led to the appearance of the TMAO reductase as an active band with a relative mobility (RF) of 0.16 in both fractions (Figure 1A1, lanes 4 and 8, TorA). Therefore, synthesis of active TMAO reductase is dependent on the intracellular molybdate availability. In addition, enzyme assays revealed that ~90 and 95% of the TMAO reductase activity was located in the periplasms of the wild-type strain or the *mod* mutant restored by molybdate, respectively. These results are in agreement

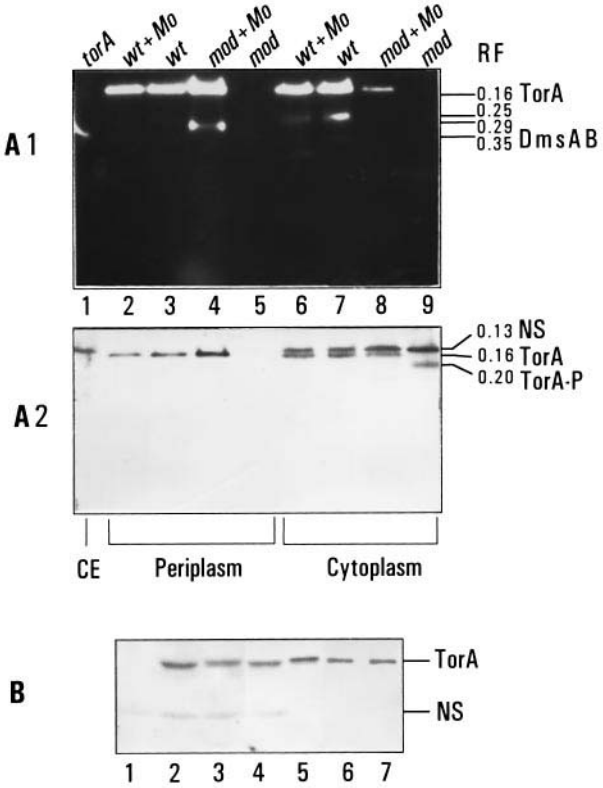

Fig. 1. Intracellular molybdate availability is essential for activity and translocation of the TMAO reductase. (**A1**) Periplasmic (5 µg of protein each) and cytoplasmic (50 µg of protein each) fractions prepared from the wild-type strain MC4100 (wt) and the *mod* mutant RK5209 (mod) grown without or with 1 mM molybdate (wt+Mo, mod+Mo) were separated on a 10% native gel and the TMAO reductase was detected by activity staining. About 70 µg of crude extract of the *torA*::Mu mutant LCB620 (torA, lane 1) was used as a control. The relative mobility (RF) of the TMAO reductase (TorA), DMSO reductase (DmsAB) and two unidentified active bands are indicated on the right. (**A2**) The same gel as (A1) was then used in immunoblot analysis employing antiserum to TorA. A non-specific contaminating band (NS), the active TMAO reductase (TorA) and its precursor depleted of molybdenum (TorA-P) are indicated on the right. (**B**) The crude extract of *torA* (lane 1), the cytoplasmic fractions of the wild-type (lane 2) or of the *mod* mutant grown with (lane 3) or without (lane 4) molybdate, the periplasmic fractions of the wild-type strain (lane 5), the crude extracts of a double mutant *torA–mod* complemented with intact *torA* gene (lane 6) or *torA* gene without the coding region for the signal sequence (lane 7) grown without molybdate were subjected to 7.5% SDS–PAGE and analyzed by immunoblot using antiserum to TorA.

with the previous observation that TMAO reductase is located mainly in the periplasm (Silvestro *et al*., 1989).

In *E.coli*, TMAO can also be reduced by the membrane-bound DMSO reductase. DMSO reductase in the crude extract of the *torA* mutant was thus detected as an active band with an RF of 0.35 by activity staining (Figure 1A1, lane 1, DmsAB). A very weak active DMSO reductase band was observed occasionally in the cytoplasmic fractions of wild-type or the *mod* mutant grown with molybdate, but was never detected in the periplasms of these strains. Two other barely detectable active bands with RFs of 0.25 and 0.29 were also found occasionally in the cytoplasm and periplasm, respectively. At present, we do not know whether they are TorA derivatives or other enzymes showing TMAO reductase activity.

Further analysis by immunoblot using antiserum against the TMAO reductase revealed that a single polypeptide with an RF of 0.16 was detected in the periplasmic fractions of the *mod* mutant grown with molybdate, as well as in the periplasm of the wild-type strain (Figure 1A2, lanes 2–4, TorA). The authenticity of this band as the *torA* gene product was established previously by immunoblot and N-terminal sequence determination (Silvestro *et al*., 1989; Méjean *et al*., 1994). As anticipated, this polypeptide was superimposable with the active band revealed by TMAO reductase activity staining and it was absent from the crude extract of the *torA* mutant LCB620 (Figure 1A2, lane 1). Most importantly, the TorA protein was absent from the periplasmic fraction of the *mod* mutant grown in the absence of molybdate (Figure 1A2, lane 5), which could be due to either of the following reasons: (i) the lack of a functional *mod* product abolishes the *torA* gene expression; (ii) TorA deficient of molybdenum is degraded in the periplasm after being translocated; or (iii) translocation of TorA lacking a functional molybdocofactor is blocked.

To assess these possibilities, we first examined the biosynthesis of TMAO reductase by studying the expression of the fusion *torA*::Mu*d*II(*lacZ*, Amp^R) in the *mod–torA* double mutant. The β-galactosidase activity was equal to 111 and 100 Miller units for the cells grown with and without molybdate, respectively. Therefore, molybdate is unlikely to affect the transcription of *torA*. As a consequence, molybdate seems to be required post-transcriptionally for the activation of TMAO reductase. Additional experiments excluded the possibility that TorA precursor lacking molybdenum was translocated and then degraded in periplasm (see below).

When cytoplasmic fractions were examined for the presence of the TorA protein by immunoblot, two polypeptides were each detected in the cytoplasmic fraction prepared from the wild-type strain and the *mod* mutant (Figure 1A2, lanes 6–9). The slower migrating band with an RF of 0.13, which was present in all cytoplasmic fractions (Figure 1A2, band NS), must be a contaminating polypeptide since it was also found in the crude extract of the *torA* mutant LCB620 (Figure 1A2, lane 1). We used this band as internal control. The second polypeptide showed two different mobilities according to the strain analyzed. In cytoplasms of the wild-type strain or of the *mod* mutant grown with molybdate, this second polypeptide detected by antibodies against TorA exhibited the same mobility (RF = 0.16) as the periplasmic TorA and it was superimposable with the active TMAO reductase band (Figure 1A2, lanes 6–8). It should be mentioned that although the amount of TorA in the *mod* mutant was comparable with that of the wild-type (Figure 1A2, lanes 8 and 6), the molybdate-restored TMAO reductase of *mod* was much less active than that of the wild-type strain (Figure 1A1, lanes 8 and 6). Enzyme assays confirmed that the V_{max} of the former was about two times lower than that of the latter (J.Buc, C.-L.Santini, R.Giordani, C.Blanco, G.Giordano and L.-F.Wu, in preparation). Because uptake of molybdate through the sulfate transporter in the *mod* mutant apparently does not suffice to generate a fully active protein, the wild-type Mod transporter may interact with cytoplasmic proteins which somehow ensure the correct formation of the active center.

