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Use of *phoA* and *lacZ* Fusions To Study the Membrane Topology of ProW, a Component of the Osmoregulated ProU Transport System of *Escherichia coli*

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The *Escherichia coli* ProU system is a member of the ATP-binding cassette (ABC) superfamily of transporters. ProU consists of three components (ProV, ProW, and ProX) and functions as a high-affinity, binding protein-dependent transport system for the osmoprotectants glycine betaine and proline betaine. The ProW protein is the integral inner membrane component of the ProU system. Its hydropathy profile predicts seven transmembrane spans and a hydrophilic amino terminus of approximately 100 residues, and it suggests the presence of an amphiphilic α-helix (L-61 to F-97) in close proximity to the first strongly hydrophobic segment of ProW. We have studied the membrane topology of the ProW protein by the *phoA* and *lacZ* gene fusion approach. A collection of 10 different *proW-phoA* fusions with alkaline phosphatase activity and 8 different *proW-lacZ* fusions with β-galactosidase activity were isolated in vivo after Tn*phoA*$_B$ and Tn*lacZ* mutagenesis of a plasmid-encoded *proW* gene. The recovery of both enzymatically active ProW-PhoA and ProW-LacZ hybrid proteins indicates that segments of ProW are exposed on both sides of the cytoplasmic membrane. To compare the enzymatic activities of each of the indicator proteins joined at a particular site in ProW, we switched the *phoA* and *lacZ* reporter genes in vitro in each of the originally in vivo-isolated gene fusions. A mirror-like pattern in the enzyme activity of the resulting new ProW-PhoA and ProW-LacZ hybrid proteins emerged, thus providing positive signals for the location of both periplasmic and cytoplasmic domains in ProW. The protease kallikrein digests the amino-terminal tail of a ProW-LacZ hybrid protein in spheroplasts, suggesting that the amino terminus of ProW is located on the periplasmic side of the cytoplasmic membrane. From these data, a two-dimensional model for ProW was constructed; this model consists of seven transmembrane α-helices and an unusual amino-terminal tail of approximately 100 amino acid residues that protrudes into the periplasmic space.

A sudden increase in medium osmolarity triggers a rapid efflux of water from the cell, resulting in a decrease in turgor and cessation of growth. To counteract these deleterious effects of high-osmolarity stress, *Escherichia coli* and *Salmonella typhimurium* have developed highly coordinated response reactions that aim to prevent the dehydration of the cytoplasm and to restore turgor and cell growth (7, 39). An important facet in the cell's adaptation response is the accumulation of a specific group of organic osmolytes, the compatible solutes (1). These compounds are amassed by the bacteria to high intracellular concentrations to reestablish the osmotic pressure gradient across the cell envelope. In addition, compatible solutes also stabilize the integrity of cell components, protect the function of proteins in solutions of high ionic strength, and do not disturb protein-DNA interactions (1). The increase in the intracellular concentration of these compounds is accompanied by an efflux of K^+ (12, 62), which is initially taken up in large quantities by the cell in response to an osmotic upshock (16). Since high concentrations of ionic osmolytes are detrimental to cell functioning, it is therefore advantageous for the bacteria to substitute compatible solutes for these ions (1).

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Glycine betaine is an important compatible solute. It is widespread in nature and has been adopted by many organisms as a preferred osmoprotectant (7, 39). The intracellular accumulation of glycine betaine by *E. coli* permits growth in a high-osmolarity environment, which would otherwise strongly inhibit the proliferation of the bacterial cells. This osmoprotectant is metabolically inert in *E. coli* (51) and can be amassed by the cells either through synthesis from the precursors choline or glycine betaine aldehyde (37) or through direct uptake from the environment (51). Two glycine betaine transport systems operate in *E. coli*: ProP, a low-affinity glycine betaine transporter that consists of a single polypeptide (8), and ProU, a high-affinity, multicomponent system (5, 14, 26, 46).

ProU is a member of the ATP-binding cassette (ABC) transporter superfamily in which substrate translocation across the cell membrane is dependent on ATP hydrolysis (13, 29). The ProU system consist of three proteins, i.e., ProV, ProW, and ProX (26). ProX is a soluble periplasmic substrate-binding protein (2, 30, 46) that recognizes glycine betaine and delivers it to a protein complex consisting of the integral inner membrane protein, ProW, and the energy-coupling component, ProV. Binding protein-dependent transport systems can mediate substrate accumulation against a steep concentration gradient (13, 29). ProX binds glycine betaine with high affinity (K_d, ~1 μM) (2, 30, 46), and thus the ProU system is well suited to scavenge glycine betaine from the environment efficiently (33) and to achieve high intracellular concentrations of this osmoprotectant. ProU also serves as a high-affinity uptake system for the effective osmoprotectant proline betaine (24, 28) and as

a low-affinity transport system for a range of other compatible solutes (24, 28, 36). The structural genes for the components of ProU are genetically organized in an operon (*proU*) (26) and are coordinately expressed in response to an increase in the osmolarity of the growth medium. In media of low or moderate osmolarity, the level of *proU* transcription is very low, but it strongly increases in proportion to rising osmolarity (5, 14, 25, 46). In addition, transport activity of the ProU system also increases at high osmolarity (5, 18). This dual osmotic control allows ProU to play an effective part in the adaptation reaction that allows *E. coli* to cope with high-osmolarity environments.

Gene fusion techniques have been successfully used as a genetic approach to analyze the topological arrangement of proteins within the bacterial cytoplasmic membrane. Two reporter proteins from *E. coli* have been particularly useful in such studies, the periplasmic alkaline phosphatase PhoA (43, 44) and the cytoplasmic β-galactosidase LacZ (19, 59). The use of these enzymes as sensors for the subcellular locations of segments of integral membrane proteins depends on their reciprocal activity in different cell compartments (44, 59). Alkaline phosphatase is enzymatically active only when it is translocated across the cytoplasmic membrane into the periplasm, where the required intrachain disulfide bond formation can occur. When the enzyme is retained in the cytoplasm, disulfide bond formation does not take place and PhoA is inactive (11). Appropriately oriented transmembrane spans of cytoplasmic membrane proteins can serve as export signals for a mutant PhoA protein lacking its own signal sequence. The alkaline phosphatase activity of such hybrid proteins thus serves as an indicator for the periplasmic or cytoplasmic location of domains from the target protein. When LacZ is used as the reporter protein, a reversed pattern of enzyme activities is observed. Fusion proteins between amino-terminal segments of membrane proteins and LacZ are active enzymatically only when the LacZ moiety of the hybrid protein remains in the cytoplasm. Fusion of LacZ to a periplasmic domain of the target protein will lead to the embedding of β-galactosidase in the membrane, resulting in the production of an enzymatically inactive fusion protein (19, 59).

The combined use of PhoA and LacZ reporter proteins with their complementary properties (40) provides an attractive system for the analysis of membrane protein topology. We have used this genetic approach to study the topological arrangement of ProW, the integral inner membrane component of the *E. coli* ProU system. Our data suggest that ProW has seven transmembrane segments and an unusual amino-terminal tail of approximately 100 amino acid residues that protrudes into the periplasm. Its membrane topology thus resembles that of a large family of eukaryotic G-coupled receptors (60).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All bacterial strains used in this study were derivatives of *E. coli* K-12. The plasmid-encoded *proW-lacZ* and *proW-phoA* fusions were maintained in strain CC118 [*araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74* Δ(*phoA*)*20 galE galK thi rpsE rpoB argE*(Am) *recA1*] (42). Strain CC311 [pOxgen::Tn*lacZ* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 galE galK thi rpsL recA1*] and strain CC321 [Δ(*ara-leu*)*7697 lacZ galE galK hsr rpsL rpoB argE*(Am) *recA1 srl*::Tn*10* (F′ *lacI*^{q} *pro zzf*::Tn*phoA*$_B$)] (40, 41) were used as the sources for the Tn*lacZ* and Tn*phoA*$_B$ transposable elements and were a kind gift from C. Manoil. The minicell-producing strain HB290 (*minB mgl rpsL*) was obtained from K. Heller. The bacterial strains were grown aerobically at 37°C in rich medium (LB) or minimal medium A (MMA) (47) supplemented with Casamino Acids (2 mg/ml) and vitamin B_{12} (5 μg/ml). Glucose (0.4%) was used as the carbon source. Low-osmolarity minimal medium (80 mosmol/kg) was prepared by diluting MMA salts with distilled water to one-third of its normal strength. High-osmolarity minimal medium (440 mosmol/kg) was prepared by adding NaCl to a final concentration of 200 mM to the low-osmolarity MMA. Osmolarities of the media were determined in an osmometer (Knauer Semimicroosmometer, type M). LB agar plates were prepared as described previously (47), except that NaCl was omitted to reduce the osmolarity of the growth medium. To avoid the toxic effects associated with overproduction of ProW and ProW–β-galactosidase and ProW-alkaline phosphatase hybrid proteins, strains that carried plasmid-encoded *proW* or *proW-lacZ* or *proW-phoA* hybrid genes were grown in low-osmolarity medium to minimize their expression from the osmoregulated *proU* promoter (39, 46). Ampicillin, chloramphenicol, and kanamycin were added to liquid and solid media at 50, 30, and 30 μg/ml, respectively. The PhoA and LacZ phenotypes of colonies were identified on agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-phosphate (XP) per ml or 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, respectively.

