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Sec-independent translocation of a 100-residue periplasmic N-terminal tail in the *E.coli* inner membrane protein proW

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The ProW protein, located in the inner membrane of *Escherichia coli*, has a very unusual topology with a 100-residue-long N-terminal tail protruding into the periplasmic space. We have studied the mechanism of membrane translocation of the periplasmic tail by analysing ProW–PhoA and ProW–Lep fusion proteins, both in wild-type cells and in cells with an impaired *sec* machinery. Our results show that the translocation efficiency is not affected by treatments that compromise the SecA and SecY functions, but that translocation is completely blocked by dissipation of the proton motive force or by the introduction of extra positively charged residues into the N-terminal tail. This suggests that the *sec* machinery can act properly only on domains located on the C-terminal side of a translocation signal, and that the N-terminal tail is driven through the membrane by a mechanism that involves the proton motive force.

Key words: *Escherichia coli*/inner membrane proteins/protein translocation/*sec* machinery

Introduction

The topology of *Escherichia coli* inner membrane proteins follows the positive inside rule; i.e. cytoplasmically oriented tails and loops contain many arginines and lysines whereas periplasmically oriented segments contain few positively charged residues (von Heijne, 1986). The topogenic effects of arginines and lysines have also been well documented experimentally (Boyd and Beckwith, 1990; von Heijne, 1994).

However, the positive inside rule does not appear to apply to the periplasmic loops longer than ~60 residues, where the frequency of positively charged residues is similar to that found in soluble periplasmic proteins (von Heijne and Gavel, 1988; von Heijne, 1994). An attractive idea is that this 'critical length' can be explained by a difference in the mechanism of membrane translocation of short and long periplasmic loops (von Heijne, 1986; Kuhn, 1988; Freudl *et al.*, 1989): short loops would be unable to make full use of the so-called *sec* machinery and their *sec*-independent translocation would be opposed by positively charged residues (Andersson and von Heijne, 1994), whereas the translocation of long loops (and of soluble periplasmic proteins) would make full use of the SecA/SecY/SecE translocase (Schatz and Beckwith, 1990; Wicker *et al.*, 1991) and there would be no such restrictions on the amino acid composition of the loop.

In consonance with this idea, where the short periplasmic segment of the phage M13 procoat protein was lengthened from ~25 to ~100 residues translocation became strongly *sec* dependent (Kuhn, 1988); likewise, when the size of a short periplasmic loop in a mutant of the inner membrane protein leader peptidase (Lep) was gradually increased from ~25 to ~65 residues, a parallel increase was seen in the degree of *sec* dependence (Andersson and von Heijne, 1993). *Sec* dependence has also been found for the large (~250 residues) C-terminal periplasmic P2 domain in Lep (Wolfe *et al.*, 1985), whereas translocation of its short N-terminal periplasmic tail is *sec* independent (Lee *et al.*, 1992).

Sec-dependent translocation has thus been demonstrated for the translocation of long internal periplasmic loops and for long C-terminal tails (C-tails), but has not been studied in proteins with long (≥60 residues) periplasmic N-terminal tails (N-tails). Such proteins are rare in *E.coli*, but a very long (~100 residues) periplasmic N-tail was recently found in the inner membrane protein ProW, a component of the ProU osmoregulatory system (Gowrishankar, 1989; Csonka and Hanson, 1991). The topology of ProW, as mapped by the PhoA–LacZ fusion technique (Manoil, 1991), is shown in Figure 1 (M.Haardt and E.Bremer, unpublished data). ProW has seven transmembrane segments, and the large N-tail is periplasmic (incidentally, this is the same topology as that of the vast family of eukaryotic G-coupled receptors). As there is no cleavable signal peptide upstream of the N-tail and there are no obvious apolar segments that could serve as internal signal sequences, the ProW N-tail represents the first example of an N-terminal periplasmic domain that is well within the *sec*-dependent length region. In contrast to known *sec*-dependent loops and C-tails, however, the ProW N-tail contains only three positively charged residues (but has 12 negatively charged ones), while a typical periplasmic domain of this size is expected to contain ~10 positively charged amino acids (von Heijne, 1986). In terms of amino acid composition, the ProW N-tail would thus be classified with the much shorter *sec*-independent loops. It is also noteworthy that neither the first cytoplasmic loop (C1) nor the first periplasmic loop (P1) contain any charged residues that could guide membrane insertion of the first three transmembrane segments.

In this communication, we report studies on the translocation of the ProW N-tail using fusions of various parts of ProW to both PhoA and Lep reporter domains. Our main

results can be summarized as follows: (i) translocation of the N-tail is not affected by treatments that impair the SecA and SecY functions but requires the proton motive force (p.m.f.), (ii) introduction of extra positively charged residues in the N-tail blocks translocation while extra negatively charged residues have no effect, (iii) fusion of an N-terminal signal peptide to the ProW N-tail does not affect the degree of *sec* dependence, (iv) a large (~180 residues, 18 Arg+Lys) periplasmic domain in the MalF protein that is located between the third and fourth of eight transmembrane segments cannot be translocated when placed in the N-tail position.

These results indicated that N-tails cannot make full use of the *sec* machinery for translocation and must thus obey the positive inside rule irrespective of their length. This not only adds to our general understanding of protein translocation and membrane protein biogenesis, but will also help to improve theoretical methods that seek to predict membrane protein topology from amino acid sequence.

Results

To study the topology and *sec* dependence of the ProW N-tail, we made fusions between various parts of ProW and two different reporter domains: mature PhoA and the normally periplasmic P2 domain of Lep (Figure 1). The alkaline phosphatase activity of the PhoA reporter depends on its periplasmic or cytoplasmic localization: high-activity fusions have a periplasmic PhoA domain while, in low-activity fusions, the PhoA domain is cytoplasmic (Manoil and Beckwith, 1986; Derman and Beckwith, 1991). The Lep P2 domain, on the other hand, is ideally suited for topological mappings based on protease accessibility in spheroplasts: the P2 domain is completely digested by proteases such as trypsin or proteinase K when it is located in the periplasm, but is protected from proteolysis by the inner membrane when it is cytoplasmic (von Heijne, 1989).

Topological mapping of ProW–PhoA and ProW–Lep fusions

In a preliminary set of experiments, we determined the minimum part of the ProW molecule that had to be included in the PhoA and Lep(P2) fusions to bring about efficient translocation of the N-tail. PhoA fusions were made in the first cytoplasmic loop and in the first, second and third periplasmic domains, and ProW–Lep(P2)d fusions were made in the first and second cytoplasmic loops and in the second periplasmic domain (Figure 1).