In contrast, in the *mod* mutant grown without supplementation of molybdate (Figure 1A2, lane 9), the anti-TorA antibodies recognized a polypeptide with a faster mobility (RF = 0.20) than that of the wild-type. Because this form of TMAO reductase accumulated in the cytoplasm, but was converted to an active, periplasmic form upon addition of molybdate to growth medium (see below, Figure 4), we designated it TorA-P, standing for precursor form of TorA. The detection of a TorA-cross-reactive protein confirms that deficiency in intracellular molybdate has no significant effect on the *torA* gene expression. This deficiency apparently increased the electrophoretic mobility of TorA-P on the native gel. Further analysis by size exclusion chromatography revealed that the non-active precursor of TorA was located in fractions corresponding to a mol. wt of ~100 kDa and the active TorA present in cytoplasm or periplasm corresponded to 200 kDa. Therefore, the precursor of TorA seems to be present as a monomer and the cytoplasmic or periplasmic active TorA may be present as a dimer. In agreement with these results, Silvestro *et al.* (1989) previously reported that the purified periplasmic TMAO reductase is a dimer. However, the cytoplasmic species of active TorA could not only represent a dimer but also a complex composed of monomeric TorA and proteins involved in TorA translocation.

The periplasmic mature TorA, the cytoplasmic active TorA and the TorA precursor exhibited similar mobility when analyzed under denaturing conditions by SDS–PAGE (Figure 1B). N-terminal sequence determination confirmed that the signal sequence has been removed from the periplasmic TorA. Actually, we had expected that removal of the signal sequence of ~4 kDa would lead to an increase in the mobility of the periplasmic form of TorA. We assume that the Mini-protein II electrophoresis system used could not resolve the difference between the 90 kDa periplasmic and the 94 kDa cytoplasmic forms of TorA. Indeed, when the precursor TorA-P was run next to TorA-SS (a construct lacking the signal peptide, see below), there was no detectable difference in mobility (Figure 1B, lanes 6 and 7). Our attempt to sequence the cytoplasmic active form of TorA was unsuccessful. However, the correct designation of these two forms of TorA was confirmed by an alternative approach. By using isoelectrofocusing (IEF) electrophoresis analysis, we observed that the cytoplasmic active TorA exhibited a more alkaline pI than the periplasmic mature TorA (data not show). The calculated pIs for them are 6.41 and 6.18,

respectively. It is thus consistent with the presence of the signal sequence conferring on the cytoplasmic species of TorA a more alkaline pI value.

Membrane fractions prepared from the wild-type strain and the *mod* mutant were also analyzed by activity staining and immunoblot after electrophoresis on either native or SDS–PAGE. Neither the TMAO reductase activity, nor the TorA protein were detected in any of these membranes under the conditions used (data not shown).

Taken together, these results suggest that deficiency in intracellular molybdate results in the accumulation of TorA in the cytoplasm in a monomeric inactive precursor form. Furthermore, re-establishment of the normal intracellular molybdate concentration then allows for acquisition of a functional cofactor, activation of the TMAO reductase, oligomerization of TorA and finally translocation of TorA. This hypothesis is supported by the additional results present in Figures 2 and 4 described below.

Effect of tungstate or depletion of molybdocofactor synthesis on the translocation of TorA

Tungsten is an element akin to molybdenum, and naturally occurring tungstoenzymes contain the same pterin-based cofactor as molybdoenzymes (Chan *et al*., 1995). In addition, tungstate might be taken up through the same Mod system as molybdate (Rech *et al*., 1996). Similarly to molybdate, we observed that addition of 1 mM tungstate to the culture resulted in activation and translocation of the TMAO reductase in the *mod* mutant (Figure 2A1, lanes 4 and 9). However, the amount of tungsten-containing TorA protein in the periplasm was ~15% of the level of molybdo-containing TorA translocated (Figure 2A2, lanes 4 and 5). Enzyme assay showed that total TMAO reductase activity in the *mod* mutant grown with tungstate was ~10% of the level obtained in the same strain grown with molybdate. Addition of tungstate to the growth medium of the wild-type strain reduced by 90% both the TMAO reductase activity and the amount of TorA protein in periplasm (Figure 2A2, lanes 1 and 2). In this case, tungstate present at high concentration may compete with molybdate for its transport system or incorporation into the cofactor. The tungsten-induced reduction of active TMAO reductase from the periplasm was expected to be paralleled by a cytoplasmic accumulation of the same TorA precursor which was recovered from the *mod* mutant (Figure 2A2, lane 8). This was indeed the case for both wild-type and the *mod* mutant when they were grown with tungstate (Figure 2A2, lanes 7 and 9). Taken together, these results indicate that tungstate is capable of substituting molybdate in both TMAO reductase activation and TorA translocation. They also suggest that formation of tungstopterin cofactor or incorporation of this cofactor into the precursor is less efficient than formation of the MPT cofactor or its incorporation.

Biosynthesis of the molybdocofactor is a multi-step process and it occurs in the cytoplasm of *E.coli* (Rajagopaln, 1996). The *moa* mutants are deficient in the early steps of the synthesis of MPT, whereas *mob* mutants are defective in conversion of MPT to MGD. As expected, *moa* and *mob* mutants were unable to synthesize active TorA (Figure 2B1, lanes 2, 3, 6 and 7). Similarly to the

