The Proton Motive Force, Acting on Acidic Residues, Promotes Translocation of Amino-terminal Domains of Membrane Proteins When the Hydrophobicity of the Translocation Signal is Low

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We have shown previously that the first transmembrane segment of leader peptidase can function to translocate the polar amino-terminal PF3 domain across the membrane into the periplasm independently of the proton motive force (pmf) (Lee, J. I., Kuhn, A., and Dalbey, R. E. (1992) J. Biol. Chem. 267, 938–943). We now show that when the first transmembrane segment lacks a strong hydrophobic character, the pmf is required for translocation. In addition, we find that the amino-terminal acidic residue proximal to the transmembrane domain plays a critical role in pmf-dependent amino-terminal translocation. Moreover, the pmf is required to hold the amino-terminal domain in the periplasm to prevent it from slipping such that the amino terminus is no longer exposed to the periplasm. In all cases, translocation occurs under conditions in which the function of the Sec machinery is impaired. These studies show that the low hydrophobicity of the first apolar domain (the translocation signal) can be compensated for by a negative charge in the amino-terminal region, upon which the pmf acts.

The mechanisms by which a protein integrates into the membrane and assumes its correct topology has been studied with great interest (1, 2). It has been shown that membrane proteins in bacteria can utilize the Sec machinery to assemble across the plasma membrane of Escherichia coli. However, there are a growing number of membrane proteins that apparently do not use the Sec machinery (3). This sec-independent class includes membrane proteins that are made without a signal sequence and that are oriented with the amino terminus on the periplasmic side of the plasma membrane (NoutCin orientation).

Although the exact mechanism by which these proteins assemble across the membrane is not known, the factors that influence the membrane topology have been elucidated. For example, it has been previously found that the asymmetric distribution of charged residues, particularly basic residues flanking a membrane-spanning domain on the cytoplasmic side, acts as a topogenic determinant for translocation (4–7). Positive charges are retained in the cytoplasm and inhibit translocation of polar regions (8–11). Negatively charged amino acids, in low abundance, do not inhibit translocation (11, 12). Several groups have postulated that the membrane topol-
have shown previously that the first hydrophobic domain (H1) of lep can function to translocate a short, polar amino-terminal 18 amino acid antigenic peptide from the phage Pf3 coat protein across the plasma membrane of *Escherichia coli* (28). We have now examined the energetic requirements necessary for the insertion of amino-terminal periplasmic domains and have determined that the hydrophobicity of the transmembrane domain and the pmf are both required to vary degrees.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids—E. coli* strain MC1061 (ΔaraC74, araD139, Δ[ara, leu7697, galU, galT, hsr, hsm, strA]) was from our laboratory. XL1-blue (supE44, hisD17, recA1, endA1, gyrA46, thi-1, relA1, lac-*) was acquired from Stratagene. Proteinase K was from Boehringer Mannheim. Tran32S-label, a mixture of 85% [35S]methionine and 15% [35S]cysteine, 1000 Ci/mol, was from ICN. Dideoxynucleotides were from Amersham Pharmacia Biotech. AmpliWax was from PerkinElmer. PAG3-purified oligonucleotides were from Integrated DNA Technologies and Qiagen midi kits were from Qiagen.

**DNA Manipulations—**Site-directed mutagenesis was accomplished using the Stratagene QuikChange protocol with a few alterations. The mutagenesis reaction was physically separated into two portions with AmpliWax beads. The lower portion contained 2.5 μl of the 10-fold reaction buffer, the mutagenic oligonucleotides (125 ng each), deoxyribonucleotide triphosphates (50 μM each, final concentration), and double distilled water to a final volume of 25 μl. An AmpliWax bead was placed on top, and a wax layer was created by heating and chilling the tube. The upper reagent, administered above the wax layer, contained 2.5 μl of the 10-fold reaction buffer, 50 ng of double-stranded DNA, 1 μl of *Pfu* DNA polymerase and double distilled water to a final volume of 25 μl. Mutagenesis was then carried out in a ProGene thermocycler following the QuikChange protocol. Following *Pfu* treatment and transformation, mutations were screened by sequencing double-stranded DNA (36) using U. S. Biochemical Corp. Sequenase version 2.0. Mutant constructs identified by sequencing were then transformed into MC1061 using the calcium chloride method (37).

