SecA-independent Translocation of the Periplasmic N-terminal Tail of an Escherichia coli Inner Membrane Protein

POSITION-SPECIFIC EFFECTS ON TRANSLOCATION OF POSITIVELY CHARGED RESIDUES AND CONSTRUCTION OF A PROTEIN WITH A C-TERMINAL TRANSLOCATION SIGNAL*

(Received for publication, August 21, 1995, and in revised form, October 6, 1995)

Paul Whitley, Guro Gafvelin, and Gunnar von Heijne‡

From the Department of Biochemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

We have shown previously that the 100-residue-long periplasmic N-terminal tail of the Escherichia coli inner membrane protein ProW can be translocated across the inner membrane in a sec-independent manner and that its translocation is blocked by the introduction of three positively charged residues near its C-terminal end (Whitley, P., Zander, T., Ehrmann, M., Haardt, M., Bremer, E., and von Heijne, G. (1994) EMBO J. 13, 4653-4661). We have now further analyzed the requirements for translocation of the N-terminal tail and found that the introduction of even a single arginine can block translocation. Position-specific differences in the effects on translocation of arginine insertions suggest that the C-terminal end of the N-terminal tail is more critical for translocation than the central and N-terminal regions. We also show that the N-terminal tail is translocated in a truncation mutant where a stop codon is placed immediately after the first transmembrane segment, provided that the transmembrane segment is flanked on its C-terminal end by positively charged residues. Thus, sec-independent translocation of a relatively large domain can be induced by a translocation signal located at the extreme C terminus of a protein.

The insertion of proteins into the inner membrane of Escherichia coli has been studied intensely over the past few years, and it is now generally accepted that hydrophobic segments drive insertion and end up spanning the membrane, whereas the orientation of the transmembrane segments is controlled by flanking positively charged residues: the “positive inside” rule (1). The details of the insertion mechanism are less clearly understood, however. In general, it seems that long periplasmic segments are translocated across the inner membrane by the so-called sec machinery also used by secretory proteins, whereas short periplasmic segments in most cases do not seem to require a fully functional sec machinery for translocation (2).

We recently described an interesting exception to this correlation between the length of a translocated domain and its sec-dependence; the 100-residue-long periplasmic N-terminal tail (N-tail) of the ProW protein can be efficiently translocated across the inner membrane under conditions where the function of SecB, SecA, or SecY is severely compromised (3). Insertion of three positively charged arginines near the C-terminal end of the ProW N-tail blocks its sec-independent translocation, whereas insertion of three negatively charged aspartic acid residues has no effect. Dissipation of the electrochemical potential across the inner membrane also blocks translocation of the N-tail, suggesting an electrophoretic component in the translocation mechanism.

We now report that insertion of a single arginine residue near the C-terminal end of the N-tail severely affects translocation, whereas insertion of two arginines blocks translocation almost completely irrespective of position. Further, we show that all of the ProW protein downstream of the most N-terminal transmembrane segment can be deleted with little effect on N-tail translocation, provided that a few positively charged residues are placed at the C-terminal end of the remaining transmembrane segment. To our knowledge, this is the first instance where the translocation of a large polar domain across the inner membrane has been shown to be induced by a translocation signal located at the extreme C terminus of the protein.

MATERIALS AND METHODS

Enzymes and Chemicals—Chicken egg white lysozyme, CCCP, and phenylmethylsulfonyl fluoride were from Sigma. Proteinase K was from Boehringer Mannheim. Enzymes used for DNA manipulation and sequencing were from Promega and Pharmacia. [35S]Methionine was from Amersham Corp.

Strains and Plasmids—All experiments were performed in E. coli strain MC1061 (ΔlacX74, araD139, Δ[ara, leu]7697, galU, galK, hsr, hsm, strA). All constructs were expressed from the pMG1 plasmid (4) by induction with arabinose.

