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Topology of the *Escherichia coli uhpT* Sugar-Phosphate Transporter Analyzed by Using Tn*phoA* Fusions

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The *Escherichia coli uhpT* protein catalyzes the active transport of sugar-phosphates by an obligatory exchange mechanism. To examine its transmembrane topology, we isolated a collection of *uhpT-phoA* fusions encoding hybrid proteins of different lengths from the N terminus of UhpT fused to alkaline phosphatase by using transposon Tn*phoA*. These fusions displayed different levels of alkaline phosphatase activity, although comparable levels of full-length UhpT-PhoA proteins were produced in maxicells of both high- and low-activity fusions. The full-length protein species were unstable and were degraded to the size of the alkaline phosphatase moiety in the case of a high-activity fusion or to small fragments in the case of a low-activity fusion. The enzyme activity present in low-activity fusions appeared to result from export of a small proportion of the fusion proteins to the periplasmic space. Although fusions were not obtained in all predicted extramembranous loops, the deduced topology of UhpT was consistent with a model of 12 membrane-spanning regions oriented with the amino and carboxyl termini in the cytoplasm.

The sequences of numerous bacterial transport proteins have been deduced from the nucleotide sequences. Although they often possess little amino acid sequence relatedness, these predominantly nonpolar proteins display a similar pattern of alternating hydrophilic and hydrophobic regions. Examination of their hydropathy profiles suggests that a substantial number of transporters, including those with different modes of energy coupling, possess 12 transmembrane segments (14, 17). Direct evidence concerning the number and extent of intramembranous regions and their orientation in the cytoplasmic membrane is generally not available. Crystallographic studies of membrane proteins are rare, and structural studies are complicated because these proteins are extensively buried within the membrane bilayer and are inaccessible to many biochemical or immunological reagents.

Boyd et al. (2) and Manoil and Beckwith (19, 20) developed a genetic probe of transmembrance topology that makes use of the ability of externally directed transmembrane segments of any integral membrane protein to serve as a signal sequence for the export of alkaline phosphatase. This approach is based on the finding that *phoA* fusions to different regions of a membrane protein display different levels of alkaline phosphatase activity. Fusions with high activity are considered to be those in which the PhoA segment is linked to a part of the target protein exposed to the periplasm, whereas low-activity hybrid proteins result from fusion to cytoplasmic domains of the target protein. Enzyme activities of fusions to membrane proteins with relatively few transmembrane regions, such as Tsr, MotB, and Lep (5, 20, 25), correlated with the transmembrane orientation determined by biochemical analysis. Alkaline phosphatase may be inactive unless exported to the periplasm because of its inability to form dimers, acquire zinc ions, or form proper intrachain disulfide bonds.

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This paper describes the properties of *phoA* fusions to *uhpT*, encoding the *Escherichia coli* sugar-phosphate transport system. UhpT is an integral component of the cytoplasmic membrane and is responsible for the accumulation of glucose 6-phosphate and other organophosphates (29). Ambudkar et al. (1) and Sonna et al. (26) demonstrated that UhpT operates by an obligatory exchange process in which the accumulation of sugar-phosphates is coupled to the electroneutral release of P_i or an organophosphate. The deduced sequence of UhpT displays substantial homology to the sequences of GlpT, the *sn*-glycerol-3-phosphate permease, and PgtP, the phosphoglycerate permease of *Salmonella typhimurium*, both of which also function as phosphate antiporters (6, 9, 10). The properties of *glpT-phoA* and *glpT-lacZ* fusions led Gött and Boos (11) to predict a two-dimensional model for GlpT having 12 membrane-spanning domains with the amino and carboxy termini facing the cytoplasm.

The properties of *uhpT-phoA* fusions extend the results obtained with GlpT and suggest an identical transmembrane structure for both GlpT and UhpT. The stability of the UhpT-PhoA fusion proteins provides additional information about the use of alkaline phosphatase fusions as a topological tool.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 strains, plasmids, and phages were previously described. All fusion plasmids were isolated and maintained in strain CC118 [Δ(*ara-leu*)*7697 araD139 ΔlacX74 galE galK ΔphoA20 thi rpsE rpoB argE*(Am) *recA1*], provided by C. Manoil (19). Strain CC202 is CC118/F42 *lacI3 zzf-2*::Tn*phoA*. Strain RK4991 is *araD139* Δ(*argF-lac*)*U169 relA1 rpsL150 thi gyrA219 non recA56 ΔuhpT2050* (29). Strain CS383 is *ompR ompC phoS* and was provided by C. Schnaitman. The *phoA* insertions were isolated in plasmid pRJK10, a pBR322 derivative carrying a 6.6-kilobase fragment with the entire *uhpABCT* region and conferring constitutive ex-

pression of the UhpT transport system (29). Phage λ Tn*phoA* was provided by C. Manoil (19).