ProW–PhoA fusions where the reporter domain was placed at residues 79, 99, 122, 149 and 209 of the ProW N-terminal domain were analysed (Figure 1). PhoA activities of fusions to amino acids 79, 99, 122, 149 and 209 were determined in whole cells and were normalized against the amount of fusion protein made as judged by [^{35}S]Met pulse-labeling and immunoprecipitation (San Millan *et al.*, 1989). The specific activities are given in Table I. Although, as shown below, the N-tail of wild-type ProW is periplasmic, PhoA fusions to this domain have low activity and are thus cytoplasmically localized. This indicates that, unlike other periplasmic domains that are tethered to transmembrane segments (e.g. ProW–PhoA fusions to residues 149 and 209), the ProW N-terminus does not contain translocation signals that induce translocation of the PhoA moiety. The ProW$_{122}$–PhoA fusion has low specific activity indicating a cytoplasmically localized fusion joint. In contrast, ProW$_{149}$–PhoA has an intermediate and ProW$_{209}$–PhoA has a high specific activity indicating periplasmic fusion joints.

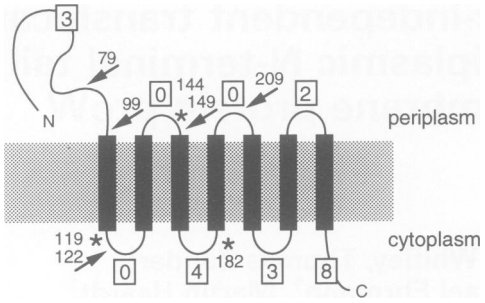

Fig. 1. Topology of ProW in the inner membrane. The number of Arg+Lys residues in the N-tail, C-tail and loops are shown in boxes. ProW–PhoA and ProW–Lep(P2) fusion joints are indicated by arrows and stars, respectively.

Table I. Specific PhoA activities and protease susceptibility of ProW-PhoA

Fusion	PhoA specific activity	ProW protease sensitivity	
		spheroplast	extract
ProW$_{1-79}$–PhoA	1	R	S
ProW$_{1-99}$–PhoA	1	R	S
ProW$_{1-222}$–PhoA	13	S	S
ProW$_{1-149}$–PhoA	55	S	S
ProW$_{1-209}$–PhoA	100	S	S

The activity of ProW$_{209}$–PhoA is 100% and corresponds to 64.3 μmol PNPP/min μg protein. Protease susceptibility in spheroplasts and whole cell extracts was carried out as described in Materials and methods. R indicates no cleavage of the fusion protein by trypsin, S indicates cleavage.

We also carried out preliminary trypsin susceptibility experiments using ProW–PhoA fusions to amino acids 79, 99, 122, 149 and 209. Cleavage sites for trypsin are located in the N-tail and in the second cytoplasmic domain of ProW (see Figure 1). PhoA itself is insensitive to protease when properly folded in the periplasm (Kamitani *et al.*, 1992). Thus, when trypsin is added to spheroplasts only those fusions that export the ProW N-tail to the periplasm are sensitive, whereas in whole cell extracts all fusions should be sensitive to trypsin. As shown in Table I, ProW–PhoA fusions to amino acid 79 and 99 were trypsin resistant while fusions to amino acid 122, 149 and 209 were cleaved in the ProW N-tail by trypsin. This indicates that the first transmembrane domain is required for translocation of the ProW N-tail while positively charged anchor domains are not necessary. These results were confirmed using ProW–LacZ fusions to amino acids 94, 99, 122, 151 and 248. Again, fusions to amino acids 94 and 99 were not cleaved by trypsin while the N-tail in the fusions to amino acids 122, 151 and 248 was trypsin sensitive (data not shown).

To obtain quantitative data on the translocation of the ProW N-tail, ProW–Lep(P2) fusions were used in pulse-