Construction of plasmids. The multicopy and low-copy-number cloning vectors pBR322 and pHSG575 have been described previously (3, 63). Plasmid pHP07 was used as the source for a restriction fragment carrying a *phoA* gene truncated at its 5′ end (27). Plasmid pGP21 is a low-copy-number vector for the construction of translational *lacZ* gene fusions (21). Plasmids pOS59 and pOS58 are derivatives of the *proU*$^+$ plasmid pOS25 (45) from which chromosomal segments flanking the *proU* operon have been removed by in vitro manipulations (see Fig. 2A). Plasmid pOS59 carries the entire *proU*$^+$ operon, whereas in plasmid pOS58, most of the *proX* gene has been removed by *Bal* 31 nuclease digestion (17). A *proW-lacZ* hybrid gene with the fusion junction in codon 153 of *proW* was constructed in vitro by ligating a 1,807-bp *Bal*I-*Hpa*I restriction fragment from plasmid pMH53 into the *Sma*I-cut low-copy-number *lacZ* fusion vector pGP21. The correct alignment of the *proW′* and *′lacZ* reading frames across the *Hpa*I-*Sma*I junction of the resulting *proW-lacZ* fusion Z153 on plasmid pMH60 was verified by DNA sequence analysis. To construct a plasmid expressing *proW* under the control of the osmoregulated *proU* promoter but lacking most of the *proV* sequences, we cleaved plasmid pOS58 with *Bal*I and *Asu*II, filled in the 5′ overhanging end with Sequenase (55), and religated the plasmid. The resulting plasmid pMH50 thus carries an in-frame deletion (*proV*^{Δ}) that removes most of the coding region (codon 51 to codon 384) of the 400-codon *proV* gene (26). Through a series of in vitro manipulations, we transferred this nonpolar *proV*^{Δ} mutation into the multicopy plasmid pBR322 along with the intact *proW*$^+$ and a truncated *proX′* gene. From the resulting plasmid pMH53 (see Fig. 2A), we transferred the *proU* material (*proV*^{Δ} *proW*$^+$ *proX′*) on a *Bal*I-*Cla*I fragment into the low-copy-number vector pHSG575 cut with *Mvn*I and *Asu*II. We verified the structure of this *proW*$^+$ plasmid, pMH54 (see Fig. 2A), by restriction analysis.

Isolation of gene fusions with Tn*phoA*$_B$ and Tn*lacZ*. Strain CC311 carries the Tn*lacZ* transposable element on the low-copy-number plasmid pOxgen, and the Tn*phoA*$_B$ element is carried by an F′ present in strain CC321 (40, 41). The *proW*$^+$ plasmid pMH54 (see Fig. 2A) was transformed into strains CC311 and CC321, and chloramphenicol-resistant colonies were selected on LB agar plates that did not contain NaCl to maintain expression of *proW* at a low level. Strains CC311(pMH54) and CC321(pMH54) were grown overnight in liquid LB medium without NaCl in the presence of chloramphenicol. The cells from 1-ml portions of such cultures were pelleted by centrifugation, resuspended in 200 μl of LB medium, and plated onto LB agar plates without NaCl that contained 200 μg of kanamycin per ml to select for increased gene dosage of the Tn*lacZ*- and Tn*phoA*$_B$-encoded kanamycin resistance gene. The plates were incubated for 2 days at 37°C. The kanamycin-resistant colonies were then pooled, and the plasmid DNA was extracted by the alkaline lysis procedure (55) and used to transform the Δ*lacX74* and Δ*phoA20* strain CC118. Transformants were selected on LB agar plates without NaCl that contained chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml). Growth on these selection plates requires the presence of pMH54 derivatives carrying Tn*lacZ* or Tn*phoA*$_B$ insertions. To score the PhoA and LacZ phenotypes of the colonies, these plates also contained XP or X-Gal, respectively. Colonies showing a PhoA$^+$ or LacZ$^+$ phenotype were purified on the same medium and then used to isolate plasmid DNA. The positions of the Tn*lacZ* and Tn*phoA*$_B$ elements in pMH54 were determined by restriction enzyme mapping. The precise DNA sequences at the junction between the *proW* gene and the Tn*lacZ* and Tn*phoA*$_B$ transposons were determined by the dideoxy chain termination method of Sanger et al. (56) with the Sequenase 2.0 kit (U.S. Biochemical Corp.). Sequencing reactions were primed with a synthetic oligonucleotide primer (5′-CGGGAAAGGTTCCGTCC-3′) that hybridized to the Tn*5* material present at the fusion junction between each Tn*lacZ*- and Tn*phoA*$_B$-generated *proW* insertion. To prevent secondary transposition of the Tn*lacZ* and Tn*phoA*$_B$ elements from plasmid pMH54, the DNA of the pMH54::Tn*lacZ* and pMH54::Tn*phoA*$_B$ insertion plasmids was cut with *Hin*dIII, and the largest restriction fragment was religated. This manipulation removed a *Hin*dIII fragment that carries the transposon-encoded *kan* gene and the structural gene for the transposase.

Reciprocal exchange of the *′phoA* and *′lacZ* reporter genes. To convert the *proW-phoA* fusions isolated by Tn*phoA*$_B$ transposition in plasmid pMH54 into *proW-lacZ* hybrid genes (40), we used in vitro recombinant DNA procedures. The pMH54::Tn*phoA*$_B$ insertion plasmid was cleaved with *Bam*HI, and the restriction fragments carrying the pHSG575 vector portion and the truncated *proV* and *proW′* material (approximately 7.6 to 8.1 kb, depending on the insertion site of the Tn*phoA*$_B$ element) was isolated. A 3,068-bp *Bam*HI restriction fragment carrying the *′lacZ* gene was isolated from a Tn*lacZ* insertion in plasmid pMH54 and ligated with the *Bam*HI fragments prepared from the various *proW-phoA* fusion plasmids. A similar strategy was used to convert *proW-lacZ* fusions into *proW-phoA* fusions. Plasmids carrying the *proW-lacZ* hybrid genes were

cleaved with *Bam*HI and *Pst*I, and the resulting restriction fragments encompassing the pHSG575 vector and the truncated *proV* and *proW'* material (approximately 5 to 5.9 kb, depending on the insertion site of the Tn*lacZ* element) was isolated. A 2.6-kb *Bam*HI-*Pst*I restriction fragment carrying the *'phoA* gene was isolated from plasmid pHP07 (27) and ligated with the *Bam*HI-*Pst*I fragment obtained from the *proW-phoA* insertions of Tn*lacZ* in pMH54. Both constructions replace the originally present indicator genes *'phoA* and *'lacZ* by *'lacZ* and *'phoA*, respectively, without altering the fusion junction originally present in the *proW-phoA* and *proW-lacZ* hybrid genes.

Western blotting (immunoblotting). To visualize the *proW-phoA*- and *proW-lacZ*-encoded hybrid proteins, Western blotting experiments (55) were performed. Overnight cultures (5 ml) of strain CC118 harboring the various pMH54-encoded *proW-lacZ* and *proW-phoA* fusions were grown in low-osmolarity minimal medium containing chloramphenicol. Expression of the *phoA* and *lacZ* gene fusions to *proW* is controlled by the osmoregulated *proU* promoter (39, 46), and we induced the expression of the *proW-phoA* and *proW-lacZ* hybrid genes by growing the cultures under high-osmolarity conditions. The cells from the overnight cultures were pelleted by centrifugation, resuspended in 5 ml of high-osmolarity minimal medium, and then diluted (1:4) in fresh high-osmolarity minimal medium. The cultures were subsequently grown for 1.5 h at 37°C until they reached an optical density at 578 nm of 0.4 to 0.5. Cells from 1-ml portions of the cultures were pelleted by centrifugation for 2 min, resuspended in sodium dodecyl sulfate (SDS) lysis buffer (60 mM Tris-HCl [pH 6.8], 10% glycerol, 3% β-mercaptoethanol, 2% [wt/vol] SDS, 0.001% bromo-phenol blue), and disrupted by boiling for 5 min. Ten micrograms of total cell protein from each culture was loaded onto an SDS-polyacrylamide gel. We used SDS–7% polyacrylamide gels to visualize ProW–β-galactosidase hybrid proteins and SDS–10% polyacrylamide gels to visualize ProW-alkaline phosphatase hybrid proteins. The electrophoretically separated total cellular proteins were transferred to a nylon membrane (Immobilon P; pore size, 0.45 μm; Millipore), and the bound proteins were then probed with a rabbit antiserum raised against β-galactosidase or alkaline phosphatase. The formed antigen-antibody complexes were visualized with goat anti-rabbit immunoglobulin G alkaline phosphatase-coupled antibody (55).

Determination of alkaline phosphatase and β-galactosidase activities. For the determination of the β-galactosidase and alkaline phosphatase activities of the strains carrying the plasmid-encoded *proW-lacZ* and *proW-phoA* fusions, we used samples of the osmotically induced cells which we prepared for the analysis of the ProW-LacZ and ProW-PhoA hybrid proteins. From each culture (optical density at 578 nm = 0.4 to 0.5), 200-μl portions were withdrawn and the cells were permeabilized by adding 2 drops of chloroform and 1 drop of a 0.1% (wt/vol) SDS solution. β-Galactosidase and alkaline phosphatase enzymatic activities were then determined spectrophotometrically with 2-nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-nitrophenyl-phosphate (pNPP) as substrates, respectively, as described previously (4, 47). All enzyme assays were performed in triplicate.