DNA Techniques—Site-specific mutagenesis was performed according to the method of Kunkel (5) as modified by Geisselsoder et al. (6). All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase. Cloning into the pMG1 plasmid was performed as described (7).

Construction of the ProW1-119 ORF and a downstream primer containing a KpnI site for cloning situated before the first codon of the ORF was used for the ProW1–182-Lep(P2) fusions, and a second downstream primer was used for polymerase chain reaction amplification of the ProW1–119 fragment. The second primer contained four

*This work was supported by grants from the Swedish Natural Sciences Research Council, the Swedish Technical Sciences Research Council, and the Swedish National Board for Technical Development (to G. V. H.). 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.

† To whom correspondence should be addressed. Fax: 46-8-15-36-79; E-mail: gunnar@biokemi.su.se.

1 The abbreviations used are: N-tail, N-terminal tail; CCCP, carbonyl cyanide p-chlorophenylhydrazone.
codons downstream of proW codon 119 coding for amino acids MKKK followed by two stop codons and a KpnI site or two codons coding for amino acids MM followed by two stop codons and a KpnI site. The amplified fragments were cloned between the XhoI and KpnI sites in the pING1 vector containing ProW1–182-Lep(P2).

Assay of Membrane Topology—E. coli strain MC1061 transformed with the pING1 vector carrying the different proW-lep constructs under control of the arabinose promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, and all amino acids (50 μg/ml each) except methionine. Overnight cultures were diluted 1:25 in 1 ml fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2%) for 5 min, and labeled with [35S]methionine (150 μCi/ml). After 1 min, nonradioactive methionine was added (500 μg/ml), and incubation was stopped by chilling on ice. Cells were spun at 14,000 rpm for 2 min, resuspended in ice-cold buffer (40% w/v sucrose, 33 mM Tris, pH 8.0), and incubated with lysozyme (5 μg/ml) and 1 mM EDTA for 15 min on ice. Spheroplasts were then incubated with either 0.3 mg/ml proteinase K or with 0.33 mg/ml phenylmethylsulfonyl fluoride for 1 h on ice. After the addition of phenylmethylsulfonyl fluoride to the proteinase K-treated sample, the periplasmically exposed ProW N-tail can be removed by protease treatment of spheroplasts, leaving a protease-resistant fragment composed of the three ProW transmembrane segments and the Lep(P2) domain.

B, topological mapping of the ProW1–182(3R)-Lep(P2) construct. Cells expressing the fusion protein were labeled by [35S]methionine for 1 min, converted to spheroplasts, treated with proteinase K, and processed for immunoprecipitation with Lep antiserum. Band a is the intact fusion protein, and band b is the protease-resistant fragment resulting from proteolytic removal of the periplasmically exposed ProW N-tail (3). Azide was added to the cells to block the function of SecA (lanes 3 and 4), and CCCP was added to dissipate the electrochemical membrane potential (lanes 5 and 6).

For all samples, the fraction of background-corrected intensities in the protease-protected fragment band b (protease-treated cells; Fig. 1B, arrow) relative to bands a and b (where band a is the full-length protein in the protease-treated sample) was calculated.

\[ f = \frac{I_b}{I_a + I_b} \]  

(Eq. 1)

All quantitations were carried out on a Fuji BAS 1000 Image reader using the MacBAS (2.1) software. For the experiments reported in Fig. 4, the translocation efficiency was determined by normalizing the intensity in the ProW band by the intensity in the band corresponding to the cytoplasmic control AraB and then comparing the amount of ProW immunoprecipitated from samples either treated with proteinase K or left untreated.

Determination of SecA and Membrane Potential Dependence—The determined SecA-dependence was determined as follows. Constructs were induced with arabinose as above, and sodium azide was added (final concentration, 2 mM) 4 min after induction. 1 min later, [35S]methionine was added, and after an additional 1-min incubation, cells were put on ice, converted to spheroplasts, protease-treated, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis as above. In parallel samples, azide-treatment was omitted.

