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The Protein Translocation Apparatus Contributes to Determining the Topology of an Integral Membrane Protein in *Escherichia coli**

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The assembly of integral membrane proteins is determined by features of these proteins and the protein translocation apparatus. We used alkaline phosphatase fusions to the membrane protein MalF to investigate the role of the protein translocation machinery in the arrangement of proteins in the cytoplasmic membrane of *Escherichia coli*. In particular, we studied the effects of *prlA* mutations on membrane protein topology. These mutations lie in the *secY* gene, which encodes a core component of the protein translocation apparatus. We find that the topology of some of the fusion proteins is changed and, in one case, is completely inverted in *prlA* mutants. We discuss the mechanism of *prlA*-mediated export and the role of the protein translocation apparatus in contributing to membrane protein topology.

Assembly of cytoplasmic membrane proteins depends on features of the protein itself and on the cell's protein translocation machinery. Long hydrophobic stretches in membrane proteins, averaging around 20 amino acids, can act as export signals promoting the translocation of hydrophilic domains across the membrane. These stretches themselves remain embedded in the membrane, acting as anchors and sometimes contributing to the protein's function. Such hydrophobic stretches also act as stop transfer sequences when they follow a hydrophilic domain that has been translocated across the membrane. Thus, these transmembrane sequences can be oriented with either their amino terminus or their carboxyl terminus in the cytoplasm.

Features of transmembrane proteins that determine their membrane topology include 1) basic amino acids in hydrophilic domains that result in a cytoplasmic location for that domain (1–3); 2) amphipathic helices in hydrophilic domains that may also contribute to anchoring those domains in the cytoplasm (4); 3) rapid folding of a hydrophilic domains (folding in the cytoplasm may prevent export of a domain, while folding of a domain in the periplasm may ensure the location of that domain (5, 6)); 4) salt bridges (or other linkages) between transmembrane segments may maintain their transmembrane configuration (7); and 5) the lipid composition of the membrane may influence topology.[1]

The efficient assembly of many membrane proteins also depends on the cell's protein translocation machinery. The assembly of the *E. coli* leader peptidase into the membrane and the translocation of a large hydrophilic domain of the cytoplasmic membrane protein, MalF, are defective in *sec* mutants that alter the translocation machinery (8, 9). For MalF, effects on assembly occur only when the defects in the secretion apparatus are severe. This stringent requirement for *sec* defects may be due to high affinity of the very hydrophobic transmembrane stretches of MalF for the secretory apparatus compared with much shorter hydrophobic regions of cleavable signal sequences (9).

This dependence for membrane protein assembly on *sec* gene products has led us to examine further the effects of mutant *sec* genes on this process. In particular, we have studied the *prlA* mutations of *E. coli* that lie in the *secY* gene, encoding a core membrane component of the bacterial protein translocation apparatus. The *prlA* mutations alter SecY so as to allow the export of proteins with defective signal sequences. In *prlA* mutants, alkaline phosphatase (AP)[2] carrying point mutations or a complete deletion of the signal sequence can be exported relatively efficiently (10). Moreover, *prlA* mutations allow the export of AP when it is fused to the cytoplasmic domains of membrane proteins (11–14). We were interested in studying the mechanism that allows AP fused to a cytoplasmic domain of a membrane protein to be translocated across the membrane in *prlA* strains.

Here we show, using a set of fusions of alkaline phosphatase to cytoplasmic domains of the MalF protein, that the topology of a number of these fusions is altered in *prlA* mutants. Further, we present evidence that, in one case, *prlA* mutations result in the inversion of the topology of a membrane protein. These results are discussed both in terms of the role of the Sec proteins in contributing to the topology of membrane proteins and the mechanism of *prlA*-mediated export.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strains and plasmids are listed in Table I. Media are as described in Ref. 15.

Screen for Mutants That Increase the AP Activity of the MalF-AP M Fusion—DHB5181 was mutagenized with nitrosoguanidine as in Ref. 15. After mutagenesis, cells were plated on NZ-amine-A plates containing 40 μg/ml 5-bromo-3-chloro-3-indolyl phosphate (a chromogenic substrate for AP). Plates were incubated for 1 day at 37 °C and 1 day at 4 °C, and dark blue colonies were picked. Elevated AP activity in the mutants was assayed. The mutants were mapped by P1 cotransduction.

Mutants mapping near *secY* were tested in two ways to see if they

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[1] W. Dowhan, personal communication.

[2] The abbreviations used are: AP, alkaline phosphatase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; IPTG, isopropyl-thio-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; MSS, membrane-spanning segment.