Media were prepared as described by Miller (22). For growth of plasmid-bearing cells, antibiotics were added to the following concentrations: ampicillin, 30 mg/liter; kanamycin, 30 or 300 mg/liter (high concentration); and tetracycline, 25 mg/liter. 5-Bromo-4-chloro-3-indolyl-phosphate (XP) was used in agar medium at 40 μg/ml.

Isolation of *phoA* fusions. Fusions of *phoA* to *uhpT* were isolated as described by Manoil and Beckwith (19) with some modifications. Tn*phoA* was introduced into strain CC118(pRJK10) either by infection with phage λ Tn*phoA* or by conjugation with strain CC202. Products of transposition were selected on medium containing kanamycin at 30 μg/ml in the case of λ Tn*phoA* infection or 300 μg/ml in the case of transposition from F′ Tn*phoA*; they were and screened for alkaline phosphatase production on XP plates. All *uhpT-phoA* fusion plasmids were reduced in size and stabilized by deletion of the *Xho*I fragments of Tn*phoA* containing the transposase and kanamycin-resistance determinants.

Recombinant DNA techniques. Standard recombinant DNA techniques were done as described previously (18, 29). Restriction enzymes T4 and DNA ligase were obtained from New England BioLabs, Inc. (Cambridge, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by their manufacturers.

Enzyme assays. Alkaline phosphatase activity was assayed as described by Brickman and Beckwith (3). β-Lactamase activity was assayed in 0.1 M phosphate buffer (pH 8.0) in the presence of the chromogenic substrate Centa (40 μg/ml; Behring Diagnostics, La Jolla, Calif.). Activity is expressed as the change in the A_{415} per minute per milliliter of sample. Glucose-6-phosphate dehydrogenase activity was measured as described by Maurer et al. (21) and is expressed as the change in the A_{340} per minute per milliliter of enzyme sample, corrected for the rate before the addition of the substrate, 10 mM glucose 6-phosphate.

DNA sequence analysis. The location of the fusion joint was determined by dideoxy sequence analysis on the double-stranded *uhpT-phoA* plasmid DNA by the protocol provided with the Sequel double-stranded DNA dideoxy sequencing kit (IBI, New Haven, Conn.). The synthetic oligonucleotide primer (5′-CCCGGTTTTCCAGAACAGGG) hybridized to the 5′ end of the *phoA* gene and provided sequence readings in the promoter-proximal direction.

Detection of UhpT-PhoA hybrid proteins. The polypeptides produced by the *uhpT-phoA* fusion plasmids were analyzed by two methods. Plasmid-encoded polypeptides were selectively labeled by the maxicell method of Sancar et al. (24) as previously described (29). Maxicells were labeled with 45 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and resolved on 11% sodium dodecyl sulfate-polyacrylamide gels (17). Samples were loaded with constant trichloroacetic acid-precipitable radioactivity.

Fusion polypeptides were detected by the Western blot (immunoblot) method of Towbin et al. (27). Similar amounts of cell protein from early-log-phase cultures were separated by electrophoresis and transferred electrophoretically to nitrocellulose membranes (Amersham). The membranes were exposed to 10 ml of blocking solution (0.67 M NaCl, 50 mM Tris hydrochloride, 0.5% Nonidet P-40, 0.1% Tween 20, 0.5% bovine serum albumin, 3% nonfat dry milk) and incubated at room temperature for 1 h. Antibody (0.1 ml of goat anti-*E. coli* alkaline phosphatase; Litton Bionetics, Kensington, Md. [>5 mg of precipitating antibody per ml]) was added in 10 ml of blocking solution. After incubation for 1 to 2 h and three washes with blocking solution, secondary antibody (affinity-purified swine anti-goat immunoglobulin G conjugated to horseradish peroxidase; Boehringer), 0.1 ml in 10 ml of blocking solution, was added for 2 to 3 h at room temperature, followed by three 10-min washes in phosphate-buffered saline and the addition of the substrate reaction solution.