To test the dependence on the electrochemical membrane potential, cells were treated as in a normal protease protection experiment except that CCCP was added to a final concentration of 50 μM 45 s before the
RESULTS

Construction of a ProW-Lep Model Protein—The wild type ProW protein has seven transmembrane segments, Fig. 1A. In order to facilitate the topological analysis of ProW mutants, we previously constructed a fusion between the N-terminal half of ProW (residues 1-182) and the C-terminal, normally periplasmic P2-domain (residues 81-323) of the E. coli inner membrane protein Lep (3). The topologies of ProW and the ProW1-182-Lep(P2) fusion proteins are shown in Fig. 1A. In the fusion protein, the Lep domain is located in the cytoplasm, and translocation of the ProW N-tail to the periplasmic side of the inner membrane can be detected by protease treatment of spheroplasts, which will remove the ProW N-tail but leave the Lep domain intact.

Somewhat atypically, the first cytoplasmic loop in ProW does not contain any positively charged residues, and we reasoned that some of the N-tail mutants discussed below might give rise to an “inverted” topology with the N-tail in the cytoplasm and the first loop in the periplasm. Because this would complicate the interpretation of the results, three positively charged arginine residues were inserted between positions 119 and 120 in the first cytoplasmic loop (mutant ProW1-182(3R)-Lep(P2)). As shown in Fig. 1B, the N-tail is translocated in this mutant as judged by its sensitivity to protease K added to intact spheroplasts (92% translocation; lanes 1 and 2), and translocation is not blocked by sodium azide (lanes 3 and 4), suggesting that SecA is not required (10). As for the original ProW1-182-Lep(P2) fusion (3), dissipation of the membrane electrochemical potential by the protonophore CCCP completely blocks translocation, leaving the entire fusion protein resistant to the externally added protease (lanes 5 and 6). We thus used the ProW1-182(3R)-Lep(P2) mutant in all subsequent experiments.

Deletion of an N-terminal Segment Has No Effect on Translocation—We have previously shown that a putative amphipathic helix, residues 77-99, located at the C-terminal end of the ProW N-tail can be deleted without affecting translocation (3). To rule out that the N-terminal extremity of the N-tail contains a translocation signal, we deleted an acidic stretch near the N terminus (residues 5-25). As shown in Fig. 2, this deletion also has no effect on translocation, strongly suggesting that the N-tail does not contain a translocation signal. This is in agreement with earlier results showing that fusions between the ProW N-tail and PhoA are located in the cytoplasm (3).

Positively Charged Residues Have Position-specific Effects on N-tail Translocation—Because we had previously found that the insertion of three arginine residues between positions 99 and 100 at the very end of the N-tail completely blocked translocation (3), we were interested to determine the minimum number of arginines required to block translocation and whether their effect on translocation depends on their position.
within the N-tail. To this end, a series of mutants with either one or two arginines inserted at various positions throughout the N-tail were constructed.

As shown in Fig. 3A, a single arginine had a very strong effect when inserted between positions 99 and 100 (5% translocation; lanes 9 and 10), a milder effect when inserted between positions 32 and 33 and positions 82 and 83 (~30% translocation; lanes 3 and 4 and lanes 7 and 8), and was well tolerated when inserted between positions 4 and 5 and positions 65 and 66 (80–90% translocation; lanes 1 and 2 and lanes 5 and 6). Two arginines blocked translocation almost completely in all positions, again with the exception of positions 65 and 66 where 30% translocation was observed. For unknown reasons, we failed to express a mutant with two arginines inserted in position 5 despite repeated attempts.

We conclude that translocation is particularly sensitive to the introduction of positively charged residues near the C-terminal region of the N-tail next to the first transmembrane segment. Nevertheless, two arginines introduced more than 60 residues away from the first transmembrane segment also block translocation almost completely.