Proteolytic digestion of ProW–β-galactosidase hybrid proteins in spheroplasts. Spheroplasts were prepared as described before (66). Strain CC118 carrying either the *proW-lacZ* (Z94) or the *proW-lacZ* (Z193) fusion was grown in high-osmolarity minimal medium for 1.5 h to induce the expression of the hybrid genes. The cells from 5-ml portions of these cultures were collected by centrifugation and resuspended in 5 ml of a solution containing 33 mM Tris (pH 8.0), 5 mM $MgSO_4$, and 100 mM KCl (TMK). Aliquots (0.5 ml) of these cells were recentrifuged and resuspended in TMK containing 40% sucrose, 5 μg of lysozyme per ml, and 1 mM EDTA. The cells were then incubated for 30 min on ice to allow the formation of spheroplasts, which was monitored microscopically. After spheroplast formation, the protease kallikrein (EC 3.4.21.35; Sigma, Braunschweig, Germany) was added to a final concentration of 1 U/ml. This solution was incubated at 25°C for 1 h, and the spheroplasts were then collected by trichloroacetic acid precipitation. Total cellular proteins were solubilized by boiling for 10 min in SDS lysis buffer, and the proteins were subsequently subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7% polyacrylamide). The ProW–β-galactosidase hybrid proteins and the reaction products of the kallikrein protease digestion were visualized by Western blotting with an antibody directed against β-galactosidase. Spheroplasts were disrupted by freezing a 500-μl cell suspension for 10 min in liquid nitrogen. Lysozyme was added to a final concentration of 2 mg/ml prior to thawing at room temperature.

Radiolabeling of proteins in minicells. Minicells were isolated from strain HB290 carrying plasmids pHSG575, pOS59, and pMH54 as described previously (53). Radiolabeling of the plasmid-encoded proteins with L-[^{35}S]methionine (1,000 mCi/mmol; Amersham, Deisenhofen, Germany) in the presence or absence of 0.3 M NaCl was performed as described previously (18). The proteins were electrophoretically separated on an SDS–12% polyacrylamide gel, and the radiolabeled polypeptides were visualized by autoradiography.

RESULTS

Hydropathy analysis of the ProW protein. To locate hydrophobic segments of the ProW polypeptide chain that could possibly function as transmembrane spans, we performed a hydropathy analysis of ProW with an algorithm developed by Kyte and Doolittle (34). The resulting profile of ProW revealed seven stretches of hydrophobic residues with lengths of 18 to 36 amino acids (aa) alternating with hydrophilic or less-hydrophobic portions of the polypeptide chain (Fig. 1A). Such a pattern is a characteristic feature of integral cytoplasmic membrane proteins. All seven of the strongly hydrophobic segments of ProW are located between aa 100 and 320 (Fig. 1A) of the 345-residue ProW protein. A notable and unusual feature of ProW is its hydrophilic amino-terminal end. Predictions of the secondary structure of the ProW amino terminus described by Garnier et al. (20) and Gibrat et al. (22) suggest the presence of two α-helix-forming regions. The first region extends from D-3 to A-45 (98% predicted α-helices), and the second region stretches from L-61 to F-97 (63% predicted α-helices). The second segment has a high potential for forming an amphiphilic α-helix (Fig. 1B) and is located immediately upstream of the first strongly hydrophobic segment of ProW (Fig. 1A). These data therefore suggest that ProW could possess seven transmembrane segments, which would position the amino-terminal and carboxy-terminal ends of the protein on different sides of the cytoplasmic membrane. Alternatively, if the amphiphilic α-helix also traverses the plane of the membrane, ProW would possess eight membrane-spanning segments, resulting in a topological arrangement with both the amino terminus and the carboxy terminus on the same side of the membrane.

Construction of a low-copy-number plasmid expressing *proW*. We chose to use the *lacZ* and *phoA* fusion technique to study the membrane topology of ProW and intended to use for these experiments the transposable Tn*lacZ* and Tn$phoA_B$ elements (40, 42). Tn$phoA_B$ differs from the previously described Tn*phoA* transposon (42) in that it carries a *Bam*HI site at the 5′ end of the *'phoA* gene (40, 41). Since such a restriction site is also present at the 5′ end of the *'lacZ* gene in Tn*lacZ*, the reporter gene of a given fusion can be readily exchanged without altering the original fusion junction (40).

Previous experiments have shown that the vast majority of Tn*5* insertions isolated in plasmids carrying a $proU^+$ locus are located at various positions within *proV*, the first gene of the *proU* operon (*proV proW proX*) (10, 18). Although the reasons for the preferential insertion of Tn*5* into *proV* are not clear, these data indicate that plasmids carrying the entire *proU* region are not suitable for in vivo mutagenesis of *proW* by Tn*lacZ* and Tn$phoA_B$ since these transposable elements are derivatives of Tn*5* (40, 42). To reduce the size of the target for the Tn*lacZ* and Tn*phoA* transposon mutagenesis, we made a series of in vitro manipulations to construct plasmid pMH53. It carries an in-frame deletion that removes most of the coding region (codon 51 to codon 384) of the 400-codon *proV* gene and thus should allow expression of *proW* under the control of the osmoregulated *proU* promoter. Plasmid pMH53 also lacks a large segment of the *proX* gene, which encodes the periplasmic substrate-binding protein of the ProU system (Fig. 2A). pMH53 was subjected to Tn$phoA_B$ and Tn*lacZ* mutagenesis, and pMH53 derivatives carrying the desired transposon insertions were characterized by restriction mapping. Thirty-one $PhoA^+$ clones carrying Tn$phoA_B$ insertions in pMH53 (isolated from 11 independent cultures) were mapped; only one plasmid carried a Tn$phoA_B$ insertion in *proW*, and this *proW-proA* fusion was out of frame. The remaining 30 plasmids harbored Tn$phoA_B$ insertions in the truncated *proX'* gene. A similar biased pattern emerged when the Tn*lacZ* insertions in pMH53 were analyzed. Twenty-five $LacZ^+$ Tn*lacZ* insertions in plasmid pMH53 (isolated from 17 independent cultures) were mapped by restriction analysis, and only two insertions were found in *proW*; both *proW-lacZ* fusions were out of frame.

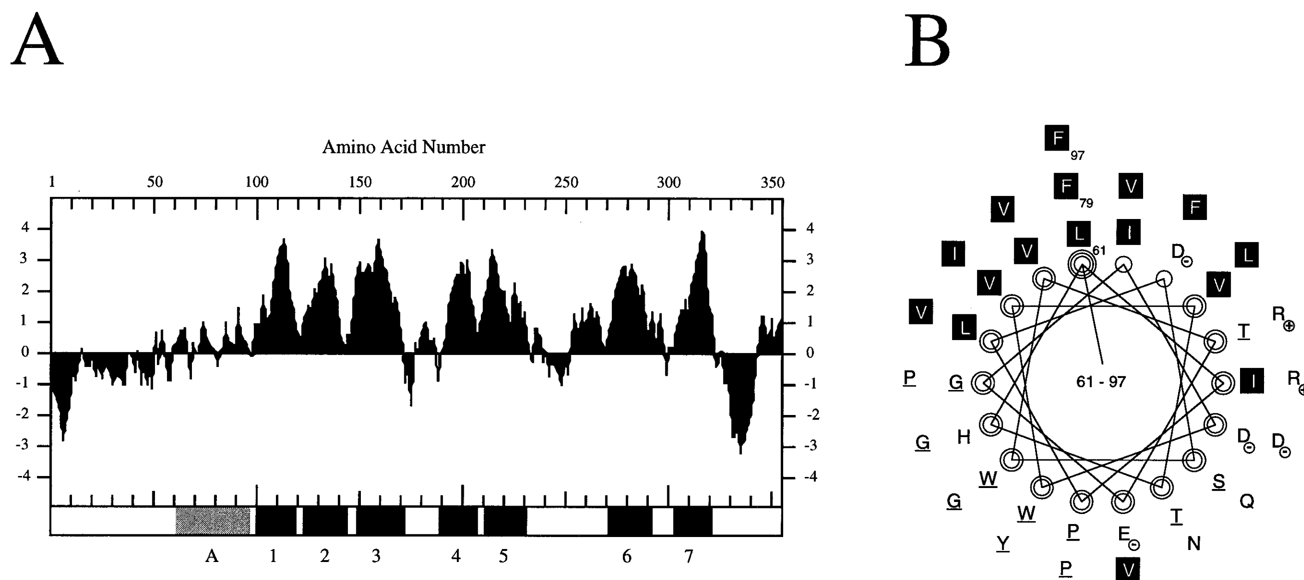

FIG. 1. Hydropathy analysis of ProW. (A) Hydropathic indices were calculated by the algorithms described by Kyte and Doolittle (34) with a sliding window of 7aa. Protein sequences that plot above the central line represent hydrophobic domains, whereas protein sequences that plot below the central line are hydrophilic. Seven protein segments of sufficient length to possibly span the cytoplasmic membrane are labeled as black boxes at the bottom of the figure. Their position within ProW and mean hydrophobicity are: segment 1, aa 100 to aa 118 (+2.3); segment 2, aa 123 to aa 142 (+1.8); segment 3, aa 149 to aa 170 (+2.5); segment 4, aa 189 to aa 206 (+1.8); segment 5, aa 211 to aa 230 (+2.3); segment 6, aa 255 to aa 290 (+1.7); segment 7, aa 303 to aa 320 (+2.5). The region represented by a shaded box and labeled A could potentially form an amphipathic α-helix (aa 61 to aa 97) with a mean hydrophobicity of +0.5. (B) Amino acids L-61 to F-97 are depicted as a α-helix with a total length of 10.3 turns. Hydrophobic amino acids with a hydrophobicity value between +4.5 and +1.8 (A, C, F, I, L, M, and V) are shown on a black background, amino acids with a hydrophobicity value between +1.6 and −1.6 (G, P, S, T, W, and Y) are underlined, and amino acids with a hydrophobicity value greater than −3.2 (D, E, H, K, N, Q, and R) (34) are shown without specific marking. The inner ring shows amino acids L-61 to H-78; the second ring shows amino acids F-79 to G-96, and the third ring shows amino acid F-97.