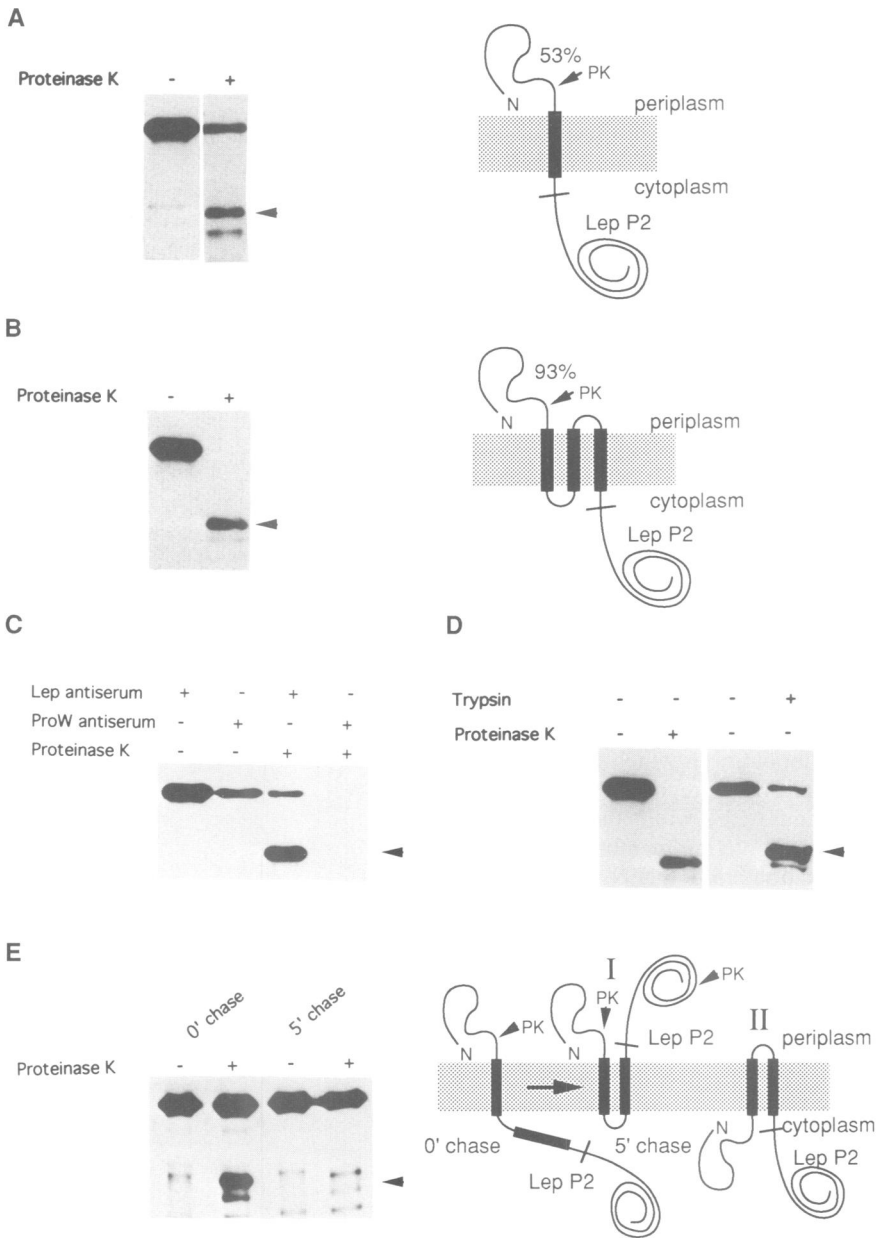

Fig. 2. Protease mapping of the topology of ProW–Lep fusion proteins in spheroplasts. (**A**) $ProW_{1-119}$–Lep. The arrowhead indicates the protease-resistant fragment composed of the first ProW transmembrane segment and the Lep P2 domain resulting from proteinase K digestion of the ProW N-tail. (**B**) $ProW_{1-182}$–Lep. The arrowhead indicates the protease-resistant fragment composed of the first three ProW transmembrane segments and the Lep P2 domain resulting from proteinase K digestion of the ProW N-tail. (**C**) $ProW_{1-182}$–Lep immunoprecipitated with Lep and ProW antiserum. (**D**) $ProW_{1-182}$–Lep treated with either trypsin or proteinase K. (**E**) Pulse–chase study of $ProW_{1-144}$–Lep. The arrowhead indicates the protease-resistant fragment composed of the first two ProW transmembrane segments and the Lep P2 domain resulting from proteinase K digestion of the ProW N-tail in molecules where the Lep P2 domain has not yet been translocated.

labeling and immunoprecipitation experiments. The results of the proteinase K mappings of ProW–Lep(P2) fusions are shown in Figure 2 and Table II. Inefficient translocation (~50%) of the N-tail was observed when the Lep P2 domain (residues 81–323) was placed after the first transmembrane segment (fusion $ProW_{1-119}$–Lep) (Figure 2A). When the fusion was made late in the second cytoplasmic loop, however, translocation of the N-tail was almost complete (~90%; fusion $ProW_{1-182}$–Lep), as seen by the disappearance of the full-length form of the fusion protein and the appearance of a protected fragment of a size expected for removal of the N-tail (Figure 2B). This band was reactive to Lep antibodies, but not to antibodies raised against the ProW N-tail (Figure 2C). We note that cleavage in the first periplasmic loop was not observed, probably because this loop is too short and does not extend far enough from the membrane surface to be accessible to the protease (cf. von Heijne, 1989). This is also shown by the fact that trypsin, which has no substrate site in the uncharged first periplasmic loop, and proteinase K produce the same protected fragment (Figure 2D).

The Lep fusion to the first periplasmic loop ($ProW_{1-144}$–Lep) was only partially protease sensitive (Figure 2E). Interestingly, a protease resistant fragment of the size expected for removal of the N-tail that could be immunoprecipitated with Lep antiserum was seen at short chase times but disappeared during a longer chase, suggesting that, in a fraction of the molecules, there is rapid

Table II. Translocation efficiency in normal cells (−azide), in treated cells (+azide), in a *secA*^{ts} strain (MM66), in a *secYs*^{ts} strain (CU164), in a *secB* null strain (CK1953), and in CCCP treated cells (+CCCP)

Fusion	−Azide	+Azide	MM66	CU164	CK1953	+CCCP
ProW$_{1-119}$–Lep	53	47	ND	ND	ND	ND
ProW$_{1-182}$–Lep	93	92	81	76	68	0
ProW$_{1-182,3R}$–Lep	14	ND	ND	ND	ND	ND
ProW$_{1-182,3D}$–Lep	79	ND	ND	ND	ND	ND
ProW$_{1-182,\Delta 77-99}$–Lep	95	94	ND	ND	ND	ND
SP–ProW$_{1-182}$–Lep	78	72	ND	ND	ND	ND

The translocation efficiency is defined as the percentage of the molecules that is sensitive to protease treatment in spheroplasts, except for the SP–ProW$_{1-182}$–Lep construct where the percentage of the molecules in which the signal peptide is cleaved is given. Whereas >80% of the cleaved form is protease sensitive under −azide and +azide conditions, only 10% of the cleaved form (3% of the total) is protease sensitive in the presence of CCCP (data not shown).
ND, not determined.

translocation of the N-tail (giving rise to the protease resistant fragment) followed by a much slower translocation of the Lep domain (molecules with both the ProW N-tail and the Lep reporter domain in the periplasm should be completely digested by protease K). The corresponding PhoA fusion ProW$_{1-149}$–PhoA is active, but in fact only has about half the specific activity of the ProW$_{1-209}$–PhoA fusion (Table I), also suggesting that Lep and PhoA fusions in the first periplasmic loop of ProW may have a mixed topology in which a fraction of the molecules have the N-tail and the reporter domain in the periplasm (topology I, Figure 2E) while the remaining molecules either do not insert into the membrane at all or have an 'inverted' orientation with the N-tail and the reporter domains in the cytoplasm and the short loop between the first and second transmembrane segments in the periplasm (topology II, Figure 2E); presumably, this loop is too short to be accessible to the added protease.

We conclude that PhoA and Lep P2 domains remain cytoplasmic when fused after the first transmembrane segment of ProW, but that the N-tail is only inefficiently translocated in those constructs. Fusions after the third transmembrane segment likewise have cytoplasmic reporter domains, and translocation of the N-tail is now almost total. Thus, fusions including the N-terminal part of ProW up to and including the second cytoplasmic loop (residues 1–182) insert efficiently into the inner membrane and have the correct topology. For this reason, most of the *sec* dependence studies were made with the ProW$_{1-182}$–Lep fusion or derivatives thereof. It is noteworthy that efficient translocation of the N-tail is only observed when at least three of the ProW transmembrane segments are present (Tables I and II), suggesting that membrane insertion is not based on a simple N-to-C-terminal insertion of one transmembrane segment at a time (Ehrmann and Beckwith, 1991; McGovern *et al.*, 1991; S.Cheng, G.Cao, P.Whitley, G.von Heijne, A.Kuhn and R.D.Dalbey, submitted; Gafvelin and von Heijne, 1994) and that the positively charged residues in the second cytoplasmic loop may be necessary for proper membrane insertion and/or orientation of the preceding parts of the molecule.