A C-terminal Transmembrane Segment Is Sufficient to Induce Translocation of the N-tail—In the ProW1–119-Lep(P2) model protein, the N-tail is followed by three transmembrane segments and a large cytoplasmic domain. Previously, we found that the N-tail was translocated, albeit with reduced efficiency (~50%), when the Lep domain was fused after the first transmembrane segment (3). This suggested to us that the first transmembrane segment itself may be sufficient to induce translocation of the N-tail.

Two ProW truncation mutants with stop codons placed in position 120 directly after the first transmembrane segment (residues 100–118) were made to test this possibility: one ending IAWQ119MM and the other ending IAWQ119MKKK. The two mutants differ by the presence of three extra positively charged lysines in the latter; methionines were included to facilitate detection by pulse labeling with [35S]methionine.

The topological analysis of the two mutants is presented in Fig. 4. The N-tail of the ProW1–119/MMK K mutant is partially translocated (65–70%) both in the absence (Fig. 4A, lanes 1 and 2) and presence (Fig. 4A, lanes 3 and 4) of sodium azide and, its translocation is thus independent of the SecA protein. Dissipation of the membrane electrochemical potential by CCCP prevents translocation (Fig. 4A, lanes 5 and 6). Note that translocation of the Sec-dependent outer membrane protein OmpA is largely blocked by both azide and CCCP, resulting in accumulation of the precursor form pro-OmpA inside the cell.

In contrast, the ProW1–119/MM mutant lacking C-terminal positively charged residues is resistant to protease digestion under all conditions (Fig. 4B), demonstrating that the N-tail is not translocated across the inner membrane. Possibly, the C-terminal extremity of the hydrophobic segment is translocated in this case, but this cannot be determined with the present assay.

We conclude that the hydrophobic segment located at the extreme C terminus of the ProW1–119 truncation mutant can act as a signal for the SecA-independent translocation of the N-tail and that its orientation can be determined by flanking positively charged residues.

**DISCUSSION**

Periplasmically exposed N-tails in E. coli inner membrane proteins contain few positively charged residues and appear to be translocated by a Sec-independent mechanism irrespective of length (3, 11). This is in contrast to long internal periplasmic loops and C-terminal tails for which translocation in most cases is Sec-dependent (12) and which can contain high numbers of positively charged residues (1, 13).

In this paper, we show that the insertion of as little as a single arginine into the ProW N-tail can dramatically affect the efficiency of translocation, whereas two arginines fully block translocation (Fig. 3). The effects on translocation vary with the position of the arginine(s), but none of the double-arginine mutations tested is completely without effect. This suggests that the entire N-tail is critical for translocation, although our data indicate that its C-terminal extremity is more important than its central and N-terminal parts. Nevertheless, deletions near the N and C termini of the N-tail have no effect on translocation (Fig. 2 and Ref. 3). Thus, the simple rule that positively charged residues are not tolerated in the N-tail irrespective of position holds remarkably well.

The ease with which the wild type N-tail can be translocated is even more dramatically illustrated by the finding that a truncation mutant composed only of the first 119 residues of ProW (i.e. the N-tail and the first transmembrane segment) followed by a methionine and three lysine inserts into the inner membrane with the N-tail exposed to the periplasm (Fig. 4). To our knowledge, this is the first demonstration that a hydrophobic segment located at the extreme C terminus of a protein can serve as a translocation signal for a large N-terminal domain. Previously, this has only been shown to be possible for small proteins such as the coat protein of phage Pf3 (14), which is
composed of a single transmembrane segment flanked by 18 N-terminal and 8 C-terminal residues. As expected from the positive inside rule (1), translocation of the N-tail in the ProW\textsuperscript{1-119} truncation mutant is only observed when the hydrophobic segment is flanked on its C-terminal side by positively charged residues. Further, translocation of the N-tail is independent of the sec-machinery, as in all other ProW constructs tested to date.