The remaining 23 Tn*lacZ* insertions had occurred in the partially deleted *proV* gene (Fig. 2A).

The strong bias for the insertion of the Tn*lacZ* and Tn*phoA* elements into the truncated *proV* and *proX* genes in the multicopy plasmid pMH53 indicated that overproduction of ProW–β-galactosidase and ProW-alkaline phosphatase hybrid proteins might be detrimental to cell viability. To minimize possible toxic effects of the *proW-lacZ* and *proW-phoA* gene products, we inserted the *proU* material ($proV^{\Delta}$ $proW^{+}$ $proX'$) present in plasmid pMH53 (Fig. 2A) into the low-copy-number vector pHSG575 (63), yielding plasmid pMH54 (Fig. 2A). To demonstrate that the *proW* gene was expressed in pMH54, we prepared minicells and visualized the plasmid-encoded proteins by radiolabeling with L-[^{35}S]methionine. Figure 2B demonstrates that ProW is synthesized in appreciable amounts in minicells harboring pMH54. As expected from the physical structure of the $proV^{\Delta}$ $proW^{+}$ $proX'$ construct carried by plasmid pMH54, no intact ProV and ProX proteins were synthesized in strain HB290(pMH54) (Fig. 2B, lanes 3 and 4) in contrast to minicells harboring the $proU^{+}$ plasmid pOS59 (Fig. 2B, lanes 5 and 6).

Isolation of *proW-phoA* and *proW-lacZ* gene fusions after Tn*lacZ* and Tn$phoA_B$ mutagenesis. We then characterized 132 $LacZ^{+}$ Tn*lacZ* insertions in the low-copy-number plasmid pMH54($proV^{\Delta}$ $proW^{+}$ $proX'$) (Fig. 2A). These $LacZ^{+}$ clones originated from 31 independent *E. coli* cultures mutagenized with the Tn*lacZ* transposon. Restriction analysis of the 132 plasmids revealed that the Tn*lacZ* element had inserted in each case into the *proU* portion of pMH54: 120 plasmids carried a copy of Tn*lacZ* inserted into the truncated *proV* gene, 12 had Tn*lacZ* insertions in *proW*, and none were found with a Tn*lacZ* element in the truncated *proX'* gene. DNA sequence analysis was subsequently used to identify the precise fusion junction among the 12 *proW*::Tn*lacZ* insertions, and eight different fusion joints were found (Table 1). In addition to the *proW-lacZ* fusions generated in vivo with Tn*lacZ*, we also constructed a *proW-lacZ* hybrid gene in vitro by recombinant DNA methods. In this gene fusion, codon 153 of *proW* was connected to a *'lacZ* indicator gene in proper reading frame (Table 1).

We also isolated 245 $PhoA^{+}$ derivatives of pMH54 carrying Tn$phoA_B$ insertions that originated from 82 independent cultures and characterized these plasmids by restriction analysis. The distribution of the insertion sites was again strongly biased. Two hundred twenty-five plasmids carried Tn$phoA_B$ insertions in the incomplete *proX'* gene, no insertion in the truncated *proV* portion was found, and 20 Tn$phoA_B$ elements were located in *proW*. DNA sequence analysis revealed 10 unique fusion junctions among these *proW*::Tn$phoA_B$ insertions (Table 1). The reading frames of *proW* and the *'phoA* and *'lacZ* indicator genes were correctly aligned in all of the sequenced *proW*-Tn*lacZ* and *proW*-Tn$phoA_B$ insertions (Table 1). When we refer to a particular ProW-LacZ or ProW-PhoA fusion protein, we use the designation Z (LacZ) or A (PhoA) followed by a number that indicates the codon of *proW* into which the Tn*lacZ* or Tn$phoA_B$ element is inserted.

Synthesis of the ProW–β-galactosidase and ProW-alkaline phosphatase hybrid proteins. Since the reading frames of the *proW* and *'phoA* and *'lacZ* genes were correctly aligned in all gene fusions characterized by DNA sequence analysis (Table 1), a hybrid protein should be produced in each case. To ensure that the expected ProW-PhoA and ProW-LacZ hybrid proteins were synthesized, we performed Western blotting experiments with all *proW-phoA* and *proW-lacZ* fusion strains. For these experiments, we induced the expression of the *proW-phoA* and *proW-lacZ* gene fusions by growing the cells in high-osmolarity minimal medium. Because toxic effects might be

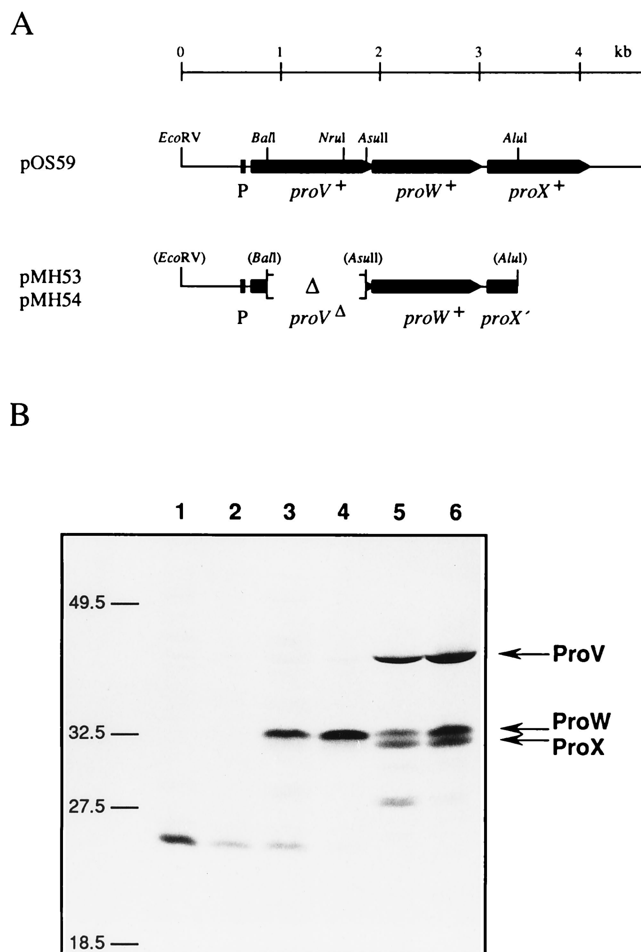

FIG. 2. Construction of a plasmid expressing *proW* and synthesis of the ProW protein in minicells. (A) The entire *proU* operon (*proV*, *proW*, *proX*) is present on plasmid pOS59. The multicopy plasmid pMH53 and the low-copy-number plasmid pMH54 are derivatives of pOS59 and carry an intact *proW* gene expressed from the osmoregulated *proU* promoter (P). Restriction sites relevant for the construction of plasmids pMH53 and pMH54 are given. Restriction sites destroyed in the course of the cloning are shown in brackets. (B) Minicells were prepared from strain HB290 carrying either the vector pHSG575 (lanes 1 and 2), plasmid pMH54 (lanes 3 and 4), or plasmid pOS59 (lanes 5 and 6). These were radiolabeled with L-[^{35}S]methionine, and the proteins were separated by SDS-PAGE (12% polyacrylamide). Radiolabeling of proteins was carried out in glucose minimal medium without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) 0.3 M NaCl as described by Faatz et al. (18). The positions of the ProV, ProW, and ProX proteins on the gel are indicated. The molecular masses (in kilodaltons) of marker proteins are indicated on the left.

associated with the induction of the hybrid proteins, we prepared overnight cultures of these strains in low-osmolarity minimal medium, diluted the cells into fresh high-osmolarity minimal medium, and grew the cultures for an additional 1.5 h. The bacterial cells were then harvested by centrifugation, total cell extracts were prepared, and the proteins were separated by SDS-PAGE. The ProW-LacZ and ProW-PhoA hybrid proteins were then visualized by immunoblotting with antisera directed against the β-galactosidase or the alkaline phosphatase portions of the hybrid proteins (Fig. 3A and B). As expected from the distribution of the *proW-phoA* and *proW-lacZ* fusion junctions along the *proW* gene (Table 1), hybrid proteins of increasing size were synthesized by our collection of fusion strains (Fig. 3A and B). Most of the hybrid proteins showed some degree of instability, yielding degradation products that corresponded to the β-galactosidase and alkaline phosphatase portions of the hybrid proteins (Fig. 3A and B). Such an instability of fusion proteins has been observed previously (23, 38, 50). Two ProW-PhoA fusion proteins (A147 and A213) were particularly unstable (Fig. 3B, lanes 4 and 9). The ProW portions of these hybrid proteins are only 2 amino acids longer (Table 1) than the reasonably stable ProW-PhoA hybrid proteins A145 and A211, respectively (Fig. 3B; lanes 3 and 8).