**Protease Mapping Studies—**MC1061 cells (1 ml) bearing the pING plasmid encoding Pf3-lep proteins were grown to the midlog phase in M9 minimal media (38) containing ampicillin (100 μg/ml, final concentration) for 5 min, the samples were acid-precipitated and incubated with or without proteinase K (1.5 mg/ml) for 1 h on ice. Another aliquot of spheroplasts was treated with 2% Triton X-100 and incubated with or without proteinase K (1.5 mg/ml) for 1 h on ice, quenched with PMSF (final concentration, 2.0 μM) and converted to spheroplasts (42). To examine translocation in the absence of a pmf, CCCP was added for 45 s prior to centrifugation (16,000 × g, 4 °C, 40 s). The spheroplasts were gently resuspended in 0.25 M sucrose, 10 mM MgSO4 and incubated with or without proteinase K (1.5 mg/ml) for 1 h on ice. Another aliquot of spheroplasts was treated with 2% Triton X-100 and incubated with proteinase K (1.5 mg/ml). After quenching the protease with PMSF (5 mM, final concentration) for 5 min, the samples were acid-purified and immunoprecipitated with antibody against lep, ribulokinase, a cytoplasmic marker, and outer membrane protein A (OmpA), as described (11). Samples were then analyzed by SDS-PAGE with a 15% gel and subjected to fluorography (39).

**Quantitation of the Translocation Data—**Fluorographs were scanned using an AppleOne Scanner. The bands were then quantitated by using the public domain program NIH Image, developed at the National Institutes of Health. The percent of amino-terminal translocation was determined by taking into account the number of methionines lost during proteolysis by proteinase K. For most of the constructs there was one methionine lost out of eight (see Equations 1, 3, and 4), whereas for Δ44–9, Δ44–22, and the constructs that contain both the amino-terminal domain and the membrane-spanning domain of Pf3 coat there was one methionine lost out of seven (see Equations 2–4).

Translocated (%) = \( \frac{(processing \% - pmf \%)}{pmf \%} \times 100 \) (Eq. 1)

Processing (%) = \( \frac{8}{7} \times \frac{processed \ Pf3-lep}{unprocessed \ Pf3-lep} \) (Eq. 2)

Translocated (%) = \( \frac{8}{7} \times \frac{processed \ Pf3-lep}{unprocessed \ Pf3-lep} \times \frac{pmf \%}{pmf \%} \times 100 \) (Eq. 3)

Translocated (%) = \( \frac{8}{7} \times \frac{processed \ Pf3-lep}{pmf \%} \times \frac{pmf \%}{unprocessed \ Pf3-lep} \times \frac{pmf \%}{pmf \%} \times 100 \) (Eq. 4)

**RESULTS**

**The Role of Amino-terminal Aspartic Acids in Amino-terminal Translocation—**The amino terminus of Pf3-lep has previously been shown to translocate in a pmf- and sec-independent manner (28). This is in contrast to amino-terminal translocation of the sec-independent Pf3 coat protein (see Fig. 1A for topology), which requires the pmf (41) and the aspartyl residue at position 18 of the amino-terminal domain (16) also present in Pf3-lep. Therefore, we have investigated the role of acidic residues in amino-terminal translocation using Pf3-leader peptidase (Pf3-lep) with an F79R mutation as our model protein (Fig. 1A). This construct contains the 18 amino acid Pf3 region, from the amino terminus of Pf3 coat, fused to the fourth amino acid of leader peptidase (lep) with a threonine linking the two domains (Fig. 1B). Pf3-lep is oriented in the plasma membrane with the amino-terminal Pf3 lep domain in the periplasm, a single hydrophobic membrane-spanning domain (H1), and the carboxy-terminal lep domain in the cytoplasm (Fig. 1A). The introduction of an arginine at position 79 of lep inhibits insertion of the second transmembrane domain and translocation of the carboxyl terminus (14, 27). The resulting protein contains only one translocated region which allows us to monitor translocation of the amino terminus of Pf3-lep across the plasma membrane via protease mapping techniques.

We conducted site-directed mutagenesis on the Pf3 domain of Pf3-lep to determine whether a similar requirement for the aspartyl residues existed for efficient translocation of Pf3-lep. We replaced the aspartyl residues at positions 7 and 18 of the Pf3 domain with alanines, either as a single mutation or as a double mutation (Fig. 1B). Amino-terminal translocation was monitored by protease mapping in the absence or presence of the protophosphorylating CCCP, which destroys the pmf.