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TABLE I
Plasmids and strains used in this study

Plasmids	Description	Reference
pDHB519	*malF*-Tn*pho*A fusion B under *tac* promoter	21
pDHB5043	*malF*-Tn*pho*A fusion C under *tac* promoter	21
pDHB5045	*malF*-Tn*pho*A fusion D under *tac* promoter	21
pDHB551	*malF*-Tn*pho*A fusion J under *tac* promoter	21
pDHB5069	*malF*-Tn*pho*A fusion M under *tac* promoter	21
pDHB5066	*malF*-Tn*pho*A fusion O under *tac* promoter	21
pDHB5065	*malF*-Tn*pho*A fusion P under *tac* promoter	21
pME2099	*malF*-Tn*pho*A fusion C△1 under *tac* promoter	23
pME2100	*malF*-Tn*pho*A fusion D△1 under *tac* promoter	23
pAID135	*phoA△2–22* under *tac* promoter	10
pl^Q/Cm	pACYC derivative containing $lacl^Q$	10
Strains	**Genotype**	**Source**
DHB4	F′ *lac-pro* $lacl^Q$*/l△(ara-leu)*7697 *araD*139 △*lac*×74 *galE galK rpsL phoR △(phoA)Pvu*ll △malF3 thi	Laboratory collection
DHB5181	DHB4 *recA*::Cm *malF*-TnphoA fusion M[a]	Laboratory collection
DHB5215	DHB4 *recA*::Cm *malF*-Tn*phoA* fusion L[a]	Laboratory collection
WP144	F′ △*lac*(U169) *malE18–1*. . . .Tn5	Laboratory collection
WP148	DHB5181, mutation in *malF*-Tn*pho*A fusion	This study
WP149	DHB5181 *prlA149*	This study
WP702	DHB4 pl^Q/Cm	This study
WP703	WP702 *prlA*. . . .Tn*10*	This study
WP704	WP702 *secB*::Tn*5*	This study
WP705	WP703 *secB*::Tn*5*	This study

[a] Integrated onto the chromosome at *phoA*.

contained *prlA* mutations. The mutants' ability to export MalE18–1 (a version of MalE with a defective signal sequence) was assessed by transducing *malE18–1* into the mutants from WP144 and plating on maltose tetrazolium medium (15); *malE18–1*, *prlA* mutants are white on this medium; $prlA^+$ cells are red. The mutants' ability to export APΔ2–22 (a signal sequenceless version of AP) was assessed by transforming the mutants with pAID135 and plating on media containing 40 μg/ml 5-bromo-3-chloro-3-indolyl phosphate and 5 mM isopropyl-thio-β-D-thiogalactopyranoside (IPTG) to induce production of APΔ2–22; *prlA* mutants are dark blue on these plates, while $prlA^+$ cells are light blue.

Alkaline Phosphatase Assays—Cells were grown in NZ-amine-A plus 200 μg/ml ampicillin to an optical density at 600 nm of approximately 0.4. The production of the MalF-AP fusions was induced for 30 min by the addition of IPTG at a final concentration of 5 mM. The cells were harvested, and alkaline phosphatase activity was assayed in duplicate as in Ref. 10 with less than 5% variation.

Proteolysis of the MalF-AP Fusions—1.5-ml cultures were grown at 37 °C in M63 minimal medium containing 0.2% glucose, 50 μg/ml each of all amino acids except cysteine and methionine, and 5 mM IPTG. After growth to an optical density of approximately 0.4 at 600 nm, the cultures were labeled for 1 min with 45 μCi/ml [^{35}S]methionine and chased with an excess of cold methionine for 30 min. 1 ml of cells was placed at 0 °C for 20 min, pelleted, and resuspended in cold spheroplast buffer containing 40% sucrose, 33 mM Tris, pH 8, 2.5 mM EDTA, and 5 μg/ml lysozyme. After 15 min at 0 °C, the spheroplasts were divided into 0.5-ml portions that were either left untreated or proteolyzed with proteinase K at a final concentration of 500 μg/ml for 20 min at 0 °C. Proteolysis was stopped with the addition of phenylmethylsulfonyl fluoride at 0.4 μg/ml. The spheroplasts were separated from cell envelope proteins by pelleting (7 min at 14,000 rpm in a microcentrifuge at 4 °C), resuspended in spheroplast buffer, and disrupted by freezing and thawing three times. Immunoprecipitation with antisera against AP and glucose-6-phosphate dehydrogenase, SDS-PAGE, and autoradiography were performed as described (16).