Enzymatic activity of ProW-LacZ and ProW-PhoA hybrid proteins. We measured the enzymatic activity of the ProW-LacZ and ProW-PhoA hybrid proteins in permealized cells of the PhoA$^-$ and LacZ$^-$ *E. coli* strain CC118. Overnight cultures of CC118 carrying the pMH54-encoded gene fusions were grown in low-osmolarity minimal medium, and the expression of the *proW-lacZ* and *proW-phoA* hybrid genes was then induced by growing the cells in high-osmolarity minimal medium to the early log phase (optical density at 578 nm = 0.4 to 0.5). The β-galactosidase and alkaline phosphatase enzyme activities of the *proW-phoA* and *proW-lacZ* fusion strains are summarized in Table 2. As expected from the LacZ$^+$ and PhoA$^+$ phenotypes of the in vivo-isolated *proW-lacZ* and *proW-phoA* fusion strains on XP and X-Gal indicator plates, each of the fusion strains exhibited high enzyme activity. This finding indicates that the segments in ProW identified with the aid of the *lacZ* and *phoA* fusion approach (Table 1) represent cytoplasmic and periplasmic domains of the ProW polypeptide chain, respectively. An exception was the in vitro-constructed *proW-lacZ* Z153 fusion strain; it exhibited only low β-galactosidase activity (Table 1), indicating that the ProW-LacZ hybrid protein Z153 was at least partially embedded in the cytoplasmic membrane.

Switching of the *phoA* and *lacZ* reporter genes. To further corroborate the data obtained with the in vivo-isolated *proW-phoA* and *proW-lacZ* fusions, we used in vitro recombinant DNA methods to switch the indicator gene in most of the originally isolated *proW*::Tn$phoA_B$ and *proW*::Tn*lacZ* fusions. The exchange of the *'lacZ* and *'phoA* genes in these hybrid genes is readily possible since an appropriately positioned *Bam*HI site is present at the junction between the Tn*5* material and the reporter genes in the Tn$phoA_B$ and Tn*lacZ* elements (9, 40, 41, 52). Such a conversion of the indicator genes does not alter the original site of the Tn$phoA_B$ and Tn*lacZ* insertion in *proW* and hence should permit evaluation of the enzyme activity of both a ProW-PhoA and ProW-LacZ hybrid protein at a given fusion junction. We use the designation ZA and AZ to refer to fusions that were constructed by changing the reporter gene from a *lacZ* fusion to a *phoA* fusion (ZA) or vice versa (AZ).

Western blotting experiments with the full set of the in vitro-constructed *proW-phoA* and *proW-lacZ* fusions showed that hybrid proteins of the expected size were produced in each case (Fig. 3C and D). Some instability of most of the hybrid proteins was noticeable. Fusion proteins with junctions at the amino-terminal part of ProW (ZA19, ZA27, ZA68, and ZA94) were produced in lower amounts than fusion proteins with junctions in the middle (ZA188, ZA193, and ZA248) or carboxy-terminal portion (ZA344) of ProW (Fig. 3D). We measured the enzyme activity in permealized cells of the *proW-phoA* and *proW-lacZ* fusion strains (Table 2). Each of the in vitro-converted fusions showed only low enzyme activity in comparison with fusions isolated with the Tn$phoA_B$ and Tn*lacZ* elements in vivo. Thus, a switch in the PhoA and LacZ reporter enzymes at a particular fusion site in ProW results in hybrid proteins with complementary enzymatic properties: ProW-PhoA fusions with high enzymatic activity yield ProW-LacZ fusions with low enzymatic activity upon exchange of the

TABLE 1. Sequences of the *proW-phoA* and *proW-lacZ* fusion junctions[a]

proW-lacZ fusion	Sequence	*proW-phoA* fusion	Sequence
Z19*	(1) AGT GCC GCG C-**CT GAC TCT** S A A P **D S**	A145*	(1) TGG TCG CAG G-**CT GAC TCT** W S Q A **D S**
Z27*	(2) TGG GGT ACA C-**CT GAC TCT** W G T P **D S**	A147	(1) CAG GCA ATG G-CT **GAC TCT** Q A M A **D S**
Z68*	(1) GAC AGT TGG G-**CT GAC TCT** D S W A **D S**	A149	(1) ATG GTG ACT C-**CT GAC TCT** M V T P **D S**
Z94*	(1) GAT TAT ATC C-**CT GAC TCT** D Y I P **D S**	A151*	(2) ACT CTG GCG C-**CT GAC TCT** T L A P **D S**
Z153	GCG CTG GTG TT-**G GGG ATC** A L V L **G I**	A209*	(2) GGT AAC GTG C-**CT GAC TCT** G N V P **D S**
Z188*	(2) GAT GCC ATG C-**CT GAC TCT** D A M P **D S**	A211	(1) GTG CCG GGC G-**CT GAC TCT** V P G A **D S**
Z193*	(1) ACG CCA GCG T-**CT GAC TCT** T P A S **D S**	A213*	(6) GGC GTG GTG G-**CT GAC TCT** G V V A **D S**
Z248*	(2) GGT GCC AGC C-**CT GAC TCT** G A S P **D S**	A295*	(3) CAG ATG GTA C-**CT GAC TCT** Q M V P **D S**
Z344*	(2) ACC ACT GGC C-**CT GAC TCT** T T G P **D S**	A303*	(1) CGT CTG GAT A-**CT GAC TCT** R L D T **D S**
		A315*	(2) GGG ATT GTG A-**CT GAC TCT** G I V T **D S**

[a] The nucleotide sequence and the deduced amino acid sequence (shown in single-letter code) at the fusion junctions of the Tn*lacZ*- and Tn*phoA*$_B$-generated *proW-lacZ* (Z) and *proW-phoA* (A) fusions are given. The inverted triangles mark the fusion joints, and the DNA sequences originating from the Tn*lacZ* and Tn*phoA*$_B$ elements are indicated in boldface type. Those *proW-lacZ* and *proW-phoA* fusions which were converted in vitro to *phoA* and *lacZ* fusions, respectively, are indicated by asterisks. The *proW-lacZ* fusion Z153 was constructed by recombinant DNA procedures; the DNA material following the fusion junction originates from the polylinker of the *lacZ* fusion plasmid pGP21. The numbers in parentheses indicate the numbers of independently isolated fusions at this junction.

reporter protein, and ProW-LacZ fusions with high enzymatic activity yield ProW-PhoA fusions with low enzymatic activity.

Models for the membrane topology of ProW. When the data obtained from the hydropathy analysis of the ProW protein (Fig. 1) and the results from the *phoA* and *lacZ* fusion analysis of the *proW* gene are combined, one can derive a topological model in which the ProW polypeptide chain transverses the membrane eight times (Fig. 4A). The eight membrane-spanning segments would include all seven strongly hydrophobic portions of ProW and also part of the extended amphiphilic α-helix (Fig. 1). The strong β-galactosidase activity of the in vivo-isolated ProW-LacZ strains with fusion junctions at either the amino- (Z19, Z27, and Z68) or carboxy-terminal end (Z344) of ProW (Table 2) suggests that both parts of the ProW protein are present in the cytoplasm (Fig. 4A). In support of this conclusion is the PhoA$^-$ character of the *proW-phoA* fusion strains (Table 2) that were derived from these *proW-lacZ* hybrids genes by a switch of the indicator gene.

However, it is well established that the topological assignments of domains from integral membrane proteins by the PhoA and LacZ fusion approach will provide correct information only if topogenic signals from the target protein are present before the fusion junction in the hybrid proteins (15). One can therefore derive an alternative model for the topology of ProW by assuming that the amphiphilic α-helix does not serve as a transmembrane span. In such a model, only the seven strongly hydrophobic segments of ProW identified by hydropathy analysis (Fig. 1A) would function as membrane-spanning regions. Consequently, the amino- and carboxy-terminal ends of ProW would be positioned on different sides of the cytoplasmic membrane. Provided that one excludes the properties of those LacZ and PhoA fusions whose fusion junction (Z19, Z27, Z68, and Z94) occurs before the first strongly hydrophobic segment in ProW (aa 100 to aa 118), the enzymatic activities of all remaining hybrid proteins suggest a topological arrangement of ProW with the amino terminus within the periplasm and the carboxy terminus in the cytoplasm (Fig. 4B).