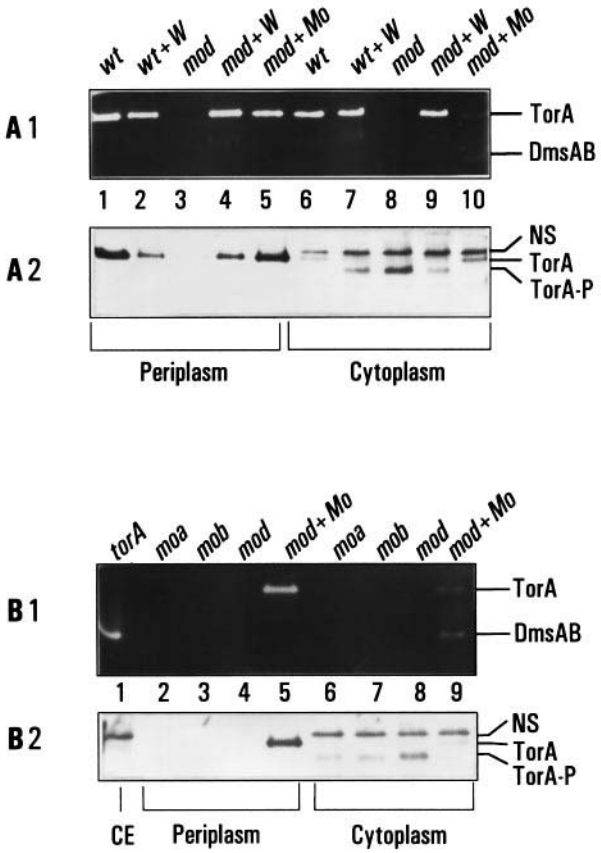

Fig. 2. Effect of tungstate or the *moa* and *mob* mutations on the translocation of the periplasmic TMAO reductase. The periplasmic (A, lanes 1–5) and cytoplasmic (A, lanes 6–10) fractions of the wild-type (wt) or the *mod* mutant (mod) grown with 1 mM molybdate (+Mo) or tungstate (+W) were analyzed by activity staining (**A1**) or immunoblot (**A2**) as described in Figure 1. Similarly, the periplasmic (B, lanes 2–5) and cytoplasmic (B, lanes 6–9) fractions prepared from *moa* (moa), *mob* (mob) or the *mod* mutant grown with (mod+Mo) or without (mod) molybdate were analyzed by activity staining (**B1**) or immunoblot (**B2**) as described in Figure 1. Crude extract (CE) of the mutant LCB620 (torA, lane 1) was used as a control. Quantification of chemiluminescence-generated bands was achieved by using the ImageQuant program (Molecular Dynamics).

mod mutant, TorA was absent from the periplasms of *moa* and *mob*, but it accumulated as precursor in the cytoplasms of these mutants. In addition, the precursor forms of TorA in the *moa*, *mob* and *mod* mutants exhibited the same mobility on a native gel (Figure 2B2, lanes 6, 7 and 8), suggesting a similar conformation for the three precursors. Taken together, these results indicate that acquisition of the functional molybdocofactor in the cytoplasm is a prerequisite for the translocation of the TMAO reductase into the periplasm.

Dissociation of activation from translocation

We then asked whether activation of TorA is dissociable from its translocation. To address this question, we removed the signal sequence of TorA by PCR. Intact *torA* gene (TorA), the *torA* gene without its signal sequence (TorA-SS) or the corresponding vector without insert were introduced into the *torA–mod* double mutant. The wild-type strain containing a chromosomal copy of the *torA* gene (TorA-C) was used as a positive control. As expected, without addition of molybdate, TorA accumulated as non-

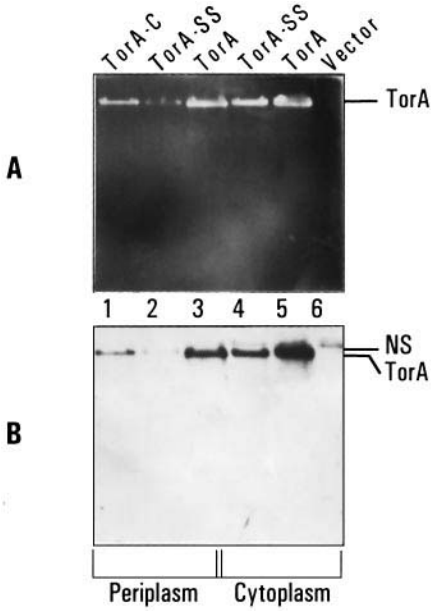

Fig. 3. Dissociation of activation from translocation of the TMAO reductase. The plasmids carrying the *torA* gene (TorA, lanes 3 and 5), the *torA* gene without the signal sequence (TorA-SS, lanes 2 and 4) or pBAD24 (Vector, lane 6) were introduced into the double *mod–torA*::Mu mutant. The wild-type strain containing the chromosomal copy of the *torA* gene (TorA-C, lane 1) was used as a control. Periplasmic (lanes 1–3) and cytoplasmic (lanes 4–6) fractions prepared from cells grown anaerobically with molybdate, were analyzed by activity staining (**A**) or immunoblot (**B**).

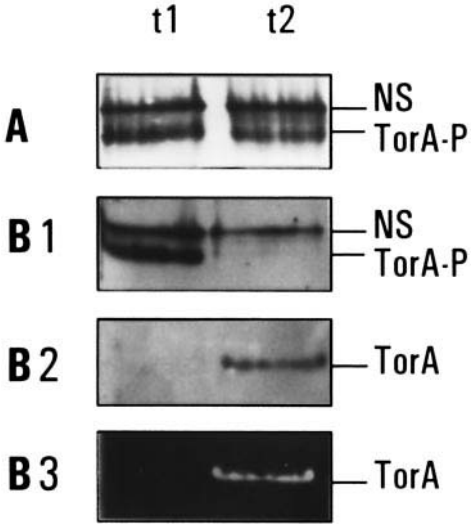

Fig. 4. Post-translational activation and translocation of the TMAO reductase. Rifampicin and chloramphenicol were added to early logarithmic phase cultures of the *mod* mutant grown anaerobically in LBGT medium without molybdate. One hour later (lane t1), molybdate (1 mM) was added to one portion of the culture (B), while another portion without addition of molybdate was kept as a control (**A**). Two hours after addition of rifampicin and chloramphenicol (lane t2), cytoplasmic (A and **B1**) and periplasmic (**B2** and **B3**) fractions were prepared and analyzed by immunoblot using antibodies against TorA (A, B1 and B2) or by activity staining (B3).

active precursor in the cytoplasm of the *torA–mod* mutant transformed with different plasmids (data not shown). Addition of molybdate in the growth medium resulted in the activation of the TMAO reductase in the strains transformed with the plasmids containing the *torA* gene, with or without the signal sequence (Figure 3A, lanes 4 and 5). The TMAO reductase was translocated normally into the periplasm in the presence of the signal sequence (Figure 3, lane 3). However, in the absence of the signal sequence (TorA-SS), TMAO reductase accumulated mainly in the cytoplasm, and only a very limited amount of the TMAO reductase was translocated into the periplasm (Figure 3, lanes 4 and 2). These results confirm that activation of TorA occurs in the cytoplasm. They also indicate that translocation is not required for activation, and that the signal sequence is essential for the translocation.

Activation and translocation of TMAO reductase is a post-translational process

We further investigated whether the activation and translocation of the TMAO reductase is a post- or co-translational process. The *mod* mutant was grown anaerobically in the absence of molybdate to early exponential phase (A_{600} = 0.3). To inhibit protein synthesis, rifampicin and chloramphenicol were then added in the LBGT growth medium of the *mod* mutant. One hour later, the growth ceased, indicating the inhibitory effects of rifampicin and chloramphenicol. The culture was divided in half; 1 mM molybdate was added to one portion to see if it was capable of triggering the activation and translocation of the TMAO reductase (Figure 4B), while another portion without molybdate was kept as a control (Figure 4A). After 1 h additional incubation, cells were harvested and analyzed for activity and translocation. In the absence of molybdate, TorA accumulated as the non-active precursor in the cytoplasm (Figure 4A, lane t1). The quantity of the precursor remained almost unchanged 2 h after the inhibition of protein synthesis (Figure 4A, lane t2). Neither TorA activity, nor TorA was detected in the periplasm during this period of incubation (data not shown). These results indicate that the precursor is relatively stable and that the amount of the precursor translocated into the periplasm would be negligible, if there was any translocation of the precursor. In contrast, 1 h after addition of molybdate, the cytoplasmic precursor of TorA had disappeared (Figure 4B1, lane t2), which was associated with the appearance of periplasmic active TorA (Figure 4B2 and B3, lane t2). Therefore, molybdate is able to trigger the post-translational activation of the cytoplasmic TorA and its translocation into the periplasm.