Urea Extraction—Cultures were grown in MOPS minimal medium (17) at 37 °C with 0.2% glucose and a 50 μg/ml concentration each of all amino acids except cysteine and methionine. They were induced with 0.04 mM IPTG for 15 min, pulse-labeled with 45 μCi/ml [^{35}S]methionine for 1 min, and chased for 2 min with an excess of cold methionine. Fractions of 0.5 ml were put on ice, and the following was added: 1 ml of 8 M urea, 0.2 ml of 1 M iodoacetamide, 0.02 ml of 0.5 M EDTA, and 0.02 ml of 0.1 M phenylmethylsulfonyl fluoride. The samples were then incubated on ice for 10 min and centrifuged at 14,000 rpm in a microcentrifuge at 4 °C for 15 min. The supernatant was taken as the urea-extractable fraction. The pellet was taken up in an equal volume of a urea solution of the same composition as the supernatant. The fractions were made 10% in trichloroacetic acid, incubated 15 min on ice, and then centrifuged. The supernatants were discarded, and the pellets were washed twice with cold 100% acetone. Immunoprecipitation was carried out as described (18). Samples were released from IGSorb by suspension in 0.05 ml of SDS sample buffer without 2-mercaptoethanol and separated into two fractions. One fraction was reduced by incubation at 80 °C for 5 min with 1⁄10 volume of 0.2 M dithiothreitol, and then 1⁄5 volume of 1 M iodoacetamide was added to each fraction followed by a 5-min incubation at 80 °C. Equal volumes of each fraction were loaded on SDS-PAGE gels.

Kinetics of DΔ1 Export—Cultures were grown in MOPS medium as described above, induced with 0.1 mM IPTG for 15 min, and pulse-labeled as described above. At 1, 2, 4, 8, and 12 min of chase, samples were removed to tubes on ice with 1⁄100 volume of 1 M iodoacetamide. After at least 5 min on ice, trichloroacetic acid precipitation, immunoprecipitation, sample preparation, and SDS-PAGE were carried out as described above.

RESULTS

prlA-mediated Export of Alkaline Phosphatase in MalF-AP Fusions—Initially, we set out to study the mechanism whereby basic amino acids act to anchor the cytoplasmic domains of membrane proteins. To do this, we sought suppressor mutations that would reduce the anchoring activity of these amino acids and allow export of a cytoplasmic domain of a membrane protein. Such suppressors might be expected to alter the mechanism that responds to the presence of the basic amino acids. Their analysis, thus, might yield insights into this mechanism.

We employed a strain in which alkaline phosphatase is fused to a cytoplasmic domain of MalF (the M fusion, Fig. 1). The AP portion of this fusion is stably anchored in the cytoplasm by the basic aminoacyl residues in the MalF cytoplasmic domain that precedes it. In the cytoplasm, AP does not assemble into an active enzyme, since the the two essential intrachain disulfide bonds in AP cannot form (19, 20). Thus, when AP is fused to cytoplasmic domains of membrane proteins, it exhibits low enzymatic activity (21). In contrast, when AP is fused to a periplasmic domain of a membrane protein, it is exported to the periplasm and becomes enzymatically active. Selecting mutants that increase AP activity of the M fusion should yield strains in which the AP is exported to the periplasmic space. Such mutants could include those that no longer recognize the basic amino acids as signals to anchor the AP in the cytoplasm. We chose the M fusion because we have previously shown that eliminating the basic aminoacyl residues in the MalF cytoplas-

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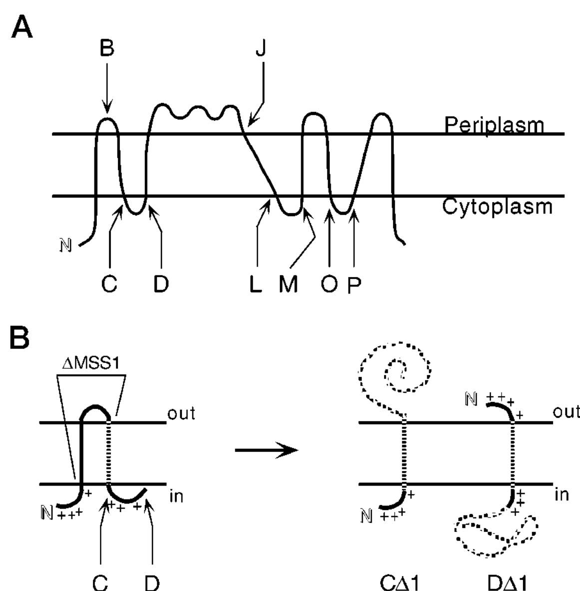

FIG. 1. **MalF-AP fusions used in the study.** *A*, location of AP fusions in MalF generated by Tn*phoA* hops into *malF* (21). *B*, representation of topology of the CΔ1 and DΔ1 fusions and how they were derived from the C and D fusions (23). The *spotted line* represents alkaline phosphatase, and the *dashed line* represents the second MSS of MalF. The second cytoplasmic domain of MalF is apparently a stronger cytoplasmic localization signal than the first cytoplasmic domain despite containing fewer positively charged residues (23).

mic domain that precedes the AP portion of this fusion results in increased export of AP (22).