Because the translocation signal is located C-terminally to the N-tail, it seems reasonable to assume that the C-terminal end of the N-tail is translocated across the membrane at an early stage, in keeping with the “helical hairpin” hypothesis (15). The position-dependent effects on translocation in the IR series of mutants analyzed here (Fig. 3) also suggest that the C-terminal end of the N-tail is more critical for translocation than other parts. Previous studies have shown that a segment encompassing the first 20 residues on the C-terminal side of a normal signal peptide in a sec-dependent protein is critical for translocation and highly sensitive to the introduction of positively charged residues (15), suggesting that the formation of a transmembrane helical hairpin may be an early step in both sec-dependent and sec-independent translocation mechanisms.

Interestingly, an insertion of two arginines more than 60 residues away from the first transmembrane segment suffices to block export of the N-tail. It would seem that the initial formation of a helical hairpin comprising the first transmembrane segment and the preceding −20 residues should not be influenced by this mutation; rather, it may be that the two basic residues prevent the ultimate translocation of the N-terminal end of the N-tail and force the chain to “slip back” through the membrane (16).

Acknowledgments—We are indebted to Carina Norström (Pharmacia Biotech AB) for expert technical assistance. ProW antiserum was a kind gift from Dr. E. Bremer (Marburg, FRG).

REFERENCES

1. von Heijne, G. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 167-192
2. von Heijne, G. (1994) FEBS Lett. 346, 69-72
3. Whitley, P., Zander, T., Ehrmann, M., Haardt, M., Bremer, E., and von Heijne, G. (1994) EMBO J. 13, 4653-4661
4. Johnston, S., Lee, J. H., and Ray, D. S. (1985) Gene (Amst.) 34, 137-145
5. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488-492
6. Geisselsöder, J., Witney, F., and Yuckenberg, P. (1987) BioTechniques 5, 786-791
7. Dalbey, R. E., and Wickner, W. (1987) Science 235, 783-787
8. Andersson, H., and von Heijne, G. (1994) EMBO J. 13, 2267-2272
9. Daniëls, C. J., Bole, D. G., Quay, S. C., and Oxender, D. L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5396-5400
10. Oliver, D. B., Cabelli, P. J., Dulan, K. M., and Jarosik, G. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8227-8231
11. Cao, G. Q., and Dalbey, R. E. (1994) EMBO J. 13, 4662-4669
12. Sääf, A., Andersson, H., Gafvelin, G., and von Heijne, G. (1995) Mol. Membr. Biol. 12, 209-215
13. Andersson, H., and von Heijne, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9751-9754
14. Kuhn, A., Rohrer, J., and Gallusser, A. (1990) J. Struct. Biol. 104, 38-43
15. Engelmann, D. M., and Stelzer, T. A. (1981) Cell 23, 411-422
16. Schiebel, E., Driessen, A. J. M., Hartfi, F.-J., and Wickner, W. (1991) Cell 64, 927-939
SecA-independent Translocation of the Periplasmic N-terminal Tail of an *Escherichia coli* Inner Membrane Protein: POSITION-SPECIFIC EFFECTS ON TRANSLOCATION OF POSITIVELY CHARGED RESIDUES AND CONSTRUCTION OF A PROTEIN WITH A C-TERMINAL TRANSLOCATION SIGNAL

Paul Whitley, Guro Gafvelin and Gunnar von Heijne

*J. Biol. Chem.* 1995, 270:29831-29835.  
doi: 10.1074/jbc.270.50.29831

Access the most updated version of this article at [http://www.jbc.org/content/270/50/29831](http://www.jbc.org/content/270/50/29831)

Alerts:

- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/270/50/29831.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 16 references, 5 of which can be accessed free at [http://www.jbc.org/content/270/50/29831.full.html#ref-list-1](http://www.jbc.org/content/270/50/29831.full.html#ref-list-1)