Energetics and kinetics of TMAO reductase translocation

Translocation of signal sequence-containing proteins across bacterial plasma membranes is powered by at least two sources of energy: the proton motive force, $\Delta\mu H^+$, and ATP hydrolysis. To assess their effect on the TorA translocation, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or the ATPase inhibitor, sodium azide, were added 5 min prior to pulse labeling of wild-type cells. Periplasmic fractions were immunoprecipitated with antiserum to TorA and analyzed by SDS–PAGE and PhosphorImager. In theory, the addition of CCCP could affect [^{35}S]methionine uptake and interfere with the pulse labeling. To assess this possibility, the influence of CCCP on the [^{35}S]methionine incorporation was analyzed. We found that the amount of [^{35}S]methionine accumulating in the whole cells which were previously treated with

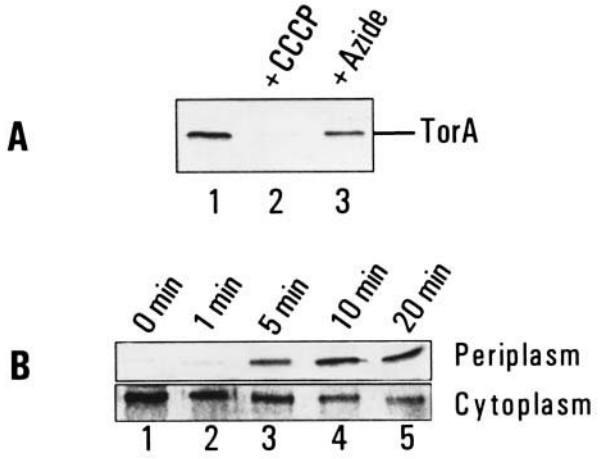

Fig. 5. Energy requirement and kinetics of TorA translocation. The wild-type strain MC4100 was grown anaerobically in M9 minimal medium until early logarithmic phase. (**A**) CCCP (100 μM, lane 2) and sodium azide (3 mM, lane 3) were added prior to pulse labeling with 50 μCi of [^{35}S]methionine for 1 min, followed by addition of 3.4 mM non-radioactive methionine, and chased for 20 min. Periplasmic fractions were prepared and subjected to immunoprecipitation using antiserum to TorA. Lane 1 is a positive control without addition of CCCP or sodium azide. (**B**) For kinetic study, cells were pulse labeled for 1 min, chased for 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4) and 20 min (lane 5). To avoid the continuous translocation during the preparation of periplasm in these time course experiments, CCCP and sodium azide were added immediately after the chase time as indicated. The amount of TorA translocated into periplasm (upper panel) or remaining in cytoplasm (lower panel) was analyzed by ImageQuant program (Molecular Dynamics).

CCCP was 90% of the level of untreated cells, while the amount of [^{35}S]methionine detected in the periplasm of the former was ~30% of the level of the latter. Therefore, the uncoupler has only a slight effect on [^{35}S]methionine uptake; however, it severely affects protein translocation into the periplasm. In the presence of 100 μM CCCP, the translocation of the TMAO reductase was almost completely blocked (Figure 5A, lane 2), showing a striking requirement for $\Delta\mu H^+$ for TorA translocation. In contrast to the CCCP effect, ~50% of the wild-type level of TorA was translocated into the periplasm in the presence of 3 mM sodium azide (Figure 5A, lane 3 compared with lane 1). ATPase might thus play a less important role than the proton motive force in the translocation of the TMAO reductase. ATPases may be involved in both molybdocofactor synthesis and acquisition, and in TorA translocation. Given that the toxic effects of sodium azide are largely due to inhibition of SecA ATPase and consequent inhibition of protein export (Pugsley, 1993), we considered the possibility that the decrease in the TMAO reductase translocation by sodium azide would result from the inhibition of the SecA ATPase. This possibility seems unlikely, since depletion of SecA does not affect the translocation of TorA (see below Figure 6C). However, we cannot exclude a direct involvement of other ATPases in the export of TorA.

To assess the kinetics of the TorA translocation, wild-type strain MC4100 was pulse labeled for 1 min and then chased for 0, 1, 5, 10 and 20 min (Figure 5B). Less than 10% of the maximal level of translocated TorA was found in the periplasm after 1 min chase (upper panel, lane 2). After 5 min chase, ~60% TorA was translocated, and reached the maximal level after 10 min chase (Figure 5B, upper panel, lanes 3–5). The increase in periplasmic TorA was paralleled by a decrease in cytoplasmic TorA (Figure 5B, upper panel versus lower panel). Compared with the export of the periplasmic maltose-binding protein MalE, which is a rapid process ($t_{1/2}$<30 s) (Randall and Hardy, 1986), the translocation of the TMAO reductase is a relatively slow one. The slow rate of translocation could be explained by the requirement for acquisition of the molybdocofactor preceding the export.

Translocation of the TMAO reductase into the periplasm is independent on the Sec system

Most membrane systems can only transport proteins that are at least partly unfolded (Schatz and Dobberstein, 1996). On the contrary, our results presented here suggest that TorA acquires the molybdocofactor and achieves its active conformation in the cytoplasm prior to translocation. These results suggest that the TMAO reductase may be translocated across the cytoplasmic membrane through an unusual mechanism. Therefore, requirement for different Sec proteins of TMAO reductase translocation was examined using various mutants and either immunoblot analysis and enzyme assay, or pulse labeling and immunoprecipitation.

The *secY39cs* mutant and its parental strain were grown in LB medium under anaerobic conditions at the permissive temperature (37°C). When cultures reached mid-log phase, they were divided in half; one portion was shifted to 23°C to inactivate the SecY, while another portion was kept at 37°C as a control. Two hours later, TMAO was added to induce the biosynthesis and translocation of TorA, and incubation was continued for 90 min. Cells were then harvested for analyzing protein translocation by immunoblot. In the absence of the inducer TMAO, TorA was not found in the cells treated under identical conditions (data not shown). Therefore, the TMAO reductase detected in these experiments was synthesized 2 h after inactivation of SecY. It is well established that translocation of the periplasmic maltose-binding protein MalE depends on the Sec system; therefore, the extent of SecY inactivation was monitored by measuring the amount of accumulated MalE precursor by immunoblot analysis. In agreement with previous work (Baba *et al*., 1990), translocation of MalE was severely impaired at the non-permissive temperature in the *secY39cs* mutant, as indicated by accumulation of its unprocessed precursor (Figure 6A1, lane 4). In the same culture, the amount of active TMAO reductase translocated into the periplasm was similar to that of the control kept at the permissive temperature or to that of wild-type cells grown at both temperatures (Figure 6A2 and A3, lanes 4 compared with lane 3 or with lanes 1 and 2). Consistently, enzyme assay showed that the specific TMAO reductase activity in the periplasm of *secY39cs* was comparable with, or even slightly higher than, that of the parental strain grown in both permissive and non-permissive conditions. Therefore, the depletion of one essential component of the membrane Sec complex is unlikely to interfere with the translocation of the TMAO reductase.