Analysis of fusion protein stability. For pulse-chase experiments with fusion-carrying strains, cells from 5 ml of early-log-phase cultures were washed and suspended in 1.1 ml of labeling mix (medium A salts [22], 2% glucose; 0.1% arginine, 0.1% lysine) and labeled for 2 min at 37°C with 0.15 μCi of [^{35}S]methionine. Aliquots were chased with unlabeled methionine (1 mg/ml). Proteins were precipitated by the addition of trichloroacetic acid to 5% and analyzed by immunoprecipitation and electrophoresis (13, 16).

Osmotic shock and cell fractionation. Cells were subjected to osmotic shock as described by Neu and Heppel (23). The cell pellet remaining after the removal of the osmotic shock fluid was suspended in 10 mM Tris hydrochloride (pH 8.0) and sonicated with three 30-s bursts. The membrane fraction was sedimented by centrifugation at 100,000 × *g* for 2 h. The membrane (suspended in 1 M Tris hydrochloride [pH 8.0]) and supernatant fractions were assayed for enzyme activity.

RESULTS

Hydropathy profile of the UhpT sequence. The distribution of amino acid residues in the UhpT sequence shows the alternation of hydrophobic and hydrophilic segments characteristic of integral membrane proteins with multiple membrane-spanning sequences (9). Analysis with the transfer energy parameters of Kyte and Doolittle (15) or Eisenberg et al. (7, 8) indicated the presence of as many as 12 possible membrane-spanning regions at least 18 amino acids in length. The hydropathy profile for UhpT was virtually superimposable on that of GlpT and was very similar to that of the shorter PgtP protein. This pattern suggests that these three homologous proteins (ca. 30% identity) possess similar transmembrane topologies. All 12 hydrophobic segments in UhpT had mean hydrophobicities of >0.5, based on the normalized consensus values of Eisenberg (7), and seven had values of >0.65 and are classified as initiator regions characteristic of integral membrane proteins.

A two-dimensional model for the transmembrane topology of UhpT (Fig. 1) was generated by defining 12 transmembrane segments based on the hydropathy analysis. The analogous segments in GlpT and PgtP are also predicted to be membrane spanning. The precise boundaries of many of these segments are not certain, except where there were clusters of positively charged residues or regions with a strong propensity for forming polar turns. The extramembranous loops having an excess of positively charged residues were placed on the cytoplasmic side, according to the proposal of von Heijne (28). There are an equal number of acidic and basic residues within the membrane-spanning regions.

Isolation of *uhpT-phoA* fusions. To gain information about the orientation of the protein in the membrane, we isolated gene fusions encoding hybrid proteins of various lengths from the amino terminus of UhpT linked to PhoA in the *uhpABCT*$^+$ plasmid pRJK10 by using transposon Tn*phoA*, a derivative of Tn*5* which carries a truncated *phoA* gene lacking normal expression and export sequences (19). Fusion of this *phoA* gene in-frame to the coding region for a

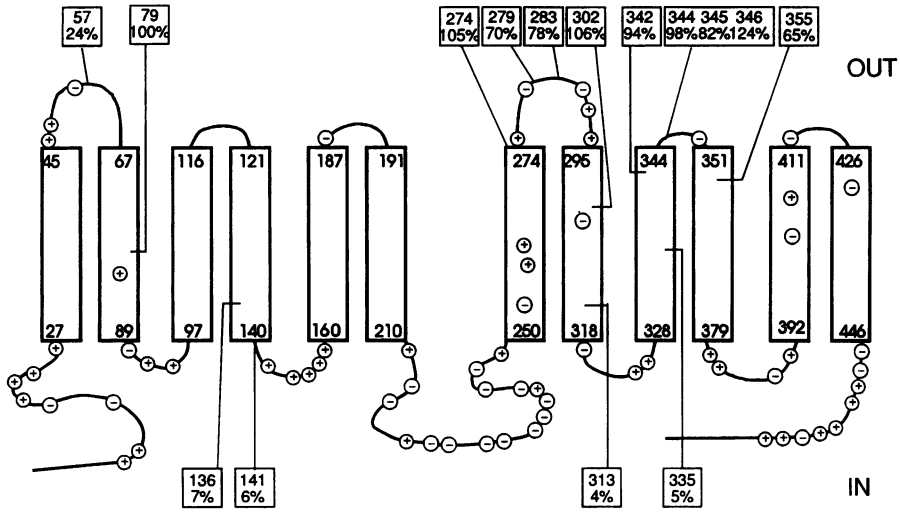

FIG. 1. Two-dimensional topological model of the UhpT transporter and properties of *uhpT-phoA* gene fusions. Each square represents a unique fusion joint and is connected to the appropriate position on the model. The upper number in each box gives the location of the last amino acid of UhpT before the fusion, and the lower number gives the alkaline phosphatase activity relative to that of fusion 79 (143 Miller units). The location of charged amino acids is indicated by the circled symbols.