After mutagenesis with nitrosoguanidine, we detected 44 mutant colonies (at a frequency of 5×10^{-5}) that exhibited increased alkaline phosphatase activity (see "Experimental Procedures"). P1 cotransduction experiments showed that 24 of 41 mutants tested carried suppressor mutations unlinked to the gene fusion. The remainder were due to alterations within the gene fusion that increased alkaline phosphatase activity. The unlinked suppressors were further mapped and all shown to lie in or close to the *secY* gene. Since it had already been shown that *prlA* mutations can promote the export of cytoplasmic alkaline phosphatase in membrane protein fusions (11–14), we guessed that our set of mutations might also be *prlA* mutations. To test this explanation, we examined the effect of the suppressor mutations on the export of two proteins with defective signal sequences, the maltose-binding protein (MalE) with the signal sequence alteration 18–1 (M18R) and AP missing its entire signal sequence. All of the *secY*-linked mutants restored export to these proteins (data not shown), suggesting that they contained typical *prlA* mutations that suppress a variety of alterations of signal sequences. Thus, the effects of these mutations were not limited to reversing the interference with export by basic amino acids, as we had hoped.

To further study the suppression by *prlA* mutants, we transferred the following MalF-AP fusion constructs into the *prlA4* mutant background: B, C, D, J, M, O, P, CΔ1, and DΔ1 (Fig. 1). For fusions C, M, O, P, and DΔ1, we found a significant increase in alkaline phosphatase activity, indicating export of AP to the periplasmic space (Table II). The absence of any increase for all of the remaining fusions, except for D, was expected, since the alkaline phosphatase is already efficiently exported to the periplasm. The D fusion, on the other hand, allows little export of alkaline phosphatase and is unaffected by the *prlA* mutation. This refractory behavior may be due to the strength of the cytoplasmic anchoring signal in this fusion, to the stabilization of the topology resulting from interactions between the two transmembrane segments, or to a combination of both.

TABLE II
AP activity of MalF-AP fusions expressed in $prlA^+$ and prlA4 cells

Fusion[a]	AP activity	
	prlA$^+$	*prlA4*
B	780	1200
C	21	120
D	13	16
J	620	770
M[b]	2.2	10
O	61	140
P	8.0	26
CΔ1	1000	1100
DΔ1	140	880

[a] Expressed from plasmids.
[b] Expressed from chromosome.

Does a prlA Mutant Alter the Topology of the MalF-AP Fusion Protein or Does It Export Cleaved AP?—We considered two explanations for the export of the AP moiety of the MalF-AP M fusion protein in *prlA* strains (Fig. 2). First, the *prlA* mutation may result in a change in the topology of the membrane protein so that AP, still attached to MalF, is now in the periplasm rather than the cytoplasm (Fig. 2*B*). Alternatively, the AP moiety in the cytoplasm may be cleaved from the fusion protein, and then the cleaved AP, lacking any export signal, is exported by the altered secretion machinery (Fig. 2*A*). This latter explanation represents a reasonable alternative, since 1) many of the MalF-AP fusion proteins are known to be unstable (21) and 2) AP without a signal sequence is exported in certain *prlA* mutants (10).

To distinguish between these two possibilities, we examined the form of AP in the strain expressing the M fusion suppressed by a *prlA* mutation. SDS-PAGE demonstrated that the M fusion protein was still intact in the *prlA* mutant background, and very little free AP could be detected (Fig. 3). When spheroplasts of this strain were treated with protease, there was a large increase in the amount of free soluble AP-sized breakdown product, indicating that the AP portion of the full-length fusion protein was in the periplasm. Such a product was not seen in the *prlA*$^+$ (wild-type) background. These results show that it is not cleaved cytoplasmic AP that is exported and, therefore, at least some aspects of the topological arrangement of the M fusion protein have been altered in the *prlA* mutant (Fig. 2*B*). Since the AP portion of the L fusion (Fig. 1) is exported to the periplasm in *prlA*$^+$ cells (16, 21), a strain expressing this fusion was included in Fig. 3 as a positive control.

We wished to determine whether the altered protein translocation machinery in the *prlA* strain had affected the membrane topology of the entire MalF-AP M fusion protein or only affected the region where AP is fused. However, due to technical difficulties in assessing the topology of the M fusion strain, we studied alterations in topology of a simpler fusion protein (see below).