Expression of Pf3-lep in exponentially growing cells was induced with 0.2% arabinose for 1 h at 37 °C. Cells producing Pf3-lep or aspartyl residue mutants were then pulse-labeled with 100 μCi of [35S]trans-labeled methionine for 1 min and then converted to spheroplasts (42). To examine translocation in the absence of a pmf, CCCP was added for 45 s prior to labeling. Protease mapping was accomplished by dividing spheroplasts into three equal fractions. The first fraction was incubated on ice for 1 h and then trichloroacetic acid-precipitated. The second fraction was incubated on ice with 1.5 mg/ml proteinase K for 1 h, quenched with PMSF (final concentration, 5 mM), followed by trichloroacetic acid precipitation. The third fraction was treated for 1 h with both 1.5 mg/ml proteinase K and 2% Triton X-100 followed by treatment with PMSF and trichloroacetic acid precipitation. After each sample was divided into two, one-half was immunoprecipitated with anti-lep domain antibody and the other half was immunopre-
Fig. 1. Mutants used to analyze the importance of acidic residues for amino-terminal translocation. A, membrane topology of Pf3-lep and Pf3 coat in the plasma membrane of E. coli. Apolar segments are represented by cylinders, and polar segments are depicted by lines. B, mutants of Pf3-lep and their membrane translocation properties. Numbers to the right of lep indicate the position of the acidic residue within the Pf3 coat amino-terminal domain in which an aspartic acid was mutated to an alanine residue. Amino-terminal translocation was determined in the presence (−CCCP) or absence (+CCCP) of a pmf.

As shown in Fig. 2A, amino-terminal translocation was observed in Pf3-lep (lanes 1–3), 7A Pf3-lep (lanes 4–6), 18A Pf3-lep (lanes 7–9), and 7,18A Pf3-lep (lanes 10–12), in the presence (−CCCP) of a pmf. This is detected by the appearance of a smaller protease-resistant fragment seen in lanes treated with proteinase K (see arrow). Since the proteinase-treated fragments differ by only 18 amino acids, there is a slight shift in molecular weight. These results show that the acidic residues are not required for translocation of the amino terminus of Pf3-lep, in contrast to Pf3-coat protein (16). When the translocation study is carried out in the absence (+CCCP) of a pmf, amino-terminal translocation is still very efficient as seen in Fig. 2B (lanes 1–3, 4–6, 7–9, and 10–12). The total amount of translocated domain in the presence or absence of a pmf was quantitated by scanning the fluorograms and using the public domain program NIH Image to determine the percent translocated, respectively. For the full-length protein (66 and 54%, respectively). Finally, translocation is roughly half as efficient as it is in the presence of the pmf (Fig. 4A). Amino-terminal translocation was quantitated and OmpA and ribulokinase controls were also carried out as before (data not shown). Although Pf3-lep with an intact transmembrane domain translocates efficiently (99%), an effect can already be seen on the translocation of the smallest deletions, Δ17-22 (lanes 1–3) and Δ4-9 (lanes 4–6), with 93 and 91% translocating, respectively. For Δ13-22 (lanes 7–9) and Δ4-12 (lanes 10–12), translocation is roughly half as efficient as it is for the full-length protein (66 and 54%, respectively). Finally, translocation is completely abolished for the larger deletions Δ9-22 (lanes 13–15), Δ4-17 (lanes 16–18), and Δ4-22 (lanes 19–21), showing that the hydrophobicity of a downstream transmembrane domain is essential for amino-terminal translocation.

In contrast, when translocation was carried out in the absence of a pmf (CCCP treated cells), only the most hydrophobic...
charged amino acids are not required for translocation of the amino terminus of Pf3-lep with a full-length transmembrane domain (Fig. 2), we wanted to test whether translocation of the amino terminus of Pf3-lep with a truncated H1 requires the amino-terminal aspartyl residues. This is of particular interest since Δ13-22 requires the presence of a pmf for translocation, as shown in Fig. 4. We used site-directed mutagenesis to create mutations within the Pf3 domain of Δ13-22, by substituting the aspartic acid residues at positions 7 and 18 of the amino-terminal domain with alanines as before (Fig. 5A). We then conducted protease mapping experiments using cells that were pulse-labeled with trans-[35S]methionine in the presence and in the absence of the pmf to determine the competency for amino-terminal translocation. Whereas the amino-terminal domains of Δ13-22 and 7A Δ13-22 translocate with equal efficiency in the presence of a pmf, at 66 and 58%, respectively, the amino-terminal domain does not translocate in the 18A Δ13-22 mutant and the double mutant 7,18A Δ13-22 (Fig. 5B). We should note that the low molecular weight bands observed in lanes 5, 8, and 11 are uncharacterized proteolytic fragments. In the absence of the pmf, translocation of all four of these proteins is abolished (data not shown). Therefore, we found that the acidic residue proximal to the transmembrane domain is absolutely required for pmf-dependent translocation of the amino-terminal domain of Δ13-22 (Pf3-lep with a truncated transmembrane domain), in agreement with the results found for Pf3 coat (16). This suggests that the pmf is acting on the aspartyl residue at position 18 of the amino-terminal Pf3 domain of Pf3-lep to drive translocation when the hydrophobicity of the membrane-spanning domain is low.