To ascertain the above conclusion, translocation of TorA was analyzed by immunoprecipitation. The *secY39cs* and

secE15cs mutants were grown at the permissive temperature (37°C) to mid-log phase, shifted to the non-permissive temperature (23°C) and subsequently pulse labeled with [^{35}S]methionine for 1 min. The extent of SecY and SecE depletion was assessed by measuring the amount of radiolabeled MalE obtained by immunoprecipitation from the periplasmic fraction of these cells. MalE was completely absent from the periplasm of the *secY39cs* mutant, and the amount of MalE translocated into the periplasm of the *secE15cs* mutant was considerably reduced (Figure 6B1, lanes 2 and 3) compared with the wild-type strain (lane 1) or with the *torA* mutant (lane 4). However, the amount of TorA in the periplasm of *secY39cs* and *secE15cs* was similar to that in the periplasm of the wild-type parental strain (Figure 6B2, lanes 2 and 3 compared with lane 1). The *secE* depletion strain CM124 is the *sec* mutant where translocation of Sec-dependent proteins seems to be most strongly affected (Traxler and Murphy, 1996). In this strain, the *secE* gene expression is under the tight control of the arabinose-inducible p*araBAD* promoter. When the cells were grown in the absence of arabinose (Figure 6B1, lane 6), the SecE depletion resulted in the complete absence of MalE from the periplasm. Similarly to the case of the *secE15cs* mutant, the depletion of SecE did not affect the TorA translocation (Figure 6B2, lane 6 compared with lane 5).

In a separate experiment, we examined the export of TorA in *secA51ts* and *secB* null mutants. SecA and SecB are required for directing exported proteins to the membrane SecYEG complex. The *secA51ts* mutant was treated like the *secY39cs* mutant described above, except that the temperature was shifted to 43°C instead of to 23°C. The periplasmic fractions were immunoprecipitated with antiserum to TorA, separated on SDS–PAGE and analyzed by PhosphorImager. TorA was translocated normally into the periplasm of the *secA51ts* mutant at both permissive and non-permissive temperature compared with the wild-type parental strain (Figure 6C, lanes 5 and 6 compared with 3 and 4). To study the effect of the *secB* null mutation on TorA translocation, CK1953 (*secB*) and its parental strain were grown in minimal medium at 37°C to mid-log phase, pulse labeled with [^{35}S]methionine for 1 min, and subsequently chased for 5 min. In agreement with the results obtained from studies with other *sec* mutants, the translocation of TorA was not affected by the *secB* mutation compared with the wild-type strain (Figure 6C, lane 2 compared with 1), whereas translocation of MalE was reduced by ~50% (data not shown). The *secB* mutant exhibits a conditional growth phenotype. While it grows like wild-type on minimal media, it is unable to form single colonies on rich media (Kumamoto

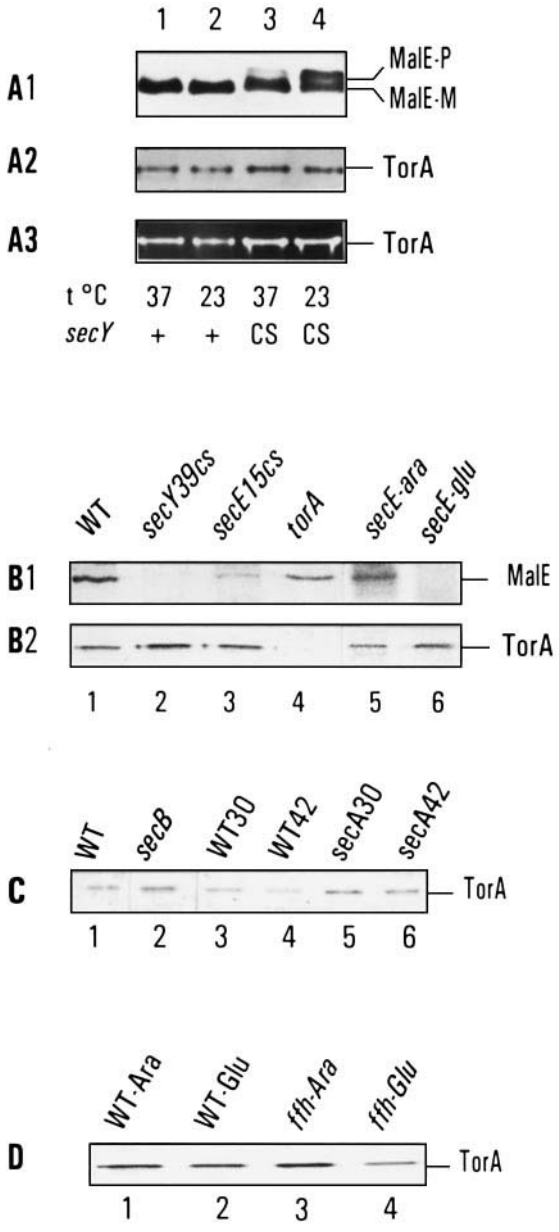

Fig. 6. Effect of depletion of the *sec* genes or *ffh* on the translocation of the TMAO reductase. (A) The *secY39cs* mutant CU164 (CS, lanes 3 and 4) and wild-type MC4100 (+, lanes 1 and 2) were grown at 37°C in LB medium until exponential phase. One portion of the culture was shifted to 23°C (A, lanes 2 and 4) to deplete SecY, whereas another portion was kept at 37°C as a control (lanes 1 and 3). Crude extract (**A1**) or periplasmic fraction (**A2** and **A3**) were separated on 10% SDS gels (A1) or native gels (A2 and A3) and analyzed by immunoblotting by using antisera to MalE (A1), or to TorA (A2), or by activity staining (A3). The precursor (MalE-P) or processed MalE (MalE-M) and the TMAO reductase (TorA) are indicated on the right. (B–D) One ml of each culture was pulse labeled for 1 min, followed by a 5 min chase. Periplasmic fractions were prepared and subjected to immunoprecipitation using antisera to MalE (**B1**) or TorA (**B2**, **C** and **D**) as described in Materials and methods. The growth conditions used were as follows. (B) Wild-type (WT), the *secY39cs* (secY39cs), *secE15cs* (secE15cs) and *torA* (torA) mutant were grown anaerobically at 37°C until early logarithmic phase. The cultures were then shifted to 23°C and continued for 1 h additional incubation. To deplete CM124 of SecE (lanes 5 and 6), cultures were diluted 1:100 from an overnight culture grown with arabinose into fresh M9 minimal medium supplemented with arabinose (secE-ara) or glucose (secE-glu) and incubated anaerobically for 6 h. (C) The wild-type strain (WT, lane 1) and the *secB* null mutant CK1953 (secB, lane 2) were grown anaerobically in minimal medium until early logarithmic phase and then pulse labeled. Similarly, the wild-type strain (WT, lanes 3 and 4) and its *secA51ts* derivative MM52 (secA, lanes 5 and 6) were grown anaerobically at 30°C to early logarithmic phase and then subjected to heat shock for 1 h (lanes 4 and 6) and pulse labeling. (D) The wild-type strain KS272 (lanes 1 and 2) and the *ffh* mutant JM113 (lanes 3 and 4) were grown anaerobically in M9 minimal medium supplemented with arabinose (0.2%) and TMAO (0.1%) until early logarithmic phase. Cells were washed and then resuspended in minimal arabinose (lanes 1 and 3) or glucose (lanes 2 and 4) media and incubated for an additional 2.5 h. The cultures were then subjected to pulse labeling. The amount of periplasmic TorA was quantified by ImageQuant program (Molecular Dynamics).

and Beckwith, 1985). We attempted to study the effect of changing the growth medium of the *secB* null mutant on the export of TorA. Even after 10 min of pulse labeling in LB medium, the TorA signal obtained was too weak to detect significant differences between wild-type and the *secB* mutant (data not shown).