Protease susceptibility of the amino-terminal end of ProW in spheroplasts. To distinguish between these models for the topological arrangement of ProW, we used protease susceptibility to probe for the presence of the ProW amino terminus in the periplasm. The protease kallikrein (EC 3.4.21.35) specifically cleaves peptide bonds following F-R and L-R residues, and the presence of two such cleavage sites are predicted for the ProW protein between residues R-80 and P-81 and R-296 and G-297 (Fig. 5). To monitor the action of the protease, we used the ProW-LacZ hybrids Z94 and Z193 as target proteins and Western immunoblot experiments with a β-galactosidase antiserum to detect possible cleavage products of these proteins. Both the Z94 and Z193 hybrid proteins contain only one of the predicted kallikrein cleavage sites (R-80 to P-81) in ProW (Fig. 5), and although β-galactosidase is predicted to contain 13 kallikrein cleavage sites, we found that this enzyme is highly resistant to the protease. If the model for the topology of ProW with the eight membrane-spanning regions is

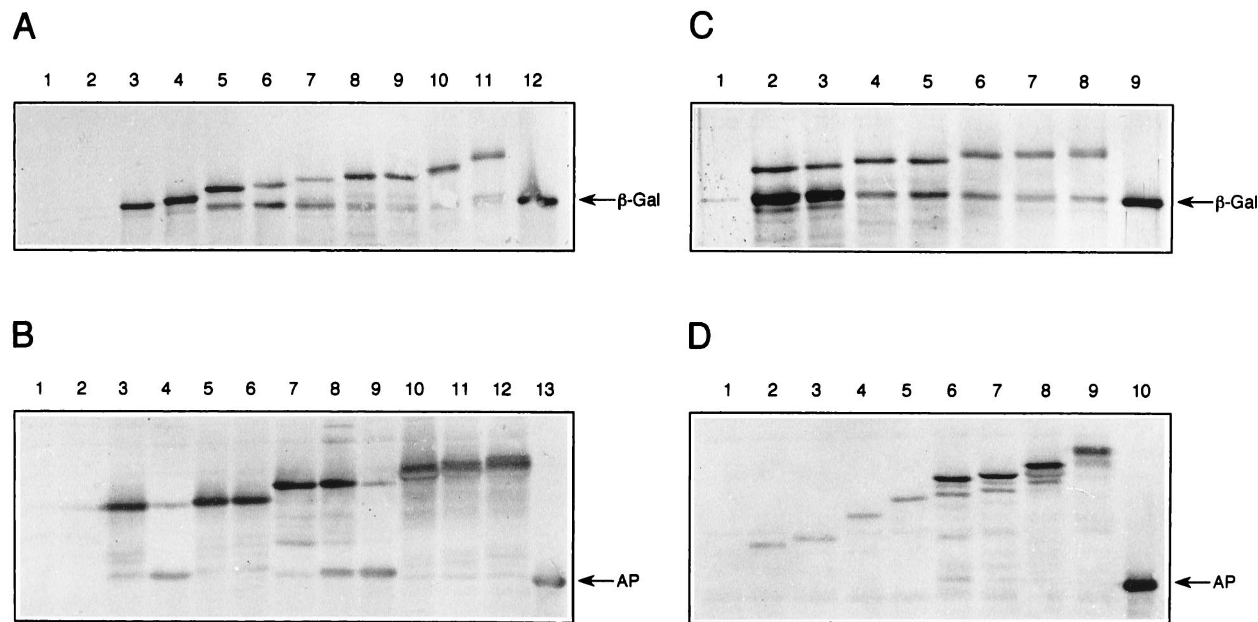

FIG. 3. Western blots of ProW–β-galactosidase and ProW-alkaline phosphatase hybrid proteins. Electrophoretically separated proteins were transferred to a Immobilon membrane, and the *proW-lacZ*- and *proW-phoA*-encoded hybrid proteins were visualized with antisera directed against the β-galactosidase (A and C) or alkaline phosphatase (B and D) moiety of the hybrid proteins. (A) Lanes: 1, protein extracts from the Δ*lacZ* Δ*phoA* strain CC118; 2, protein extracts from strain CC118 carrying the *proW*$^+$ plasmid pMH54; 3 to 11, protein extracts of strain CC118 carrying the plasmid pMH54-encoded *proW-lacZ* fusions Z19, Z27, Z68, Z93, Z153, Z188, Z193, Z248, and Z344, respectively; 12, purified β-galactosidase. (B) Lanes: 1 and 2, protein extracts from strain CC118 and its plasmid pMH54-harboring derivative; 3 to 12, protein extracts of strain CC118 carrying the plasmid pMH54-encoded *proW-phoA* fusions A145, A147, A149, A151, A209, A211, A213, A295, A303, and A315, respectively; 13, purified alkaline phosphatase. (C) Lanes: 1, protein extracts from strain CC118 carrying plasmid pMH54; 2 to 8, protein extracts of strain CC118 carrying the plasmid pMH54-encoded *proW-lacZ* fusions which were constructed in vitro (AZ145, AZ151, AZ209, AZ213, AZ295, AZ303, and AZ315, respectively) from the *proW-phoA* fusions isolated in vivo by Tn*phoA*$_B$ transposition; 9, purified β-galactosidase. (D) Lanes: 1, protein extracts from strain CC118 carrying plasmid pMH54; 2 to 9, protein extracts of strain CC118 carrying the plasmid pMH54-encoded *proW-phoA* fusions which were constructed by replacing the *'lacZ* sequences from the Tn*lacZ*-generated *proW* fusions with the *'phoA* gene (ZA19, ZA27, ZA68, ZA94, ZA188, ZA193, ZA248, and ZA344, respectively); 10, purified alkaline phosphatase. The arrows point to the positions of β-galactosidase (β-Gal) and alkaline phosphatase (AP) on the Western blot.

correct, then both the Z94 and Z193 hybrid proteins should be resistant to kallikrein when the protease is added to the periplasmic side of the membrane (Fig. 5A). If, however, ProW features a topological organization with seven transmembrane spans (Fig. 5B), cleavage of the Z193 protein in spheroplasts by kallikrein is predicted, whereas the Z94 hybrid should be resistant.

Synthesis of the ProW-LacZ polypeptide Z94 or Z193 was induced by growing the cells in high-osmolarity medium. The cells were harvested by centrifugation and either converted to spheroplasts or lysed to produce whole protein extracts. Kallikrein was added, and the reaction products of the Z94 and Z193 proteins were subsequently visualized by Western blotting (Fig. 5). The Z94 protein yielded two cross-reacting bands, namely, the full-length hybrid protein and a breakdown product with an electrophoretic mobility similar to that of authentic β-galactosidase. The full-length Z94 protein was cleaved by kallikrein in whole-cell extracts but not in spheroplasts (Fig. 5, lanes 1 to 4), indicating a cytoplasmic location of the Z94 ProW-LacZ hybrid protein. In contrast, the Z193 hybrid was susceptible to the protease in both whole-cell extracts and spheroplasts (Fig. 5, lanes 5 to 8), demonstrating that part of the ProW moiety of the hybrid protein was exposed on the periplasmic side of the membrane. Kallikrein degraded the Z193 protein to a defined product in spheroplasts of about the size predicted from the position of the kallikrein cleavage site at the amino terminus of the ProW moiety in the hybrid protein. An intact cytoplasmic membrane then prevented the further proteolytic breakdown of the Z193 protein to a fragment with the size of authentic β-galactosidase (Fig. 5, lanes 5 to 8). The experimentally observed pattern of resistance and sensitivity of the Z94 and Z193 proteins to kallikrein in whole-cell extracts and in spheroplasts thus followed the predictions made from the ProW topology model with seven transmembrane spans (Fig. 5B).

DISCUSSION

We have investigated the membrane topology of the *E. coli* ProW protein by use of the genetic *phoA* and *lacZ* gene fusion approach (40, 44, 59). ProW is the integral inner membrane component of ProU, an ATP-binding cassette uptake system (26) for the osmoprotectants glycine betaine and proline betaine (24, 28, 30, 46). The high-affinity transport of these compatible solutes across the cytoplasmic membrane requires the productive interactions of ProW with two other components of the ProU system, namely, the periplasmic substrate-binding protein ProX and the energy-coupling protein ProV, which is located on the cytosolic side of the membrane (45). The contact of ProW with proteins located in different cell compartments implies that segments of the ProW polypeptide chain are exposed on both sides of the cytoplasmic membrane. In analogy with the biochemically well-studied binding protein-dependent transport systems for maltose and histidine (9, 52), one can assume that the docking of the substrate-loaded ProX protein to ProW triggers a conformational change in the ProX, ProW, and ProV protein complex that leads to the activation of the ATPase activity of ProV and results in the concomitant ProW-mediated translocation of the substrate into the cell. Indeed, consumption of ATP has been experimentally demon-

TABLE 2. Enzyme activities of ProW–β-galactosidase and ProW-PhoA hybrid proteins[a]

Fusion[b]	LacZ activity[c]	Fusion[b]	PhoA activity[d]
Z19	5.59	ZA 19	0
Z27	5.55	ZA 27	0
Z68	5.52	ZA 68	0
Z94	3.47	ZA 94	0
AZ145	1.37	A145	9.5
		A147	12.2
		A149	11.2
AZ151	1.39	A151	10.6
Z153	0.10		
Z188	4.70	ZA188	0.4
Z193	3.85	ZA193	0
AZ209	1.36	A209	17.9
		A211	21.5
AZ213	1.05	A213	24.7
Z248	3.76	ZA248	0.3
AZ295	0.64	A295	17.5
AZ303	0.73	A303	21.2
AZ315	1.12	A315	14.6
Z344	3.17	ZA344	0

[a] The β-galactosidase and alkaline phosphatase activities were measured in strain CC118 containing the plasmid pMH54-encoded *proW-lacZ* and *proW-phoA* fusions. All enzyme assays were done in triplicate.

[b] The designations Z and A refer to *proW-lacZ* and *proW-phoA* fusions, respectively. The designations ZA and AZ refer to fusions which were constructed by exchanging the reporter gene from a *lacZ* fusion to a *phoA* fusion (ZA) or vice versa (AZ). The numbers following these designations indicate the codon in *proW* carrying the fusion junction.

[c] LacZ activity is expressed as micromoles of ONPG hydrolyzed per minute per milligram of protein. The LacZ activity of strain CC118(pMH54) without fusions is equal to zero.

[d] PhoA activity is expressed as micromoles of pNPP hydrolyzed per minute per microgram of protein. The PhoA activity of strain CC118(pMH54) without fusions is equal to zero.

strated during glycine betaine transport through ProU, with an apparent stoichiometry of two molecules of ATP hydrolyzed per molecule of substrate transported (48). Since most binding protein-dependent transport systems form a complex with a stoichiometry of two ATPases and two hydrophobic integral inner membrane components (13, 29, 58), it is likely that both ProW and ProV function as homodimers.