Role of PMF in Sustaining Amino-terminal Translocation—We wanted to determine if the pmf was also required to sustain amino-terminal translocation of Pf3-lep in which the hydrophobicity of apolar domain 1 had been decreased. In this study, we characterized Pf3-lep with a full-length transmembrane segment, Δ4-9, which is dependent on the pmf for translocation, and Δ13-22, which had an even stronger defect in translocation as shown in Fig. 4. We pulse labeled a 2-ml culture for 1 min using 200 μCi of trans-[35S]methionine followed by a 5-min chase of cold methionine (500 μg/ml). One-third of the sample was then chilled, and the remaining cells were treated with 5 μl of 10 mM CCCP for 1 min. At this time, one-half of the remaining sample was then removed and chilled as above, whereas the remaining cell culture was treated with 2-mercaptoethanol to inactivate the CCCP and allow the pmf to regenerate (43). These samples were then washed to remove the 2-mercaptoethanol and incubated at 37 °C for 10 min to re-establish the pmf (see figure legend). All the samples were

FIG. 2. Protease mapping to monitor amino-terminal translocation of the Pf3-lep mutants in the presence or absence of a pmf. A, exponentially growing cells (1 ml) of MC1061 with plasmids expressing the parent Pf3-lep or Pf3-lep mutants were pulse-labeled with 100 μCi of trans-[35S]methionine for 1 min and then converted into spheroplasts as described under “Experimental Procedures.” B, for those samples treated with CCCP, 5 μl of 10 mM CCCP was added 45 s prior to adding [35S]methionine and analyzed in the exact manner as before. Aliquots of the spheroplasts (100 μl) were incubated on ice with or without proteinase K (final concentration 1.5 mg/ml) on ice for 1 h. A lysis control was included by adding proteinase K (1.5 mg/ml final concentration) and Triton X-100 (2% final concentration). Proteinase K was inactivated with 5 mM PMSF for 5 min. The samples were acid-precipitated and then immunoprecipitated with antisera to leader pep-tidase (lep), OmpA, and ribulokinase (Rib).

FIG. 3. Summary of deletions within the H1 of Pf3-lep. Amino-terminal translocation was monitored in cells with or without a pmf. The summed hydrophobicity H is also indicated.
then converted into spheroplasts and protease-mapped as described previously. We found that Pf3-lep with a full-length apolar domain 1 was unaffected by this treatment (Fig. 6A, lanes 1–9). To our surprise, we found that both D4-9 and D13-22, which had undergone amino-terminal translocation (91 and 66%, respectively) after a 5-min chase (Fig. 6, B and C, lanes 1–3; denoted as Pulse 2*CCCP), were not as accessible to protease when the pmf was abolished following pulse labeling (Fig. 6, B and C, lanes 4–6; denoted as Pulse 1*CCCP). We found that the extent to which the amino terminus had “slipped” out of the periplasmic space was correlated with the total amount of hydrophobicity in the apolar domain 1. Although full-length Pf3-lep does not “slip” out from the periplasmic space (hydrophobicity of 246 kcal/mol) when the pmf was abolished, only 62% of Δ4-9 with a total hydrophobicity of 28 kcal/mol (Fig. 3) and none of Δ13-22 with a total hydrophobicity of 25 kcal/mol remain translocated. We are convinced that this effect was due to the abolishment of the pmf because when we allow the pmf to regenerate in the presence of 2-mercaptoethanol for 10 min, translocation of the amino-terminal domains is recovered to the same extent as before and are digested by protease (81 and 50%, respectively) Fig. 6, B and C, lanes 7–9 (denoted as P/+CCCP 10’ Recover). The results indicate that when the transmembrane domain is not adequately hydrophobic, as in the cases of Δ4-9 and Δ13-22, the pmf is required not only for initiation of amino-terminal translocation but also to sustain translocation of the Pf3 region. We hypothesize that the pmf holds the translocated amino-terminal domain with the proximal acidic residue in the periplasm and therefore prevents this domain from “slipping” out from the periplasmic space.