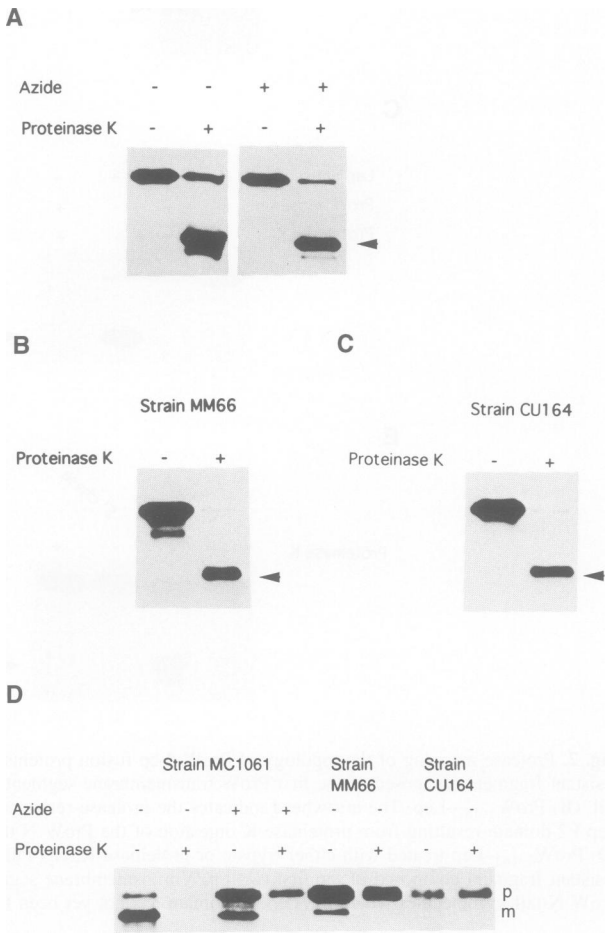

Fig. 3. Translocation of the ProW N-tail is *sec* independent. (**A**) Protease mapping of ProW$_{1-182}$–Lep in the absence and presence of sodium azide to block SecA function. (**B**) Protease mapping of ProW$_{1-182}$–Lep at the non-permissive temperature (42°C) in the *secA*^{ts} strain MM66. (**C**) Protease mapping of ProW$_{1-182}$–Lep at the non-permissive temperature (23°C) in the *secY*^{cs} strain CU164. (**D**) Protease resistance of proOmpA (p) and mature OmpA (m) immunoprecipitated from the same experiments as in (A)–(C). Note that only the mature form is accessible to the protease, and that translocation and signal peptide cleavage are severely inhibited.

Translocation of the ProW N-tail is not affected under conditions of impaired SecA and SecY function

SecA dependence of the translocation of the ProW N-tail in ProW–Lep(P2) fusions was assessed both under conditions where the ATPase activity of SecA was blocked by addition of sodium azide to the growth medium (Oliver *et al.*, 1990) and by expression of the fusion proteins at the non-permissive temperature in strain MM66 which is

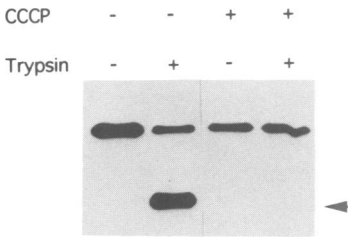

Fig. 4. Translocation of the ProW N-tail requires a p.m.f. Protease mapping of $ProW_{1-182}$–Lep in the absence and presence of the protonophore CCCP to dissipate the p.m.f.

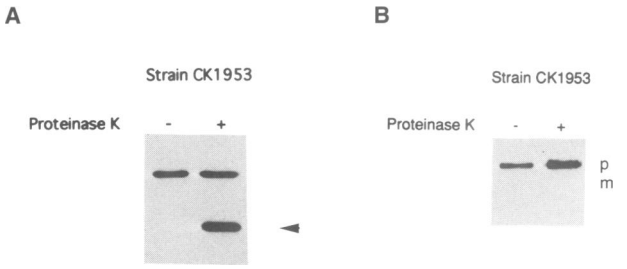

Fig. 5. Translocation of the ProW N-tail is weakly dependent on SecB. (**A**) Protease mapping of $ProW_{1-182}$–Lep in the *secB* null-strain CK1953. (**B**) Protease resistance of proOmpA (p) and mature OmpA (m) immunoprecipitated from the same samples as in (A). Note that translocation and signal peptide cleavage are completely inhibited.

$secA^{ts}$ (Schmidt *et al.*, 1988). As shown in Figure 3A and B and Table II, translocation of the N-tail in $ProW_{1-119}$–Lep and $ProW_{1-182}$–Lep was not affected when the function of SecA was perturbed. Only 20–30% of the SecA dependent outer membrane protein proOmpA was cleaved to the mature form and translocated under these conditions (Figure 3D).

SecY dependence was checked by expression at the non-permissive temperature in strain CU164 which is $secY^{cs}$ (Baba *et al.*, 1990); again translocation of the N-tail was very efficient (Figure 3C) while translocation of proOmpA was almost completely blocked (Figure 3D).

Translocation of the N-tail requires the proton motive force

Sec-dependent translocation requires the presence of a p.m.f. across the inner membrane (Daniels *et al.*, 1981; Zimmermann and Wickner, 1983; Bakker and Randall, 1984), while *sec*-independent translocation of short loops can be either p.m.f.-dependent or p.m.f.-independent depending, at least in part, on the number of negatively charged residues present in the loop (Andersson and von Heijne, 1994). The p.m.f. dependence of $ProW_{1-182}$–Lep was assayed by expression in the presence of CCCP, a protonophore that dissipates both the electrical and the pH components of the p.m.f. (Daniels *et al.*, 1981). As shown in Figure 4, CCCP completely blocked translocation of the N-tail. A possible explanation for the p.m.f. dependence is the presence of 12 negatively charged residues in the N-tail, though this requires further work to be verified.