Reversal of the Topology of a Membrane Protein Promoted by the prlA-altered Secretion Machinery—For these studies, we chose the MalF-AP DΔ1 fusion protein (Fig. 1). The D fusion protein has AP fused to the second cytoplasmic domain of MalF and exhibits very low AP activity (Table II). The DΔ1 fusion protein was derived from the D fusion by the deletion of the first membrane-spanning segment of MalF (MSS1) and the short periplasmic domain that follows it (Fig. 1*B*; Ref. 23). The construct was made in such a way that MSS2 is now bounded by two hydrophilic domains that correspond to the first two cytoplasmic domains of MalF. The second cytoplasmic domain is then followed by AP. Both of these hydrophilic domains are enriched for basic amino acids. Since each of these domains could, in principle, act as a cytoplasmic anchor, it was not

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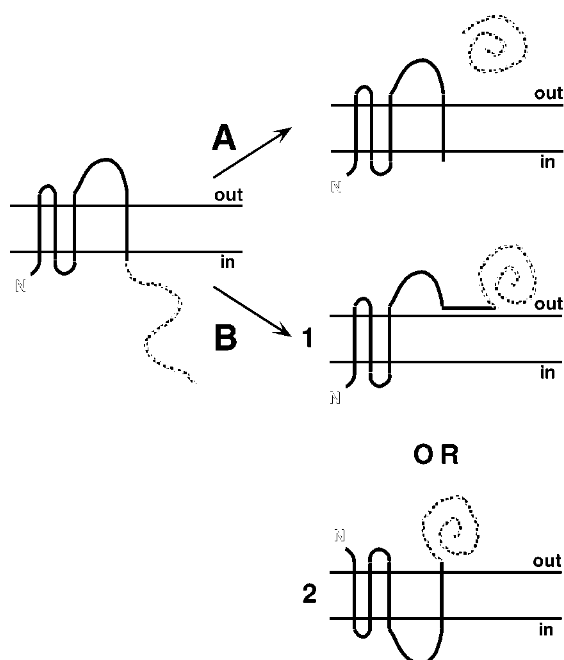

FIG. 2. **Two possible mechanisms of *prlA*-mediated suppression of the M fusion.** *A*, the AP (*spotted line*) is cleaved from the rest of the fusion (*solid line*) in the cytoplasm and then exported to the periplasm. *B*, AP is exported to the periplasm as part of an intact fusion protein. The fourth MSS of the fusion protein may be exported to the periplasm (*B1*), or the topology of the entire fusion protein may be inverted (*B2*).

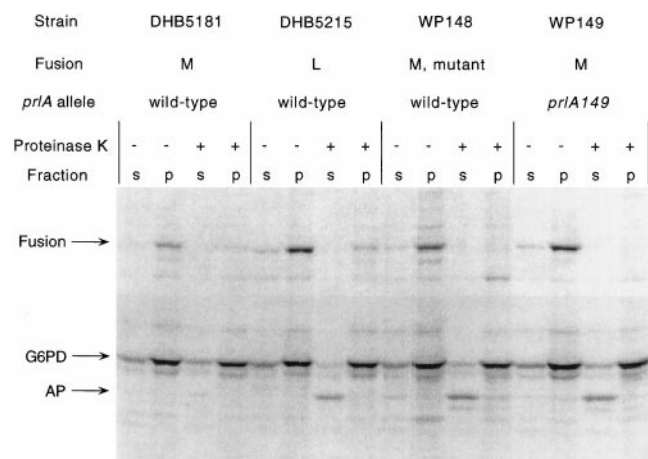

FIG. 3. **The topology of the M fusion is altered in a *prlA* mutant.** Pulse-labeled spheroplasts (*p*) were separated from soluble proteins (*s*) and either left untreated (−) or treated (+) with proteinase K. Glucose-6-phosphate dehydrogenase (*G6PD*) and AP were immunoprecipitated and separated with SDS-PAGE. WP148 and WP149 are two of the mutants that were isolated in the screen described under "Experimental Procedures." WP148 expresses a mutant version of the M fusion, which has increased AP activity.

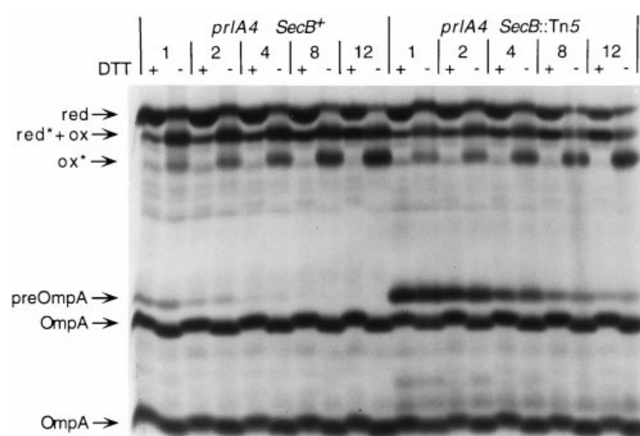

FIG. 4. **The AP portion of DΔ1 is rapidly exported to the periplasm.** Both *prlA4 secB*$^{+}$ and *prlA4 secB*::Tn*5* cells expressing DΔ1 were pulse-labeled and chased for the indicated number of minutes. Samples were divided in half, either left untreated (−) or reduced with dithiothreitol (+), and immunoprecipitated as indicated under "Experimental Procedures." The locations of oxidized (*ox*) and reduced (*red*) full-length DΔ1 and oxidized (*ox**) and reduced (*red**) breakdown product of DΔ1 are shown.

immediately obvious what the topology of this protein would be. However, analysis of the protein showed 1) that the AP moiety is in the cytoplasm, thus exhibiting very low enzymatic activity and 2) that the amino terminus of the fusion protein is exposed to proteolytic attack on the periplasmic surface of the cytoplasmic membrane (23). These findings led to the conclusion that the DΔ1 fusion protein has the topology indicated in Fig. 1*B*. We proposed that the net positive charge in the second cytoplasmic domain predominated in orienting the protein in the membrane (23).