The hydropathy analysis of ProW revealed seven hydrophobic segments with lengths between 18 and 36 aa residues. Each of these regions has an average hydrophobicity of ≥1.6 (Fig. 1). These segments thus conform to the requirements of membrane-spanning regions according to the criteria defined by Kyte and Doolittle (34). None of these strongly hydrophobic segments of ProW is present within the first 100 amino-terminal residues. A comparison of the hydropathy profile of ProW to that of 35 other integral inner membrane components of binding protein-dependent transport systems (13, 29, 57) showed that this hydrophilic amino-terminal segment is a distinct feature of ProW. Secondary-structure predictions (20, 22) of the amino-terminal portion of ProW suggest the presence of an amphiphilic α-helix (Fig. 1B) that possibly could also function as a transmembrane span. The computer analysis therefore indicates that the ProW polypeptide chain transverses the plane of the cytoplasmic membrane either seven or eight times and consequently suggests two different models for the location of the amino- and carboxy-terminal ends of ProW.

To determine the number and topological arrangement of the membrane-spanning regions of ProW, we isolated in vivo a series of enzymatically active *proW-lacZ* and *proW-phoA* fusions by mutagenizing a low-copy-number $proW^{+}$ plasmid (pMH54) with the Tn*lacZ* and Tn$phoA_{B}$ transposable elements. Use of a low-copy-number plasmid with a *proU* construct in which most of the coding regions for *proV* and *proX* were deleted (Fig. 2) proved to be crucial for the successful isolation of both *lacZ* and *phoA* in-frame fusions to *proW*. The distribution of the insertions of the Tn*lacZ* and Tn$phoA_{B}$ transposons into pMH54 was highly biased, indicating that the synthesis of ProW-LacZ and ProW-PhoA fusion proteins is detrimental to cell growth. The physical structure of the Tn*lacZ* and Tn$phoA_{B}$ elements has been designed by C. Manoil (40, 41) such that the combined use of the *phoA* and *lacZ* fusion techniques for the topological analysis of membrane proteins is greatly facilitated. A switch in the compartment-specific reporter proteins PhoA and LacZ can be efficiently accomplished with this fusion system either by in vivo genetic recombination procedures (40) or, as used here, by simple in vitro recombinant DNA methods. Such an exchange in the reporter enzymes makes it feasible to compare the activities of each of the PhoA and LacZ indicator enzymes attached to a particular site of the target protein. Both positive and negative signals for the location of the junction sequences in either periplasmic or cytoplasmic domains are thus obtained. When this combined *phoA* and *lacZ* fusion approach was applied to the topological analysis of ProW, hybrid proteins with the same fusion junction that exhibited reciprocal enzymatic activities were produced. Thus, as predicted from the compartment-dependent properties of the alkaline phosphatase and β-galactosidase moieties of these hybrid proteins (19, 44, 59), a mirror image in the enzymatic activities of the ProW-PhoA and ProW-LacZ proteins was observed without exception. Essentially all of the produced fusion proteins were unstable to some degree (Fig. 3), making it somewhat difficult to estimate the enzymatic activities of these hybrids from the total alkaline phosphatase or β-galactosidase activity in permealized cells alone. However, the complementary properties of each pair of ProW-PhoA and ProW-LacZ proteins (Table 2) allowed a clear assignment to a periplasmic or cytoplasmic location of the fusion junction of the hybrid proteins.

The results obtained from the characterization of the 34 *phoA* and *lacZ* fusions to *proW* and the data derived from the hydropathy and computer analyses are consistent with an eight-helix model for the membrane topology of ProW and suggest a cytosolic location for both the amino- and carboxy-terminal ends of ProW (Fig. 4A). However, it is well recognized that the assignment of cytoplasmic or periplasmic domains of a target protein by the *phoA* and *lacZ* fusion approach will provide only correct topological information if topogenic signals from the target protein are present before the fusion junction to the reporter enzyme (15, 44, 59). There is no cleavable signal peptide in ProW upstream of the amino-terminal tail, and there are no obvious apolar segments that could serve as internal signal sequences (6, 65) prior to the first strongly hydrophobic region between residues 100 and 118 (Fig. 1A). Thus, the enzyme activities of either ProW-PhoA or ProW-LacZ proteins with fusion junctions in the extended hydrophilic amino-terminal segment of ProW might erroneously indicate a cytoplasmic location of this region. Our protease accessibility experiments with the $LacZ^{+}$ ProW-LacZ fusion proteins Z94 and Z193 strongly suggest that this is indeed the case and that the amino terminus of ProW is located in the periplasmic space (Fig. 5). A similar situation was encountered by Lacatena et al. (35) during the topological analysis of the human β_2-adrenergic receptor (huβ_2AR) by the *phoA* fusion technique. When expressed in *E. coli*, the huβ_2AR protein adopts its natural conformation with seven transmembrane spans and exhibits an extended amino-terminal region in

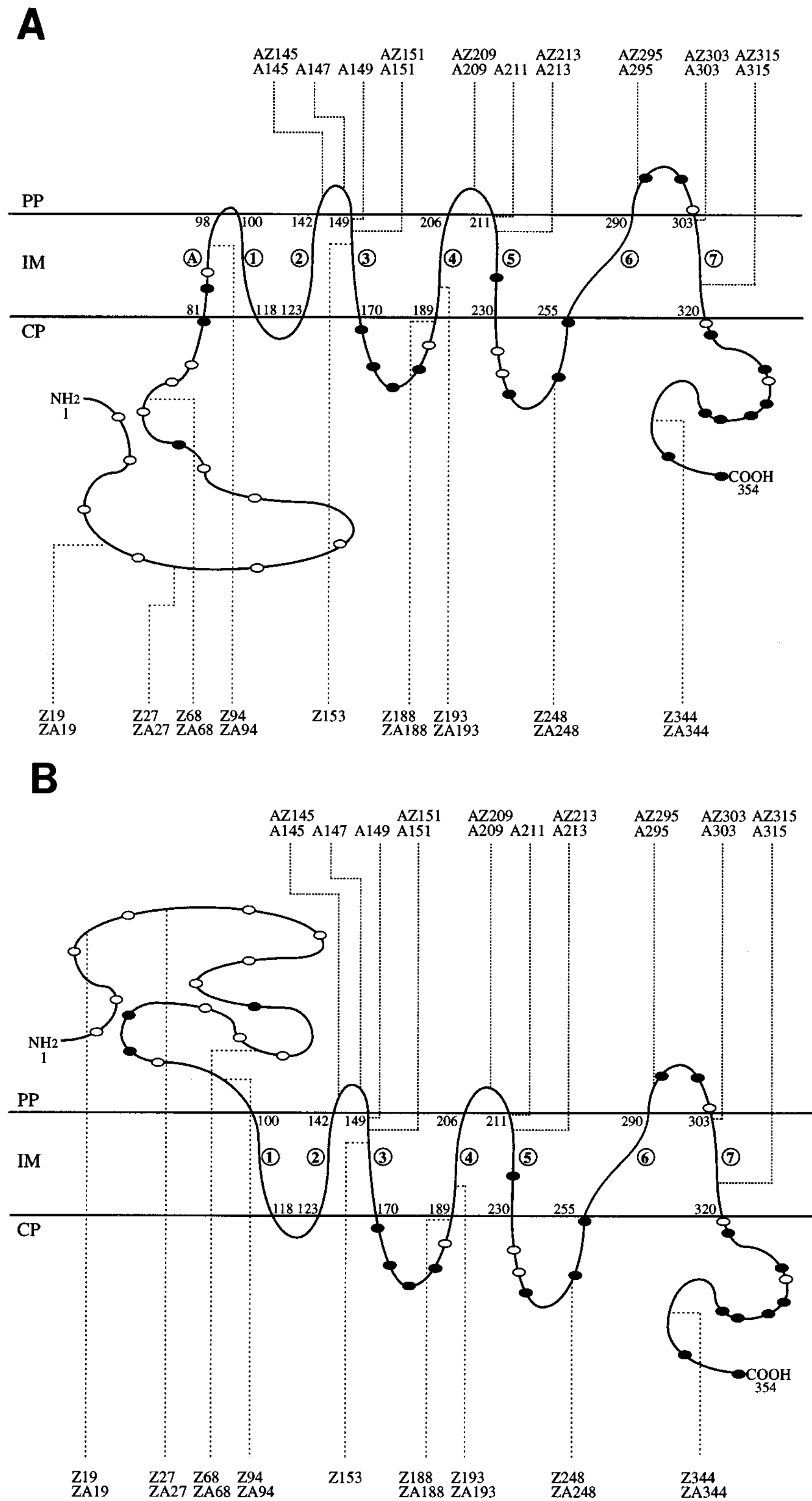

FIG. 4. Models for the topological organization of ProW. Two models (A and B) for the two-dimensional topology of ProW within the inner membrane are shown. The first and last residues of the predicted transmembrane segments of ProW are indicated. The positions of the alkaline phosphatase and β-galactosidase fusions to ProW are given. The designations Z and A refer to ProW-LacZ and ProW-PhoA fusions, respectively, and are followed by an allele number that gives the *proW* codon carrying the fusion junction. The designations ZA and AZ indicate *lacZ* and *phoA* fusions which were converted in vitro into *phoA* and *lacZ* fusions, respectively. The *proW-lacZ* fusion Z153 was constructed by recombinant DNA methods. Positively charged residues (R, K) (⬬) and negatively charged residues (D, E) (⬭) of ProW are indicated. IM, inner membrane; PP, periplasm; CP, cytoplasm.