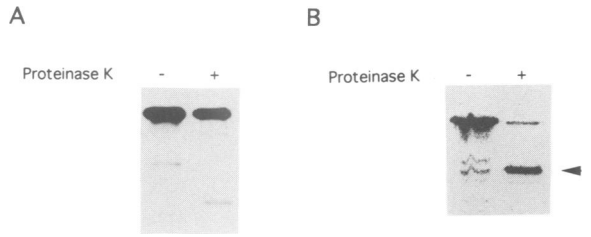

Fig. 6. Positively but not negatively charged residues block translocation of the ProW N-tail. (**A**) Protease mapping of $ProW_{1-182,3R}$–Lep. (**B**) Protease mapping of $ProW_{1-182,3D}$–Lep.

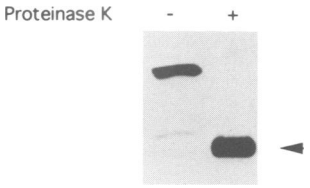

Fig. 7. The amphiphilic segment 79–99 in the ProW N-tail is not required for translocation. Protease mapping of $ProW_{1-182,\Delta 77-99}$–Lep.

Translocation of the N-tail is largely SecB independent

Some but not all secretory and outer membrane proteins in *E.coli* depend on the cytoplasmic chaperone SecB for efficient translocation (Strobel *et al.*, 1993). When $ProW_{1-182}$–Lep was expressed in the *secB* null-strain CK1953 (Kumamoto and Beckwith, 1985), a slightly less efficient translocation of the N-tail was observed (Figure 5 and Table II) though the impairment was much less marked than for the SecB-dependent control protein proOmpA. It is thus possible that the N-tail interacts with SecB, but this interaction is clearly not necessary for translocation. It should be noted that the Lep P2 domain does not depend on SecB for translocation (R.Dalbey, personal communication; H.Andersson and G.von Heijne, unpublished observation).

Positively but not negatively charged residues block translocation of the N-tail

Since translocation of the ProW N-tail is independent of SecA and SecY, and since the N-tail contains only three positively charged residues, it was of interest to check whether the addition of extra positively or negatively charged residues would in any way affect the translocation efficiency. A string of three positively charged arginine residues was thus inserted between residues 99 and 100 in the N-tail of $ProW_{1-182}$–Lep (mutant $ProW_{1-182,3R}$–Lep); as seen in Figure 6A and Table II, translocation was blocked in this mutant. In contrast, when three negatively charged aspartic acid residues were inserted in the same position (mutant $ProW_{1-182,3D}$–Lep), translocation was unaffected (Figure 6B).

The amphiphilic segment 77–99 is not required for translocation

Although stretches of hydrophobic residues are the primary translocation signals in the signal peptides of secretory proteins and in integral membrane proteins, chain segments

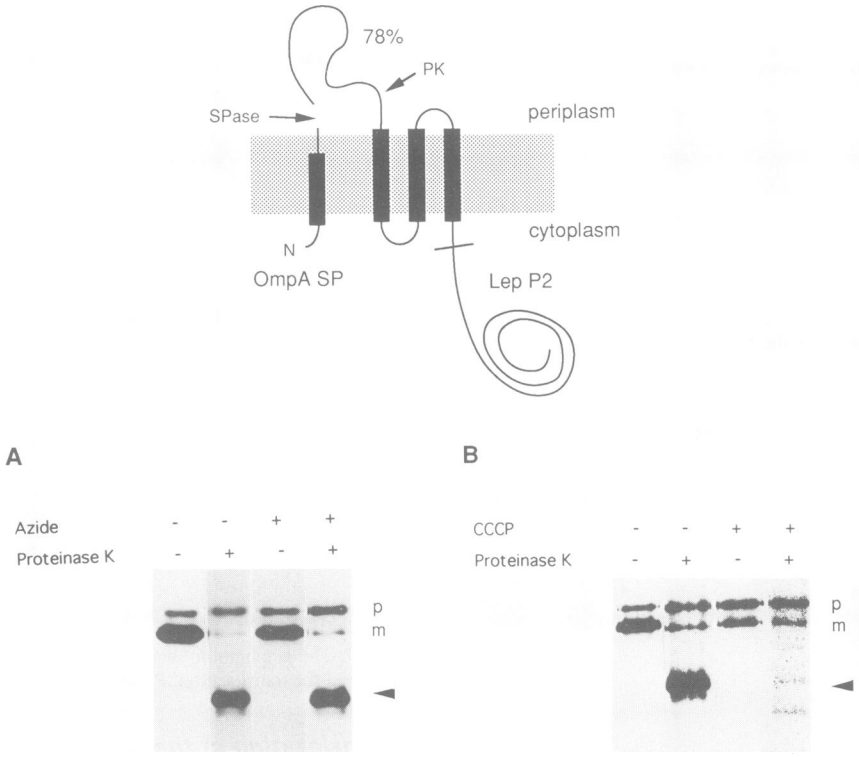

Fig. 8. Addition of the proOmpA signal peptide upstream of the ProW N-tail does not affect *sec* or p.m.f. dependence. (**A**) Protease mapping of SP–$ProW_{1-182}$–Lep in the absence and presence of sodium azide. The precursor (p) and mature (m) forms of the protein are indicated. (**B**) Protease mapping of SP–$ProW_{1-182}$–Lep in the absence and presence of CCCP. Note that, in both panels, only the mature form of the protein is sensitive to protease.

with a potential to form amphiphilic α-helices have also been implicated in the membrane insertion process of certain inner membrane proteins (Saier *et al.*, 1988, 1989; Yamada *et al.*, 1991). In the ProW N-tail, residues 77–99 just upstream of the first transmembrane segment have a high potential for forming an amphiphilic helix, and would be an obvious candidate for serving as some kind of internal amphiphilic signal sequence. However, translocation of the N-tail is unaffected by deletion of this stretch (mutant $ProW_{1-182,\Delta 77-99}$–Lep (Figure 7 and Table II) and remains SecA independent (data not shown).

Addition of an N-terminal signal peptide

One of the unusual features of the ProW N-tail is that, despite its length, it lacks an N-terminal, cleavable signal peptide. To check whether the presence of a signal peptide would make any difference in terms of *sec* or p.m.f. dependence of the N-tail, the proOmpA signal peptide was added in front of the N-tail in the $ProW_{1-182}$–Lep fusion. The SP–$ProW_{1-182}$–Lep construct was efficiently translocated and the signal peptide was removed both in the absence and presence of azide, whereas translocation and cleavage of the signal peptide was largely blocked by addition of CCCP (Figure 8 and Table II). Thus, the addition of the proOmpA signal peptide does not affect the translocation behaviour of the ProW N-tail: translocation still requires the p.m.f. and remains unaffected when SecA function is perturbed. It is interesting to note that translocation appears to be more sensitive to CCCP treatment than is signal peptide cleavage, suggesting that, even in the presence of CCCP, the signal peptide may insert sufficiently far into the translocation machinery to be cleaved by the signal peptidase, but that further translocation of the ProW N-tail is efficiently blocked.