In the presence of the *prlA4* mutation, a strain bearing the DΔ1 fusion protein now exhibits high levels of AP activity (Table II). We asked whether the exported AP in this strain was still attached to the membrane protein or was the result of export of the AP moiety after cytoplasmic cleavage. This strain showed an added complexity in that, as we describe below, the exported AP still attached to MSS2 was slowly cleaved in the periplasm to release free soluble AP. This property made it difficult to evaluate the mechanism of export of DΔ1 using the same technique that we used to assess the M fusion. Instead, we made use of the fact that AP can only form disulfide bonds when it is in the periplasm but not in the cytoplasm. These disulfide bonds provide an accurate measure of the cellular location of AP.

Experiments on the kinetics of export of AP as assayed by disulfide bond formation are presented in Fig. 4. There, we show 1) that the initial export of AP is very rapid, with a substantial proportion of the protein becoming oxidized (disulfide bonded) after a 1-min pulse-label and 1-min chase and 2) that the disulfide-bonded AP is still attached to MSS2. Thus, as with the M fusion, the export of the AP in the DΔ1 fusion is not due to export of an AP moiety that was cleaved from the fusion protein in the cytoplasm. However, it is also clear that the exported AP is slowly cleaved in the periplasm from the fusion protein. The cleaved material is marked with an *asterisk* in Fig. 4. Note that the reduced breakdown product and oxidized full-length protein run with the same mobility. In addition, two forms of mature OmpA are seen, because a fraction of this protein retained some secondary structure.

We were interested in determining the topological arrangement of the DΔ1 fusion in the *prlA* background. We imagined two possible topologies for this protein that would depend on the mechanism of AP export (Fig. 5). In the first, the fusion protein initially inserts into the membrane in the same way as in a wild-type background, but the altered protein translocation machinery promotes export of AP. This export might lead to the structure depicted in Fig. 5*A*, in which both amino terminus and carboxyl terminus of the hybrid protein are on the periplasmic face of the membrane. Such a protein would not be expected to be stably inserted in the membrane. In the second, the fusion protein, before any interaction with the membrane, is recognized by the *prlA*-altered secretion machinery differently than in the wild-type situation so that MSS2 is seen as an export signal allowing translocation of the AP moiety that follows it. The DΔ1 protein, in this case, would end up

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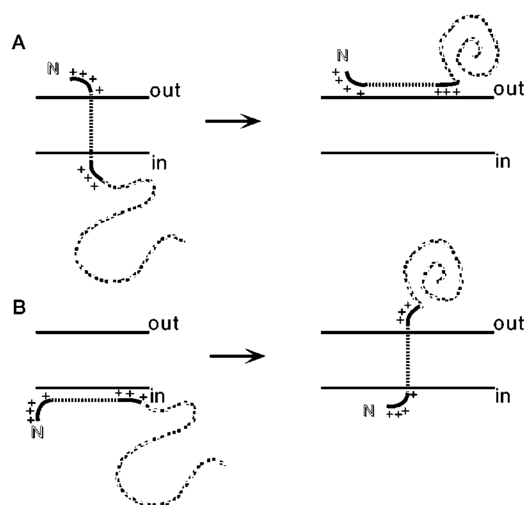

FIG. 5. **Two models of *prlA*-mediate export of DΔ1.** The DΔ1 fusion is represented as in Fig. 1*B*. *A*, DΔ1 inserts into the membrane before being engaged by the Sec machinery and is subsequently exported completely out of the cytoplasm. *B*, DΔ1 does not insert into the membrane before being engaged by the Sec apparatus.