**PMF-dependent Translocation of the Amino Terminus of Pf3-lep When H1 of Lep Is Replaced with the Transmembrane Segment of the Pf3 Coat Protein—** Although the Pf3 coat protein requires a pmf, Pf3-lep does not. One possible reason for this difference is that the transmembrane domain of Pf3 coat is less hydrophobic than that of lep. In view of our results with respect to hydrophobicity, we wanted to determine the outcome of replacing the H1 of lep, with a summed hydrophobicity of −46 kcal/mol, with that of the Pf3 coat protein, which has a hydrophobicity of −39 kcal/mol. Oligonucleotide-directed mu-
...trogenesis was used to create this mutant. The Δ4-22 Pf3-lep mutant, in which the first transmembrane domain of lep was deleted, was used as a template for mutagenesis. The insertion of the Pf3 coat transmembrane domain was completed in two rounds of mutagenesis in which nine amino acids were first inserted, followed by the insertion of the remaining nine amino acids. The completed mutant was sequenced and the trans-encoded proteins for 1 h. The cells were treated with azide for 5 min, followed by pulse labeling for 1 min, and then analyzed by protease mapping; azide has been shown to inhibit the SecA ATPase activity (44). We show that azide treatment does not affect amino-terminal translocation of either of these proteins (Fig. 8, A and B, lanes 4–6). Furthermore, translocation is not affected in SecA- or SecY- cells when grown at the non-permissive temperature, 42°C. As shown in Fig. 8A, Pf3-lep translocates its amino terminus to the same extent in cells with impaired SecA (lanes 4–6) or SecY (lanes 7–9) as in cells with functional SecA and SecY (Fig. 2A, lanes 1–3). Similar results are found with Δ13-22 (Fig. 8B, lanes 4–9). Under these experimental conditions where the function of SecA or SecY is impaired, the translocation of OmpA is completely blocked as indicated by the fact that pro-OmpA is protease-resistant. These results suggest that amino-terminal translocation occurs in a Sec-independent manner when the transmembrane segment has either high or low hydrophobicity.

**DISCUSSION**

In this report we have examined why amino-terminal translocation of some proteins, such as Pf3 coat (41) and ProW (45), requires the pmf and leader peptidase (28) does not. Our results show that when the hydrophobic character of a transmembrane segment is high, amino-terminal translocation occurs independently of the pmf. However, translocation is dependent on the pmf, which acts on a negative charge when the hydrophobic character of the transmembrane segment is low.

As a general rule, we found that amino-terminal transloca-
were analyzed for protease mapping as described in Fig. 2. For SecA ts studies, E. coli CJ105 and CJ107 are derivatives of HJM114 (F<sup>+</sup> lac pro<sup>+</sup> lac proph) and have been described (46). Cornet et al. (40) was used to determine the total hydrophobicity of the Pf3-lep mutants are plotted against the hydrophobicity of the respective H1 regions. The hydrophobicity scale of 35-S methionine for 1 min. Aliquots were analyzed for protease mapping as described in Fig. 2. For SecA<sup>ts</sup> studies, E. coli CJ105 cells bearing the plasmid encoding Pf3-lep and Δ13-22 were grown to the mid-log phase at 30 °C, shifted to 42 °C, and then labeled with 100 μCi of trans-<sup>35</sup>S methionine for 1 min. For SecY<sup>ts</sup> studies, E. coli CJ107 cells bearing the plasmid encoding Pf3 lep or Δ13-22 were grown and labeled as described for the SecA<sup>ts</sup> studies. Following pulse labeling, the cells were converted to spheroplasts and analyzed as described in Fig. 2. CJ105 and CJ107 are derivatives of HJM114 (F<sup>+</sup> lac pro<sup>+</sup> lac pro) and have been described (46). Strikingly, we observed an increased pmf requirement for translocation when the hydrophobicity of apolar domain 1 was decreased. Even the deletion of four amino acids had a noticeable effect on translocation, where translocation decreased from 99 to 91%. Almost equally surprising was the finding that amino-terminal translocation could be supported, although less efficiently, by a mutant that contained a stretch of only nine uncharged amino acids, Δ13-22. However, translocation was blocked by decreasing the hydrophobic character further. The other intriguing finding (see Fig. 9) is that to achieve approximately 50% amino-terminal translocation, a summed H1 hydrophobicity of −21 kcal/mol is required in the presence of the pmf, compared with a summed hydrophobicity of −32 kcal/mol in the absence of the pmf. This suggests that the pmf contributes energy that is equivalent to −111 kcal/mol of hydrophobicity, i.e. four leucine residues.