The large periplasmic domain of MalF cannot be translocated when in an N-tail position

It appears from the results presented above that the unique translocation behaviour of the ProW N-tail is to a first approximation a result of its reduced content of positively charged residues. This suggests that other large periplasmic domains with a more normal content of positively charged residues would be impossible to translocate if placed in an N-tail position. As a first test of this corollary, we studied the translocation of the large periplasmic loop in the MalF protein that is normally located between the third and fourth of the eight transmembrane segments present in this molecule [residues 92–275; this segment contains 18 positively and 21 negatively charged residues (Froshauer *et al.*, 1988) but its translocation is nevertheless only weakly affected when SecA function is blocked (McGovern and Beckwith, 1991; our unpublished results)]. Residues 2–99 of wild-type MalF were deleted, thus removing all three transmembrane segments on the N-terminal side of the large loop. As expected, the protein was completely resistant to proteolysis (Figure 9) indicating that translocation of the large loop was completely prevented by the deletion.

Discussion

This paper presents the first analysis of the translocation behaviour of a very long periplasmic N-tail (~100 residues)

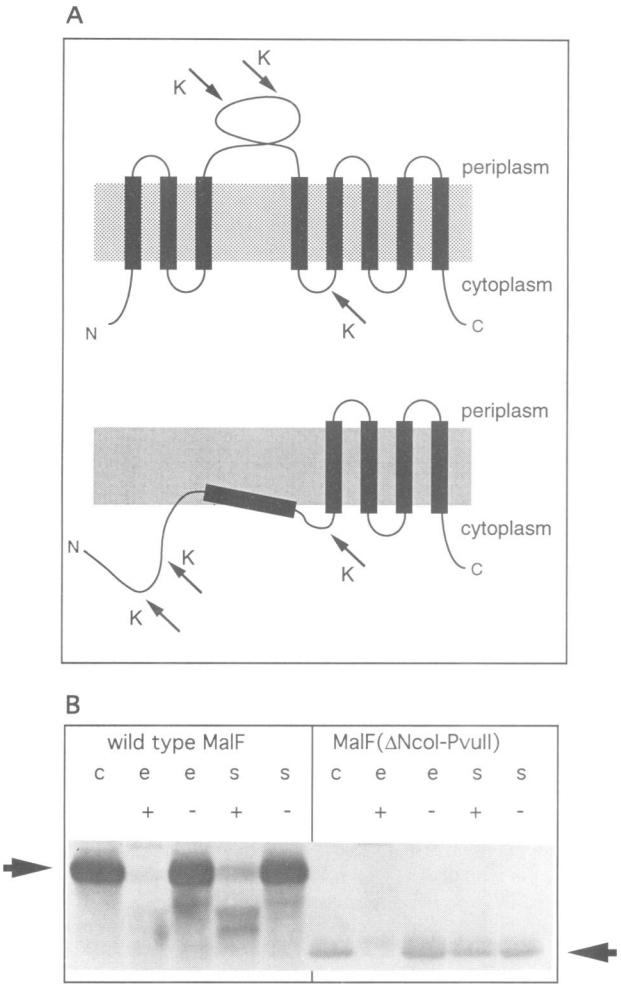

Fig. 9. The large periplasmic domain of MalF cannot be translocated when in an N-tail position. (**A**) Topologies of the MalF wild-type (top) and MalF(Δ*NcoI*–*Pvu*II) mutant (bottom). Potential kallikrein-sensitive sites are indicated by 'K'. (**B**) Kallikrein sensitivity of the MalF wild-type (left) and MalF(Δ*NcoI*–*Pvu*II) mutant (right). The absence and presence of the protease are indicated by '−' and '+'. c, cells; e, extract; s, spheroplasts.

in an *E.coli* inner membrane protein. Our results show that translocation of the ProW N-tail is largely or even completely unaffected by conditions under which the functioning of the *sec* machinery is severely compromised. We conclude that SecA, SecY and SecB all seem to be dispensable for translocation, or at least that whatever function they may have can be fulfilled by the residual activity present under the conditions employed [see Andersson and von Heijne (1993) for a discussion of this point]. A few other bacterial inner membrane proteins with fairly long periplasmic N-tails are also known: the longest one (~40 residues) is found in the F_1–F_0 ATPase *a* subunit (Lewis *et al.*, 1990). Again, the number of positively charged residues in this N-tail is small whereas negatively charged residues are more abundant (one Arg versus five Asp+Glu). It is attractive to suggest that the *sec* machinery can act properly only on domains located on the C-terminal side of translocation signals (i.e. cleavable or non-cleavable signal sequences), and that N-tails thus cannot use the classical *sec* pathway.

Long N-tails are more common in eukaryotic membrane proteins, and efficient translocation of the ~60-residue-long glycophorin C N-tail has been demonstrated in *E.coli* (Hennessey *et al.*, 1993). Interestingly, the glycophorin C N-tail contains only two positively charged but seven negatively charged residues, and is thus similar to the N-tails of bacterial proteins in this regard. The *sec* dependence of glycophorin C expressed in *E.coli* has not been studied.

On the other hand, translocation of the ProW N-tail is completely dependent on an intact p.m.f. The p.m.f. is known to be required for *sec*-dependent translocation (Daniels *et al.*, 1981; Zimmermann and Wickner, 1983; Bakker and Randall, 1984) and we have recently found that it seems to oppose the translocation of positively charged residues and to facilitate the translocation of negatively charged ones during *sec*-independent translocation (Andersson and von Heijne, 1994). The p.m.f. dependence of the ProW N-tail may thus be related to its net surplus of negatively over positively charged residues. A possible scenario is that the N-tail may be driven across the membrane by the p.m.f. acting on the negatively charged residues and that most or all of the functions of the ATP-dependent SecA/Y/E translocase (Wickner *et al.*, 1991) may be dispensable. This idea is supported by our observations on the fusion between the proOmpA signal peptide and ProW: translocation of the N-tail is *sec* independent but p.m.f. dependent even when the proOmpA signal peptide is present. The signal peptide presumably mediates an initial targeting to the *sec* machinery, but the subsequent translocation event is not measurably affected when the SecA function is inhibited.