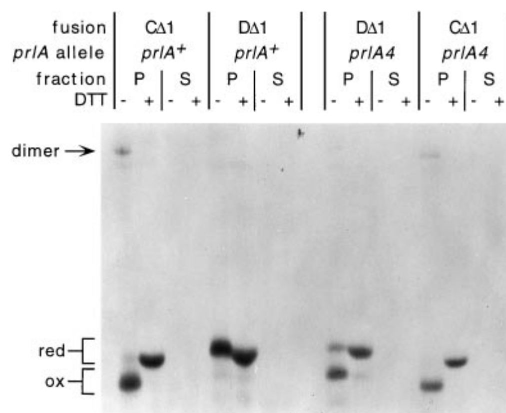

FIG. 6. **The DΔ1 fusion is an integral membrane protein in *prlA* mutants.** Urea extraction of pulse-labeled *prlA*$^+$ and *prlA4* cells producing either the CΔ1 or DΔ1 fusion was performed as described under "Experimental Procedures." The urea-extractable material (*S*) was separated from the nonextractable protein (*P*), either left unreduced (−) or reduced (+) with dithiothreitol, and separated by SDS-PAGE as described under "Experimental Procedures." The locations of the reduced (*red*) and oxidized (*ox*) fusion protein are shown. The location of what may be a dimer of fusion protein is also shown.

inserted in the membrane with the opposite topology from that which it exhibits in a wild-type background (Fig. 5*B*).

To distinguish between these possibilities, we have used urea extraction to determine whether the fusion protein is stably anchored in the membrane. It has been shown that urea treatment of membranes does not release proteins that are stably integrated membrane proteins, but rather it will cause release of a protein that is only weakly inserted (24). The DΔ1 fusion protein in the *prlA* background is resistant to urea extraction, suggesting that it is stably anchored in the membrane via the full insertion of its MSS in the membrane (Fig. 6). Control experiments showed that the conditions we used extracted 97% of wild-type AP, while OmpA was resistant to extraction (data not shown). Longer exposures of the gel in Fig. 6 revealed small amounts of AP-sized breakdown product in the supernatant of the *prlA4* strain expressing the DΔ1 fusion. This probably reflects the slow cleavage of AP from DΔ1 after DΔ1 is inserted into the membrane (Fig. 4). Other fractionation methods, including extraction of spheroplasts or membranes with urea or extraction with 0.1 N NaOH gave similar results; all full-length fusion protein was associated with the membrane fraction. Extraction with octyl glucoside, on the other hand, solubilized about half of the full-length fusion protein, as is seen with other integral membrane proteins under similar conditions (data not shown).

SecB Dependence of Inversion of Membrane Protein Topology—SecB is a chaperone required for the efficient export of certain secreted proteins. AP is not one of these proteins (25). However, in a *prlA* strain that exports AP lacking its signal sequence, the export *is* SecB-dependent (10). This change in SecB dependence is thought to be due to the slower post-translational export of alkaline phosphatase that occurs in *prlA*-suppressed signal sequence mutants. We asked whether, in a *prlA4* mutant, the translocation of the AP portion of the M and DΔ1 fusions is SecB-dependent. Whereas a *secB*$^+$ *prlA4* strain expressing the M fusion has 10 units of AP activity (Table II), an isogenic *secB* strain has only 1.7 units, indicating that export of the AP moiety of the M fusion requires SecB. In contrast, the translocation of AP in the DΔ1 fusion is largely independent of SecB (Fig. 4). The kinetics of OmpA export are slowed in the cells missing SecB (Fig. 4).

DISCUSSION

Membrane Protein Topology and the Sec Machinery—Our results show that the topology of a membrane protein can be altered by mutations that alter the bacterial protein export machinery. The AP moiety of the MalF-AP M and DΔ1 fusions is localized to the cytoplasm in wild-type cells and the periplasm in *prlA* mutants. For both fusions, the export of AP caused by the *prlA* mutation was not due to cleavage of AP from the hybrid protein in the cytoplasm and subsequent translocation of the free signal sequenceless AP; the exported AP is still part of the hybrid protein. Thus, in both cases, the arrangement of the hybrid protein in the membrane of *prlA* mutants must be at least partly altered from the arrangement it assumes in wild-type cells.

In the case of DΔ1, our results suggest that the topology of the protein has been completely and efficiently inverted. A transmembrane segment that in the wild-type protein and in the DΔ1 fusion acts as a stop-transfer sequence now appears to be acting as an export signal.

These results suggest that the protein translocation apparatus contributes to determining the topology of the DΔ1 fusion; the *prlA*-altered Sec machinery may be unable to recognize some of the topogenic determinants in DΔ1. The mechanism of *prlA*-mediated export of DΔ1 is discussed below.

An alternative explanation of our results is that the DΔ1 fusion is targeted for export in *prlA* mutants by its AP moiety, since the mature domain of AP is known to contain targeting information that allows it to be exported in *prlA* mutants (10, 26). However, there is a noticeable difference in the kinetics of *prlA*-mediated export of DΔ1 (Fig. 4) and that of AP with an altered or missing signal sequence. In the latter case, the export is quite slow, with half-times of export of about 5 min. In contrast, the AP moiety of the DΔ1 fusion is translocated quite rapidly, with about 50% being localized to the periplasm within a minute. Thus, it seems unlikely that the AP portion of DΔ1 is responsible for determining the topology of this fusion in *prlA* mutants.