Depletion of Ffh showed a slight effect on the translocation of TorA

The signal recognition particle (SRP), comprised of Ffh and Ffs, and its receptor FtsY function as an alternative protein translocation pathway in *E.coli* (Murphy and Beckwith, 1996; Schatz and Dobberstein, 1996). Given that translocation of the TMAO reductase is independent of the Sec system, it would be plausible if SRP was required for the targeting of TorA. We examined the translocation of TorA in the wild-type strain KS272, and the *ffh* mutant JM113, whose *ffh* expression is under control of an arabinose-inducible promoter (MacFarlane and Müller, 1995). TorA export was analyzed in pulse–chase labeling experiments after the cells had grown for three generations in the absence of arabinose. Growth with glucose or arabinose had no effect on the translocation of TorA in the wild-type strain (Figure 6D, lanes 1 and 2). However, the amount of TorA in the periplasm of the *ffh* mutant grown with glucose was ~70% of the level of the wild-type strain or 60% of the level of the same strain grown with arabinose (Figure 6D, lane 4 compared with lanes 2 and 3). In a separate experiment, enzyme assays showed that the specific TMAO reductase activity in the periplasm of the *ffh* mutant grown with glucose was reduced by ~20% compared with that grown with arabinose. Therefore, depletion of Ffh seems to interfere slightly with the translocation of TorA.

Discussion

Translocation of polypeptides across biological membranes is a fundamental step in the transfer of exported proteins from their site of synthesis to their final destinations. *Escherichia coli* exports proteins from the cytoplasm to both the periplasm and outer membrane by a mechanism involving a common Sec proteinaceous export apparatus (Pugsley, 1993; Murphy and Beckwith, 1996). It is generally accepted that preventing protein folding or protein unfolding prior to translocation is a necessary step for the transport of proteins across the cytoplasmic membrane of *E.coli* or the membranes of organelles such as the endoplasmic reticulum or mitochondrion (Pugsley, 1993; Rapoport *et al*., 1996; Schatz and Dobberstein, 1996). However, several lines of evidence in this study show that the TMAO reductase seems to be exported into the periplasm in a stably folded conformation and that the translocation of the TMAO reductase into the periplasm is independent of the Sec machinery.

The poor intracellular molybdate availability in the *mod* mutant or the abolition of molybdocofactor synthesis in the *moa* or *mob* mutants results in the accumulation of the inactive TMAO reductase in a monomeric precursor form in the cytoplasm. Four observations indicate that cytoplasmic acquisition of the molybdocofactor is a prerequisite for the TMAO reductase translocation. First, the precursor is detected only in the cytoplasm, but it is absent from either cytoplasmic membrane or periplasm. The absence of the precursor from the membrane rules out the possibility that the release of the precursor from the membrane translocase complex would be dependent upon cofactor insertion, in which case unprocessed precursor would block the export system and result in cytoplasmic accumulation of the precursor. Second, the quantity of the cytoplasmic TorA precursor in the *mod* mutant remains unchanged for 2 h, or even 4 h (data not shown), after the inhibition of protein synthesis. This result indicates that the cytoplasmic precursor is relatively stable and, more importantly, that the precursor is unlikely to be translocated into the periplasm and then degraded there in the absence of the molybdocofactor. Third, removal of the signal sequence from TorA does not have an effect on the cytoplasmic activation of the TMAO reductase, although it severely affects the translocation of the TMAO reductase. Finally, no molybdocofactor export system has been found in any of the bacteria analyzed. Since the molybdocofactor synthesis machinery is located in the cytoplasm, such a system would be absolutely necessary if the acquisition of the cofactor occurred in the periplasm.

Consistently, we previously reported that the formation of the nickel center of hydrogenase 2 (HYD2) of *E.coli* occurs in the cytoplasm, and that incorporation of nickel into the precursor is a prerequisite for the translocation of HYD2 across the cytoplasmic membrane (Rodrigue *et al*., 1996). The incorporation of nickel is associated with the removal of a C-terminal pre-sequence of the large subunit of HYD2 by a specific protease, which makes the process irreversible. In addition, acquisition of nickel results in a conformational change of its large subunit into a stably folded form, as monitored by the decrease of sensitivity to proteolytic degradation. Taken together, these results indicate that the acquisition of the molybdocofactor or the formation of the nickel center are essential for the translocation of these two metalloenzymes. Apparently, they cross the cytoplasmic membrane in stably folded conformations with their metallocenter functionally formed.

This observation is in contrast to the mechanism of protein export through the SecYEG complex. According to the prevailing model, only unfolded or partially folded proteins are competent for translocation. Indeed, we have demonstrated that the translocation of the TMAO reductase and that of the nickel-containing HYD2 (A.Rodrigue and L.-F.Wu, in preparation) are independent of the Sec system. These results suggest an unusual mechanism used for the translocation of these two metalloenzymes of *E.coli*.

In addition to being anaerobic metalloenzymes, both the TMAO reductase and HYD2 contain a special signal sequence. The signal sequences of periplasmic or membrane-bound Fe- or NiFe-hydrogenases show two particularities compared with classical signal sequences (Przybyla *et al*., 1992; Voordouw, 1992; Wu and Mandrand, 1993). First, these signal sequences possess a perfectly conserved motif Arg-Arg-X-Phe-X-Lys (RRxFxK), and second they are unusually long, ranging from 30 to 50 residues. The signal sequence of the TMAO reductase is very similar to those of hydrogenases. It is composed of 39 residues and contains a partially conserved RRxFxK motif, of which the lysine is substituted by an alanine residue in TorA (Méjean *et al*., 1994). Recently, this class of particu-

lar signal sequences has be extended to double-arginine signal sequences that are found on precursors of bacterial periplasmic proteins binding seven types of redox cofactors (Berks, 1996).

Nivière *et al.* (1992) have constructed a fusion protein between the NiFe-hydrogenase signal sequence from *Desulfovibrio vulgaris* and β-lactamase from *E.coli*. The resulting fusion protein was exported successfully in *E.coli* and the export was increased under anaerobic conditions. By using site-directed mutagenesis, these authors demonstrated that the first conserved arginine is essential for the export of the fusion protein. However, it was not analyzed whether the fusion protein is exported by the Sec pathway.