Our results are largely corroborated by the findings reported in the accompanying paper (Cao and Dalbey, 1994), in which N-tails of different lengths were added to the periplasmic N-terminus of Lep. Short N-tails of up to 38 residues lacking Lys and Arg residues were efficiently translocated under conditions of impaired SecA and SecY function, whereas, in contrast to the ProW result, longer N-tails were only inefficiently inserted. When extra positively charged residues were added to the 38-residue-long N-tail, translocation was blocked. Translocation of a 138-residue-long N-tail became possible when a signal peptide was added; however, again in contrast to the corresponding ProW result, translocation was now found to depend strongly on SecA. Thus, although both studies agree that N-tails cannot make normal use of the *sec* machinery for translocation, the ProW N-tail is sufficiently different from the 'artificial' N-tails used by Cao and Dalbey to allow its efficient, *sec*-independent translocation even though it is more than twice as long as the longest efficiently inserted artificial N-tail.

Finally, we note that the results presented here should be taken into account when trying to predict the topology of inner membrane proteins from their amino acid sequence. Thus, in the TOP-PRED algorithm (von Heijne, 1992), possible topologies derived from a hydrophobicity plot are ranked in order of increasing bias in the distribution of positively charged residues between the periplasmic and cytoplasmic loops. Since the positive inside rules does not apply to loops longer than ~60 residues (von Heijne, 1994), such loops are excluded from the charge-bias calculation. However, the results obtained for the ProW N-tail suggest that N-tails obey the positive inside rule irrespective of length, and that they must therefore always

be included in charge-bias calculations. Indeed, TOPPRED correctly predicts the topology of ProW even when the charges in the N-tail are included (data not shown).

Materials and methods

Enzymes and chemicals

Trypsin, soybean trypsin inhibitor, chicken egg white lysozyme and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Kallikrein was from Boehringer Mannheim. Other enzymes were from New England Biolabs, Promega and Pharmacia.

Strains and plasmids

Escherichia coli strains used were DHB4 [*araD*139 Δ(*ara–leu*)7697 Δ*lacX*74 Δ*phoA* (*Pvu*II) *phoR* Δ*malF*3 *galE galK thi rpsL* F′*lacI*^{Q} *pro*) (Boyd *et al.*, 1987), CC118 (*araD*139 Δ(*ara–leu*)7697 Δ*lacX*74 Δ*phoA* 20 *galE galK thi rpsE rpoB argE*$_{am}$ *recA1*) (Manoil and Beckwith, 1985), CU164 (*F*−, *secY39(Cs)*, *zhd-33::*Tn*10*, *araD139*, Δ(*argF–lac*)*U169*, *rpsL150*, *relA1*, *flbB5301*, *deoC1*, *ptsF25*, *rbsR*) (Baba *et al.*, 1990), CK1953 (MC4100 *secB::Tn5*) (Kumamoto and Beckwith, 1985), MM66 (MC4100 *geneX-am*, *supF-ts*, *tet-r*) (Schmidt *et al.*, 1988) and MC1061 (Δ*lacX74*, *araD139*, Δ(*ara, leu*)*7697*, *galU*, *galK*, *hsr*, *hsm*, *strA*). Leader peptidase mutants were expressed from the pING1 plasmid (Johnston *et al.*, 1985) by induction with arabinose. Plasmid pDHB32 contains the *E.coli malF* gene under tac promoter control (Boyd *et al.*, 1987).

DNA techniques

Site-specific mutagenesis was performed according to the method of Kunkel (1985), as modified by Geisselsoder *et al.* (1987). All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase. Cloning into the pING1 plasmid was performed as described (Dalbey and Wickner, 1987).

Construction of malF ΔNcoI–PvuII

malF Δ*Nco*I–*Pvu*II is derived from pPFP9 (Uhland and Ehrmann, 1994). pPFP9 has bp 4–297 of *malF* replaced by *phoA* lacking its signal sequence and its 3′ stop codon. pPFP9 has a polylinker containing *Sal*I, *Xba*I, *Bam*HI, *Sma*I and *Kpn*I restriction sites 5′to phoA and a *Bst*EII site 3′ to phoA. To construct *malF* Δ*Nco*I–*Pvu*II, pPFP9 was cut with *Xba*I and *Bst*EII, filled using Klenow enzyme and ligated. Constructs were checked by identification of truncated MalF by Western blotting and restriction analysis. The *Bst*EII site was regenerated after ligation while *Bam*HI, *Sma*I and *Kpn*I sites were deleted.

Construction of ProW–PhoA fusions to amino acids 79, 99 and 122

These fusions were constructed by amplification of *proW* fragments using one primer (TAT CGA ATC AAA GCT GCC G) located upstream of the *proW* promoted and individual primers carrying *Bam*HI sites at specific fusion joints, i.e. CGC GGA TCC AAA TGG GTA ACG ACC CAG for ProW$_{79}$–PhoA, CGC GGA TCC TGC TGG AAA CCG TTG AGG for ProW$_{99}$–PhoA and CGC GGA TCC CCG GAA ATC TGC CAG for ProW$_{122}$–PhoA. Each PCR product was gel purified and cut with *Eco*O109 and *Bam*HI and ligated into the vector fragment of pProW$_{149}$–PhoA that was cut with *Eco*O109 and *Bam*HI. Constructs that carried the desired insert were verified by nucleotide sequencing of the entire *proW* coding region up to the fusion joint. The construction of fusions ProW$_{149}$–PhoA and ProW$_{209}$–PhoA will be described elsewhere (M.Haardt and E.Bremner, in preparation).

Construction of ProW–Lep(P2) fusions

ProW–Lep(P2) fusions were constructed by PCR amplification of the chromosomal *proW* gene using a common primer containing a *Xho*I site for cloning situated before the first codon of the *proW* ORF and individual primers containing *Kpn*I sites at specific fusion joints. The PCR fragments were cloned using the *Xho*I and *Kpn*I sites into an arabinose inducible vector (based on pING1) containing the *lep* gene to create in-frame fusion with amino acid 81 of Lep. The fusion joints of the ProW–Lep(P2) fusion proteins were as follows (residues introduced by the cloning procedure underlined; numbers refer to the wild-type ProW and Lep sequences): ..AWQ$_{119}$MVPY$_{81}$EP.. (ProW$_{119}$–Lep); ..WSQ$_{144}$VPY$_{81}$EP.. (ProW$_{144}$–Lep); ..IRP$_{182}$VPY$_{81}$EP.. (ProW$_{182}$–Lep).