These findings raise the question of the role of the Sec machinery in the topological arrangement of membrane proteins under wild-type conditions. In most cases, the protein translocation machinery, if it is involved, would be only one of the several factors that contribute to the assumption and stability of membrane protein topology we have outlined in the intro-

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duction. Thus, alteration of this machinery by *prlA* mutations may not affect topology for most proteins. However, it could be that for membrane proteins of simple structure, for instance with a single transmembrane segment, some confusion of signals could take place in *prlA* strains, leading to a mixed population of topological structures. An effect of the altered secretion machinery on topology would then suggest that this machinery may be a contributor to the orientation of transmembrane segments of membrane proteins, in general. Such experiments may be limited by the folding properties of the normal cytoplasmic domains of these proteins.

How Does the prlA Alteration of the Export Machinery Promote Export of AP in a Protein Such as DΔ1?—To consider this question, we take into account studies with the *E. coli* cytoplasmic membrane protein leader peptidase, which suggest the following properties of the assembly system for membrane proteins (27). When a hydrophilic domain of a membrane protein is less than 25 amino acids, the translocation of the domain across the membrane does not depend on the *sec* gene products. However, for domains larger than 25 amino acids, translocation does require the bacterial export machinery. Such long hydrophilic domains simply cannot pass through the lipid bilayer.

A feature of the *sec* machinery that is also relevant to this discussion is the effect of basic amino acids on the exportability of proteins with signal sequences. Mutations that alter the amino acid sequence at the beginning of the mature protein to yield a net positive charge cause a defect in export (28–30). But these altered proteins can be exported in strains carrying *prlA* mutations. The altered secretion machinery ignores the ordinarily inhibiting effect of the basic amino acids. This effect is not specific to the mechanism by which basic amino acids block export, since these *prlA* mutations also suppress mutants with altered or missing signal sequences (10). We have proposed that *prlA* mutants alter the export machinery so that it no longer requires a signal sequence-induced change in conformation and now allows export of any unfolded protein (26).

To explain the export of DΔ1, we picture the following steps in its assembly in a wild-type background. The positive charges at the carboxyl terminus of the hydrophobic MSS prevents DΔ1 from being engaged by the export machinery or being properly oriented in the machinery. Instead, this hydrophobic sequence inserts into the lipid bilayer as a result of its natural affinity for the membrane. Ordinarily, the orientation of this MSS is determined by the "positive-inside rule," whereby positively charged hydrophilic domains of membrane proteins tend to be localized to the cytoplasm. However, in DΔ1, there are positively charged residues at both ends of the MSS. Our previous studies have shown that the N terminus of DΔ1 is translocated into the periplasm, despite its net positive charge (23). This translocation may not be *sec*-dependent, since it has been suggested that N-terminal export of this sort does not utilize the *sec* machinery (31, 32).

What Happens to DΔ1 in the prlA Mutant Strains?—Two possibilities are as follows.

1) The *prlA*-altered secretion machinery is now primed to accept unfolded proteins and begins to incorporate alkaline phosphatase with its attached hydrophobic export signal. Since the *prlA*-altered export machinery no longer rejects proteins with basic amino acids following this hydrophobic sequence, the protein is aligned with the machinery with its export signal in the portion of the complex where signal sequences normally lie. Previous studies with *prlA* mutants have shown slow export of proteins with defective or missing signal sequences (10). However, DΔ1 still maintains an intact highly hydrophobic sequence, which may add to the facilitation of export by contributing to the activation of the apparatus already primed by the *prlA* mutation.

2) Alternatively, the *prlA*-altered machinery may act to export AP after DΔ1 has assumed the orientation in the membrane found in the wild type. If this is the case, the translocation of the AP to the periplasmic face of the cytoplasmic membrane will bring with it at least a portion of the carboxyl terminus of the MSS. This would lead to the likely membrane-unstable structure shown in Fig. 5*A*.

Our results with urea extraction and other fractionation techniques support the first model, in which the DΔ1 protein is stably inserted in the membrane, with its amino terminus in the cytoplasm (Fig. 6).

The Role of SecB in the Export of MalF-AP Fusions—Export of wild-type alkaline phosphatase is not SecB-dependent (25). However, the export of signal sequenceless AP or AP in the M fusion in a *prlA* mutant is SecB-dependent (10). SecB is required to maintain certain exported proteins in a translocation-competent conformation (33). We suggest that export of wild-type AP is so rapid that SecB is not required; translocation outpaces folding. However, in the signal sequenceless AP or in the M fusion, the export is so slow that SecB is needed to maintain the cytoplasmically accumulating form in a conformation that can be exported. If this explanation is correct, it is the rapid export of AP seen in the DΔ1 fusion in a *prlA* background that avoids the requirement for SecB.

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