Cytosolically synthesized chloroplast proteins are imported into the thylakoid lumen by two completely different mechanisms (Robinson and Klösgen, 1994). A subset of lumenal proteins, which contain a thylakoid transfer signal similar to the bacterial classical signal sequence, are transported by the Sec system. The transport of the rest of the known lumenal proteins requires neither the Sec system nor ATP, but instead is dependent on the thylakoidal ΔpH. The ΔpH-dependent system and the export system used by the TMAO reductase and HYD2 share several very important common features. First, the translocation of the TMAO reductase is independent of the Sec system and it is driven mainly by the proton motive force. Second, like the signal sequence of the TMAO reductase or HYD2, signals of the thylakoidal proteins for the ΔpH-driven system contain a common twin-arginine motif immediately before the hydrophobic region (Chaddock *et al*., 1995). A Sec-avoiding motif (twin-Arg in the N-domain, Lys in the C-domain) has been identified recently in the twin-arginine signal sequence of the ΔpH-dependent thylakoid lumen proteins (Bogsch *et al*., 1997). Finally, a tightly folded lumenal protein containing the twin-arginine signal is translocation-competent for ΔpH-driven translocase (Creighton *et al*., 1995). Similarly, we found that both the TMAO reductase and the HYD2 are translocated in tightly folded conformations. Collectively, these common points might suggest that the two twin-arginine pathways are mechanistically related.

The acquisition of molybdocofactor or nickel is a prerequisite for the translocation of the TMAO reductase and HYD2, respectively. To account for these data, we propose a shelter-model to explain discrimination between the precursors without molybdocofactor or nickel and cytoplasmic active translocation-competent forms. The particular signal sequence on a nascent chain of the redox enzymes is not accessible due to binding of a specific shelter protein. Alternatively, binding of a specific chaperone protein to multiple sites on the precursor, which keeps the precursor in a conformation competent for the incorporation of metal, could also shelter the signal sequence from binding of a signal recognition factor. The precursor would thus not be competent for being targeted to the translocation machinery. Acquisition of cofactors or formation of a metallocenter trigger a conformational change of the precursor. Such a conformational change displaces the shelter protein or chaperone and unveils the signal sequence. A specific signal recognition factor then binds to the particular signal sequence and targets it to the translocation machinery. This model accounts for the following findings. (i) Acquisition of molybdocofactor or nickel occurs in the cytoplasm and is a prerequisite for translocation. (ii) Acquisition of nickel or activation of the TMAO reductase in the cytoplasm result in a conformational change of both enzymes into more compact forms. (iii) Activation of TorA is dissociable from the translocation and the signal sequence is not required for the activation, but it is essential for the translocation. According to this model, it is conceivable to obtain mutants defective in shelter protein or chaperone. In these mutants, precursor without metallocenter or cofactor would be capable of being translocated.

The SRP is a soluble ribonucleoprotein complex which functions as a Sec-alternative protein targeting pathway in both eukaryotic organisms and bacteria (Rapoport *et al*., 1996; Schatz and Dobberstein, 1996). Earlier studies showed that depletion of Ffh or FtsY, two core components of *E.coli* SRP, apparently affects the export of several different periplasmic and outer membrane proteins (Phillips and Silhavy, 1992; Luirink *et al*., 1994). However, more recent reports point towards a specific involvement of the *E.coli* SRP in the integration of hydrophobic membrane proteins, such as lactose permease (MacFarlane and Müller, 1995). This finding has now been extended to leader peptidase (de Gier *et al*., 1996) and SecY (Seluanov and Bibi, 1997). By using a genome-wide screen approach, Ulbrandt *et al.* (1997) reported that *E.coli* SRP is required for the insertion of a subset of inner membrane proteins, whereas most preproteins and some inner membrane proteins utilize SRP-independent targeting pathways effectively. The defect in preprotein export in *ffh* and *ftsY* is considered as a second effect of the depletion of these proteins which are essential for cellular viability (Ulbrandt *et al*., 1997). In this study, we found that the translocation of TorA is partially blocked by Ffh depletion. Further investigation is required in order to produce conclusive results regarding the involvement of SRP in metalloenzyme translocation.

Our studies with two metalloenzymes provide the first examples showing that stably folded proteins are capable of crossing the bacterial cytoplasmic membrane. Similarly, several lines of evidence show that peroxisomal matrix proteins can be imported into the lumen of the organelle in a stably folded conformation (Subramani, 1996). Two peroxisomal targeting signals (PTS) which direct proteins to the peroxisome have been identified. PTS1 is a conserved, carboxy-terminal tripeptide SKL that constitutes the major peroxisome targeting signal. PTS2 is composed of a conserved nonapeptide with the sequence (R/K)(L/V/I)X5(H/Q)(L/A), and it is used by a smaller subset of matrix proteins (Subramini, 1993). PTS2 is naturally located at the amino-terminus of the proteins, but also functions at internal locations in passenger proteins. Although several potential cytosolic, membrane and intraperoxisomal PTS receptors have been identified, the mechanism for protein uptake into peroxisomes remains obscure. Neither PTS1 nor PTS2 were found in the sequence of TorA. We could not, however, exclude the possibility that the translocation of TorA uses the same mechanism, but with different components, as that for peroxisomal protein import.

Materials and methods

Strains, plasmids and growth conditions

The strains CK1953 (*secB*::Tn*5)* (Kumamoto and Beckwith, 1985), MM52 (*secA51ts*) (Oliver and Beckwith, 1981), CU164 (*secY39cs*, *zhd-*

33::Tn*10*) (Baba *et al*., 1990), PS622 (*zijRK498::Tn5 secE15cs*) (Schatz *et al*., 1989), RK5209 (*mod*), RK5200 (*moa*), RK5208 (*mob*) (Stewart and MacGregor, 1982), LCB620 [*torA*::Mu*d*II(*lacZ*, Kan^R)] (Méjean *et al*., 1994) are derivatives of MC4100 (F′ *lac*Δ*U169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*) (Casadaban, 1976). The bacterial strain KS272 [F$^-$ Δ*lacX74 galE galK thi rpsL* Δ*phoA*(*Pvu*II)] was a gift from L.-M.Guzman (Guzman *et al*., 1995); JM113 (P_{ara}-*ffh*) from J.MacFarlane (MacFarlane and Müller, 1995); and the *secE* depletion strain CM124 [*secE*Δ*19–111 pcnB80 zadL*::(*Tn*10 Tc^S Str^r) *phoA*Δ*Pvu*II *lac*Δ*X74 galE galK rpsL recA::cat*/pCM22 Para-*secE*$^+$] from J.Beckwith (Murphy and Beckwith, 1994). The double *mod–torA* mutant was constructed by P1 transduction (Miller, 1972), with LCB620 as donor strain and RK5209 as recepient.