Cloning of the proOmpA signal sequence in front of ProW

The proOmpA signal sequence (21 amino acids) was amplified by PCR using two primers both containing *Sal*I restriction sites for cloning. The *Sal*I digested PCR fragments were cloned into a *Xho*I site at the 5′ end of the ProW coding region. The fusion joint thus created was proOmpA..TVAQA↓SSMADQ..ProW (amino acids introduced by the cloning procedure underlined; the normal proOmpA signal peptidase cleavage site is indicated by the arrow).

Labelling of cells, immunoprecipitation, gel electrophoresis and Western blotting

Protein was labelled in cultures, growing exponentially (OD_{600} = 0.2) in MMA, 0.2% glucose, 1 μg/ml thiamine, 50 μg/ml ampicillin or 15 μg/ml chloramphenicol, supplemented with each common amino acid except cysteine and methionine. Cells (2 ml) were exposed to [^{35}S]methionine at 75 μCi/ml for 2 min and subsequently cooled on ice for immunoprecipitation. Immunoprecipitation and 10% SDS–PAGE were carried out as described by Ito *et al.* (1981) and Laemmli (1970), respectively. To precipitate or detect ProW–PhoA fusion proteins, SecA, or MalFΔ*Nco*I–*Pvu*II, monoclonal antibodies against PhoA (Caltag, South San Francisco) or polyclonal antibodies against the ProW N-terminus, against SecA, or against the second periplasmic domain of MalF (Traxler and Beckwith, 1992) were used, respectively. Western blotting was carried out as described (Sambook *et al.*, 1989) using cell lysates of log phase cultures. For Western blots, antibodies were diluted 1:20 000.

Assay of alkaline phosphatase

PhoA activity in strains was assayed by measuring the rate of *p*-nitrophenylphosphate hydrolysis by permeabilized cells as described earlier (Michaelis *et al.*, 1983). PhoA activity (units) is expressed as hydrolysis of μmol PNPP per min and mg of total cellular protein. To ensure reproducibility, assays were carried out at least four times.

Specific PhoA activity was calculated as folows. Pho activity of whole cells was normalized against the amount of proteins expressed (San Millan *et al.*, 1989). Protein expression was determined by immune precipitation of radiolabelled fusion protein. Incorporated label was determined by exposing dried gels to a phosphor screen for 16–24 h at room temperature. Screens were scanned on a Fuji PhosphorImager and the data were analyzed using the GelAnalyze software. In order to exclude pipetting errors, expression of a soluble cytoplasmic protein, SecA, was determined by immunoprecipitation using polyclonal antibodies against SecA.

Assay of membrane topology

Escherichia coli strains CC118 and DHB4 were transformed with plasmids encoding ProW–PhoA fusions and pDHB32 or pDHB32 Δ*Nco*I–*Pvu*II, respectively. Cells were grown in LB supplemented with either 30 μg/ml chloramphenicol or 200 μg/ml ampicillin until OD = 0.6. Cells were harvested in ice-cold buffer (40% w/v sucrose, 33 mM Tris pH 8.0) and incubated with lysozyme (50 μg/ml) and 5 mM EDTA for 30 min on ice. Aliquots of the cell suspension were incubated 1 h on ice, either with or without 0.1 mg/ml TPCK–trypsin or kallikrein. Samples were acid-precipitated (trichloroacetic acid, 10% final concentration, 10 min on ice), washed twice in cold acetone, aspirated and resuspended in 100 μl of sample buffer. Samples were subjected to SDS–PAGE and analysed by Western blotting as described above. Whole cell extracts were prepared by freeze–thaw treatment (three times) of spheroplasts using liquid nitrogen.

Escherichia coli strains transformed with the pING1 vector carrying mutant leader peptidase (*lep*) genes 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 labelled with [^{35}S]methionine (150 μCi/ml). After 1 min, non-radioactive methionine was added (500 μg/ml) and incubation was stopped by chilling on ice. Cells were spun at 16 000 *g* 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 treated with either proteinase K or trypsin. For proteinase K treatment, samples were incubated either with 0.3 mg/ml proteinase K or with 0.33 mg/ml PMSF for 1 h on ice. For trypsin treatment, samples were incubated 1 h on ice, either with 0.75 mg/ml TPCK–trypsin

or with (0.75 mg/ml TPCK-trypsin + 1.0 mg/ml trypsin inhibitor + 0.33 mg/ml PMSF). After addition of trypsin inhibitor and PMSF, samples were acid precipitated (trichloroacetic acid, 10% final concentration), resuspended in 10 mM Tris pH 7.5–2% SDS, immunoprecipitated with antisera to Lep, OmpA (an outer membrane control, not shown) and AraB (a cytoplasmic control, not shown), washed, and analyzed by SDS–PAGE and fluorography.

Determination of sec dependence

The degree of SecA dependence was determined as follows: constructs were induced with arabinose as above, and sodium azide was added (2 mM final concentration) 4 min after induction. One minute later, [^{35}S]Met was added, and after an additional 1 min incubation cells were put on ice, converted to spheroplasts, protease-treated, immunoprecipitated and analysed by SDS–PAGE as above. In parallel samples, azide treatment was omitted. For all samples, the fraction of background-corrected counts in the protease-protected fragment band *b* (protease-treated cells; Figure 3A, arrow) relative to bands *a*+*b* (where band *a* is the full-length protein in the protease-treated sample),

$$f = \frac{C_b}{C_a + C_b},$$

was calculated, and the degree of *sec* dependence was defined as $(1-f^{+az}/f^{-az})$. All quantitations were carried out on a Fuji PhosphorImager using the Gel Analyze software.

A further check for SecA dependence was performed using the SecA dilution strain MM66. Overnight cultures of MM66 transformed with the appropriate pING plasmids were grown at 30°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, and all amino acids (50 μg/ml each) except methionine. Cells were backdiluted, grown at 30°C for a further hour and then shifted to 42°C for 3.5 h to deplete SecA. Fusion protein synthesis was induced for 5 min by the addition of arabinose and then the cells were pulse labelled for 1 min and processed for protease accessibility as described above.

To check for dependence on SecY, the appropriate pING plasmid was transformed into the *secY*^{cs} strain CU164 which was then grown for 3.5 h at the permissive temperature (37°C) as above, shifted to the non-permissive temperature (23°C) and induced for Lep synthesis by addition of arabinose and incubated for an additional 5 min, pulse-labelled with [^{35}S]Met for 1 min, and processed for the protease accessibility assay as above.

To test dependence on 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 addition of [^{35}S]methionine (Daniels *et al.*, 1981; Andersson and von Heijne, 1994).

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