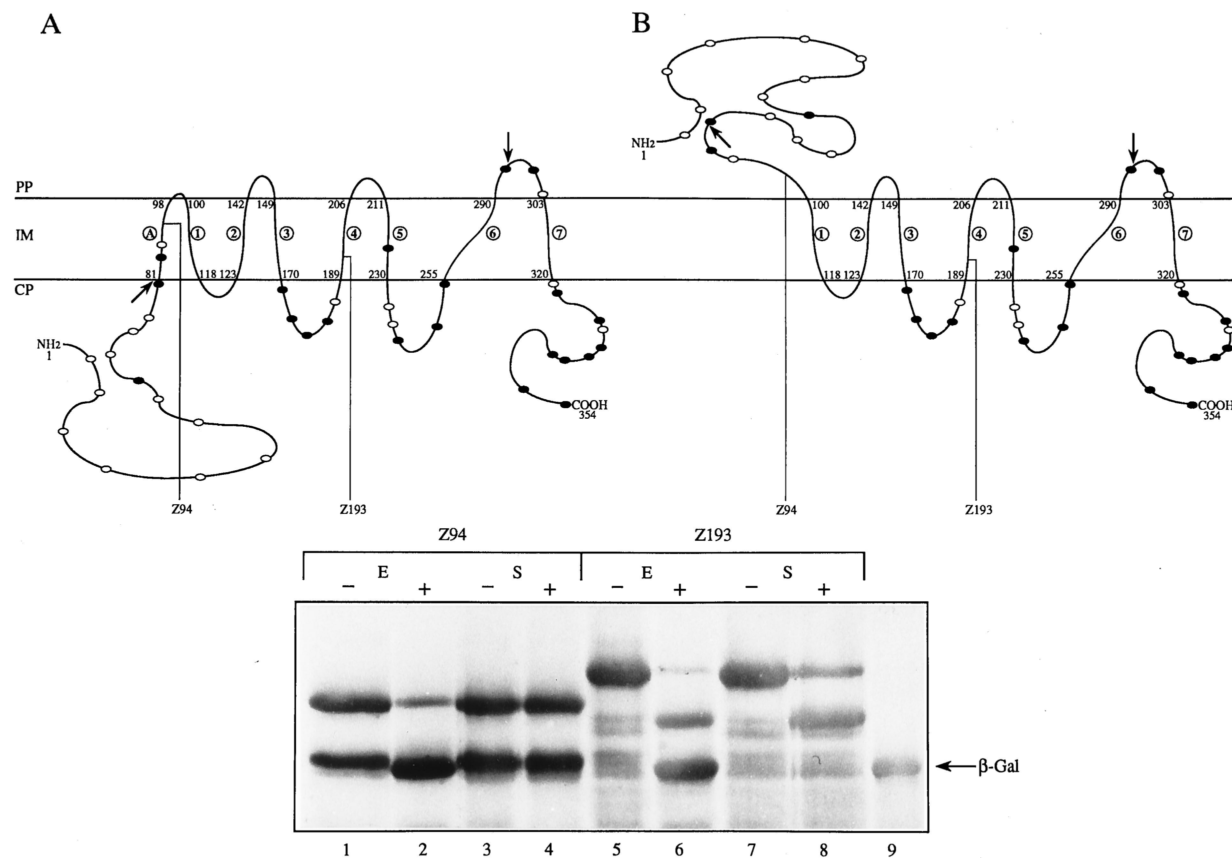

FIG. 5. Protease susceptibility of ProW-LacZ hybrid proteins in spheroplasts. Two different models (A and B) for the topology of ProW are shown. The fusion junctions of the ProW-LacZ hybrid proteins Z94 and Z193 are indicated. The arrows point to the two sites that are predicted from the amino acid sequence of ProW to be susceptible to the protease kallikrein. The other symbols are as described in the legend to Fig. 5. The lower part of the figure shows a Western blot of protein extracts prepared from strain CC118 carrying the plasmid-encoded *proW-lacZ* gene fusions Z94 and Z193. Spheroplasts (S) and cell extracts (E) were prepared and incubated at 25°C in the absence (−) or presence (+) of the protease kallikrein. The hybrid proteins and their degradation products were visualized with an antiserum raised against β-galactosidase. Lanes: 1 to 4, ProW–β-galactosidase fusion Z94; 5 to 8, ProW–β-galactosidase fusion Z193; 9, purified β-galactosidase.

the periplasmic space. Alkaline phosphatase fusions to segments between the amino terminus and the second external domain of the huβ_2AR protein yielded data that were inconsistent with the known topology of huβ_2AR in the mammalian membrane and other experimental findings. The amino-terminal portions of ProW and the huβ_2AR proteins thus lack the topogenic information required to correctly position these domains in the periplasm when fused to reporter enzymes. The membrane topology of proteins with extended hydrophilic amino termini thus appears to rely on information present in the distal portions of the polypeptide chain. We obtained in vivo the first *proW-phoA* hybrids with high PhoA activity in a region that immediately followed the second strongly hydrophobic segment of ProW. These hybrid genes yielded fusions with moderate β-galactosidase activity when their indicator portion was switched to *lacZ* (Table 2), implying that the region between aa residues 142 and 149 represents a periplasmic domain of ProW (Fig. 4). No *lacZ* fusions were obtained in the loop connecting the transmembrane segments one and two (Fig. 4B). Thus, our assignment of a cytoplasmic location of this segment lacks positive experimental confirmation. Our difficulty in obtaining unambiguous topological information for the region amino-terminal to the second transmembrane span in ProW is probably connected with a lack of topogenic signals that would direct the ProW-PhoA and ProW-LacZ hybrid proteins to their proper cellular location.

The combined results from the ProW hydropathy profile, the enzymatic properties of the ProW-PhoA and ProW-LacZ fusion proteins, and the proteolytic susceptibility of the amino-terminal segment of ProW to kallikrein in spheroplasts strongly suggest a topological model in which the amino and carboxy termini of ProW are located on different sides of the cytoplasmic membrane and are connected by a ProW segment which spans this membrane seven times (Fig. 6B). The proposed topological organization of ProW shows striking similarities to that of a superfamily of eukaryotic receptor proteins known as the seven-membrane-receptor family consisting of G-coupled receptor proteins that allow eukaryotic cells to sense and respond to a wide variety of stimuli (60). We have recently shown that the translocation of the amino-terminal tail of ProW across the inner membrane is independent of the general protein secretion machinery (Sec) but is strictly dependent on the proton motive force. Insertion of this portion of ProW into the periplasm is efficiently blocked when additional positive charges are introduced into the amino terminus (65). The model for the topology of ProW is also consistent with the positive inside rule of von Heijne and Gavel (64), in which cytoplasmic domains of membrane proteins are enriched in lysine and arginine residues (Fig. 5B). Only a single charged amino acid (R-224) is located in one of the proposed transmembrane spans of ProW, and we have recently found that this membrane-embedded residue plays a critical role in the ProU-

mediated glycine betaine uptake (54). The integral membrane components from binding protein-dependent transport systems display a conserved peptide motif (the EAA motif) that is located approximately 100 residues from the carboxy terminus (58) and might constitute an interaction site with the ATP-binding components of the ABC transport systems (32). The corresponding segment of ProW is located in one of the predicted hydrophilic cytoplasmic loops (Fig. 5B), fully consistent with the proposed position of the EAA-region in the other membrane proteins of bacterial ATP-binding cassette transport systems (58).

The majority of the inner membrane components of binding protein-dependent transport systems are predicted to have six membrane-spanning segments with their amino and carboxy termini located in the cytoplasm (29). Examples that do not conform to the six-helix paradigm include the MalF protein of the maltose transport system from *E. coli* with eight transmembrane segments (15, 19) and the HisQ and HisM proteins from the histidine transport system of *S. typhimurium* with five transmembrane segments (32). We can currently only speculate about a possible function of the extended amino-terminal domain of ProW. The rate of glycine betaine transport mediated by the ProU system can be induced, even under conditions when de novo protein synthesis is inhibited, by increasing the osmolarity of the medium (5, 18). This activation of preexisting ProU components does not depend on an osmotically enhanced binding of glycine betaine by the periplasmic substrate-binding protein ProX (30, 46). Mechanosensitivity is known to alter the activity of ion channels both in prokaryotic and eukaryotic organisms (49, 61). It is thus tempting to speculate that the amphiphilic α-helix within the periplasmic ProW amino-terminal tail may serve as a mechanosensitive detector for alterations in membrane tension that occur during rapid changes in the environmental osmolarity. We have recently identified in the gram-positive soil bacterium *Bacillus subtilis* a transport system, OpuA, for the osmoprotectant glycine betaine that is structurally closely related to the *E. coli* ProU system (31). The amino acid sequences of ProW and the analogous OpuAB protein are rather well conserved, but OpuAB lacks most of the extended hydrophilic amino-terminal region that distinguishes ProW from other inner membrane components of binding protein-dependent transport systems (31). Similar to ProW, an amphiphilic α-helix is predicted for OpuAB in a region just upstream of the first strongly hydrophobic transmembrane segment, but it is unknown whether the transport activity of preexisting OpuA components can also be enhanced in response to increases in the environmental osmolarity. The model proposed here for the membrane topology of the *E. coli* ProW protein provides a base for further investigations to identify residues involved in substrate recognition and translocation and to clarify the function of the exceptional amino-terminal periplasmic domain of ProW.

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