The intact *torA* gene or the *torA-ss* gene without its signal sequence were amplified by PCR using TORAEND (5′-aaatcttctctcatccgccaaaacagccaa-3′) and TORANCO1 (5′-gaaggaagaaaaaccatggacaataacgat-3′), or TORAEND and TORANCO2 (5′-cgtgcgactgcggcgcccatggcgactgac-3′) as primers and pTorA (Méjean *et al*., 1994) as template, respectively. The reaction was performed by using the Expand High Fidelity PCR System according to the manufacturer's instruction (Boehringer Manheim). The amplified fragments were purified, double digested by *Nco*I and *Hin*dIII and then cloned into the corresponding sites of the plasmid pBAD24 (Guzman *et al*., 1995). The presence or absence of the signal sequence in the resulting plasmids was verified by automatic DNA sequencing using the Applied Biosystems sequencer. In the presence of the signal sequence, introduction of an *Nco*I site (CCATGG) at the initiation codon level of *torA* substitutes the Asn2 by Asp. In the case of absence of the signal sequence, an *Nco*I site was introduced into *torA* at the amino acid positions 41 and 42, which converts Ala42 to Met. Therefore, the truncated TorA-SS starts from Met42, and lacks the 39 residue signal sequence and two amino acids of the mature part of TorA. Expression of the *torA* and the *torA-ss* genes was under the tight control of the P_{ara} promoter as described by Guzman *et al*. (1995).

The bacteria were grown routinely in Luria-Bertani (LB) medium, on LB plates or in minimal M9 medium (Miller, 1972). Anaerobic growth was achieved normally in LB medium supplemented with glucose (0.2%) (LBG) and TMAO (0.1%) (LBGT) in stoppered bottles or tubes filled to the top or on LB plates in GasPak anaerobic jars (BBL Microbiology Systems). As required, ampicillin (100 μg/ml), rifampicin (200 μg/ml), chloramphenicol (300 μg/ml), TMAO (1 mg/ml), sodium molybdate (1 mM) or sodium tungstate (1 mM) were added. Cultures were incubated at 37°C in most cases, or at 30°C for thermosensitive strains (*moa*, *mob*, *mod* and *secA*). As required, 23 and 42°C were used as non-permissive temperatures for cold-sensitive or thermosenstive mutants, respectively. Pre-cultures were inoculated from a single colony and used at 100-fold dilution.

Preparation of subcellular fractions

Periplasm and spheroplasts were prepared by the lysozyme/EDTA/cold osmoshock method (Osborn *et al*., 1972). Spheroplasts were washed once and disrupted by two passages through a French press. Cell debris was discarded by centrifugation at 18 000 *g* for 15 min. The supernatant was centrifuged further twice at 120 000 *g* for 90 min. The supernatant was saved as cytoplasmic fraction. The pellet obtained from the first ultracentrifugation was washed in 40 mM Tris–HCl (pH 7.6) containing 1 mM benzamidine–HCl and 15% sucrose. The resulting pellet was washed once and saved as membrane fraction.

Enzyme assays

TMAO reductase activity was measured at 37°C by following spectrophotometrically the oxidation of reduced benzyl viologen at 600 nm coupled to the reduction of TMAO as described by Takagi *et al*. (1981). One unit of specific activity was defined as 1 μmol of TMAO reduced per mg protein per min. Alternatively, protein fractions were separated on 10% non-denaturing polyacrylamide gels (Laemmli, 1970), and TMAO reductase activity was visualized by an activity staining method which is based on a methyl viologen-linked TMAO reduction (Silvestro *et al*., 1989). It should be noted that activity staining is a very sensitive but not quantitative method. Very weak TMAO reductase activity can be detected by this method, but it is not an appropriate method for accurate quantity comparison. β-Galactosidase activity was assayed in cells treated with SDS and chloroform by following the hydrolysis of *o*-nitrophenyl-β-galactopyranoside at 420 nm according to Miller (1972).

Electrophoretic and immunological procedures

Proteins were separated by polyacrylamide gel electrophoresis in the presence or in the absence of SDS (SDS–PAGE and native-PAGE, respectively) on 10% acrylamide gels (Laemmli, 1970). Immunoblot was performed by using the ECL method according to the manufacturer's instruction (Amersham). Quantification of the chemiluminescence-generated bands was performed by using the ImageQuant program (Molecular Dynamics). Accurate quantification of TorA antigen present in different cellular fractions was performed by rocket immunoelectrophoresis as described previously (Silvestro *et al*., 1989).

Isoelectrofocusing electrophoresis was performed on a pH 4–7 linear Immobiline DryStrip IEF gel (18 cm) by using the Multiphor II system according to the manufacturer's instructions (Pharmacia). Samples were prepared in 8 M urea, 4% CHAPS, 65 mM dithioerythritol, 1% Triton X-100 and 0.8% Bio-lyte 3–10.

For immunoprecipitation experiments, cells were grown anaerobically in 5–10 ml of M9 minimal medium, supplemented with 19 amino acids (50 μg/ml for each one, without methionine), 0.2% glucose (or arabinose, as required), 1 mg/ml TMAO, 1 μM sodium molybdate and thiamine (16.5 μg/ml), at the permissive temperature to early exponential phase (A_{600} = 0.3). The cultures were then shifted to the non-permissive temperature and incubated for an additional 1 h. For the *ffh* mutant and the wild-type strain KS272, cells were grown in M9-arabinose medium, centrifuged and washed, and then resuspended in fresh medium containing either arabinose or glucose as energy and carbon sources. The expression of *ffh* is under the control of the P_{ara} promoter, and it is thus repressed in the M9-glucose medium. In all cases, 1 ml cultures of a cell density of 2.5×10^8 cells/ml were pulse labeled by the addition of 50 μCi of [^{35}S]methionine/ml and then chased by the addition of non-radioactive methionine to a final concentration of 3.4 mM. Pulse labeling and chase times are indicated in Results. Cells were harvested by centrifugation, washed once with 0.1 M Tris-acetate buffer (pH 8.2), and a periplasmic fraction was prepared by the chloroform extraction method (Ames *et al*., 1984). Alternatively, to analyze a parallel evolution, periplasmic and cytoplasmic fractions were prepared by the lysozyme/EDTA/cold shock method (Randall and Hardy, 1986). Since immunoblot experiments using our antiserum against TorA showed that native gels gave higher sensitivity and lower background than SDS-denatured gels, the periplasmic fractions were transferred directly, without denaturation, to tubes containing 10 μl of a protein A–Sepharose slurry that had been reacted with antisera to TorA or MalE as described by Randall and Hardy (1986). Immunoprecipitation was performed according to Randall and Hardy (1986). Samples were separated by 10% SDS–PAGE. Dried gels were digitized by Storm PhosphorImager and signals were analyzed by ImageQuant program (Molecular Dynamics).

Size exclusion chromatography

Size exclusion chromatography was achieved by using two Superose 6 columns in series according to the manufacturer's instructions (Pharmacia). Columns were equilibrated with 40 mM Tris–HCl (pH 7.6), 200 mM NaCl, 1 mM benzamidine, and calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.5 kDa). Two hundred μl of the periplasmic fraction (~0.5 mg of protein) or cytoplasmic fraction (~2 mg of protein) from the wild-type strain or the *mod* mutant grown with or without molybdate were applied onto the columns and were eluted at 0.2 ml/min with the same buffer used in the equilibration of columns. Fractions were collected, separated on a native gel and analyzed by activity staining and immunoblot.

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