Our results also demonstrate that the amino-terminal aspartyl residue flanking the transmembrane segment is essential for pmf-dependent translocation of Δ13-22. This confirms that aspartic residues can play an active role in amino-terminal translocation of its amino terminus across the membrane independently of the pmf. This may explain the pmf requirement of Pf3 coat (16) since the Pf3 coat has a transmembrane segment that is less hydrophobic than the first transmembrane segment of lep. Indeed, we observed pmf-dependent translocation of the amino terminus of Pf3-lep when H1 of lep was replaced with the transmembrane segment of the Pf3 coat protein. The pmf dependence was even more dramatic when the H2 and H3 domains of lep were deleted preventing them from aiding translocation of the amino-terminal domain (Fig. 7).

Fig. 9 illustrates the results in which the translocation data of the Pf3-lep mutants are plotted against the hydrophobicity of the respective H1 regions. The hydrophobicity scale of 35-S methionine for 1 min. Aliquots were analyzed for protease mapping as described in Fig. 2. For SecA<sup>ts</sup> studies, E. coli CJ105 cells bearing the plasmid encoding Pf3-lep and Δ13-22 were grown to the mid-log phase at 30 °C, shifted to 42 °C, and then labeled with 100 μCi of trans-<sup>35</sup>S methionine for 1 min. For SecY<sup>ts</sup> studies, E. coli CJ107 cells bearing the plasmid encoding Pf3 lep or Δ13-22 were grown and labeled as described for the SecA<sup>ts</sup> studies. Following pulse labeling, the cells were converted to spheroplasts and analyzed as described in Fig. 2. CJ105 and CJ107 are derivatives of HJM114 (F<sup>+</sup> lac pro<sup>+</sup> lac pro) and have been described (46). Strikingly, we observed an increased pmf requirement for translocation when the hydrophobicity of apolar domain 1 was decreased (Figs. 3 and 4). The only mutant that was able to translocate in a pmf-independent manner, Δ17-22, had a very small deletion in the first transmembrane domain. This suggests that it is the overall high hydrophobic content of transmembrane segment 1 of the native leader peptidase that enables translocation of its amino terminus across the membrane independently of the pmf. This may explain the pmf requirement of Pf3 coat (16) since the Pf3 coat has a transmembrane segment that is less hydrophobic than the first transmembrane segment of lep. Indeed, we observed pmf-dependent translocation of the amino terminus of Pf3-lep when H1 of lep was replaced with the transmembrane segment of the Pf3 coat protein. The pmf dependence was even more dramatic when the H2 and H3 domains of lep were deleted preventing them from aiding translocation of the amino-terminal domain (Fig. 7).

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translocation as previously suggested (16). It is striking that the pmf is not only required for initiation of translocation but also to stabilize the amino-terminal domain of Δ4-9 and Δ13-22 in the periplasm to prevent it from slipping away from the periplasmic space so that it is not accessible to protease (Fig. 6, B and C). In contrast, slipping is not observed for Pf3-lep which has a transmembrane segment with high hydrophilicity (Fig. 6A). How the pmf holds the amino-terminal domain on the periplasmic side of the inner membrane is not known at this time. Although it is possible that the amino-terminal polar domain is merely slipping into the membrane bilayer, we think that this would be unlikely because it would be energetically unfavorable for the polar domain to reside within the apolar membrane bilayer.

One possible role of a pmf is that it affects translocation, via direct interaction of the negatively charged residues of the inserting membrane protein, with its electrical potential component (positive outside, negative inside). Our data do not directly address this possibility. However, if such a simple electrophoretic mechanism was operating in E. coli it does not explain why the acidic residue at position 18 would be required and not the one at position 7. A second possibility is that the pmf acts via a transmembrane pH gradient. If this were the case, a protein machinery, which protonates and deprotonates amino acid residues, would most likely be involved.

In conclusion, the present data provide strong evidence that the pmf, acting on an acidic residue, can compensate for the low hydrophobicity of the translocation signal. This indicates that hydrophobic forces and proton motive forces work together in the translocation process.

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