periplasmic protein or for a periplasm-facing segment of a cytoplasmic membrane protein allows export of the PhoA moiety, resulting in high phosphatase activity. Fusions to cytoplasmic domains of the target protein exhibit lower enzymatic activity (2, 5, 20).

Transposition of Tn*phoA* into pRJK10 gave rise to colonies with a range of alkaline phosphatase activities, monitored with the chromogenic substrate XP. Insertions into *uhpT* were identified by restriction mapping of the plasmid with *Eco*RV and *Cla*I and by testing for the inability to complement the *uhpT* deletion in strain RK4991. Only about 5% of the transposition products produced blue colonies on XP plates. All dark blue isolates carried insertions in *uhpT*, whereas light blue and white isolates contained insertions in *uhpT* or other *uhp* genes.

The location of the fusion junctions in 20 independently isolated *uhpT-phoA* fusions was determined by nucleotide sequence analysis (Fig. 1). Three white Pho$^-$ fusions were out-of-frame, while the remaining 17 Pho$^+$ fusions were in-frame. Fusions are designated by the residue number of the last amino acid encoded by *uhpT*. There was considerable clustering of insertion sites, with two fusions at amino acid 79 and two at amino acid 345; six fusions had junctions between amino acids 335 and 346.

Enzyme activities of *uhpT-phoA* fusions. The alkaline phosphatase activities of the *uhpT-phoA* fusions (Fig. 1) correlated with the intensity of blue color on XP plates. Fusions at amino acids 79, 274, 279, 283, 302, 342, 344, 345, 346, and 355 gave similar high levels of enzyme activity. Fusions at amino acids 136, 141, 313, and 335 gave low levels of enzyme activity (less than 10% of that of the high-activity strains), and the fusion at residue 57 gave intermediate activity. The fusions with high PhoA activity occurred in or near the regions between predicted hydrophobic segments 1 and 2, 7 and 8, and 9 and 10. The low-activity fusions occurred between segments 4 and 5 and segments 8 and 9. The occurrence of the high-activity fusions at the even-numbered hydrophilic segments suggests that these regions normally face the periplasm.

Production and stability of UhpT-PhoA hybrid proteins. When the hybrid proteins encoded by *uhpT-phoA* plasmids were metabolically labeled in a maxicell system, a polypeptide of the size expected for the full-length hybrid protein was produced for each fusion up to amino acid 335 (data not shown). The amount of UhpT-related proteins was low relative to that of other plasmid-encoded proteins. Strains with longer fusions had lower levels of total incorporation and grew at substantially reduced rates. Roughly similar amounts of the fusion proteins were seen regardless of the alkaline phosphatase activity of the strain. In addition to the fusion protein, an additional polypeptide migrating at an M_r of 48,000, near the size expected for the PhoA moiety, was present in maxicells of strains with high alkaline phosphatase activity. Thus, during the limited labeling conditions in maxicells, comparable amounts of the fusion proteins were produced, suggesting that differences in PhoA enzyme activity were not the result of differences in the rates of synthesis of the fusion proteins.

Steady-state levels of the *uhpT-phoA* gene products were detected by Western blotting with antibodies against PhoA (data not shown). Strains with the high-activity fusions possessed small amounts of immunoreactive polypeptides in the size range of full-length fusion proteins, but substantial reactivity was seen at the position corresponding to intact PhoA. Thus, all UhpT-PhoA fusion proteins are subject to proteolytic cleavage, although it is not known whether cleavage occurs only at the fusion junction or throughout the length of the UhpT portion. Strains with the low-activity fusions had very low or undetectable amounts of intact PhoA. This result suggests that the decreased enzyme activity results from the decreased stability of the PhoA moiety of the fusion protein.

The stability of several representative UhpT-PhoA fusion proteins was examined. Strains were labeled with [^{35}S]methionine for 2 min and chased with nonradioactive methionine for various periods, and the labeled polypeptides were visualized after immunoprecipitation (Fig. 2). The strain expressing the high-activity fusion at residue 302 produced a polypeptide with an M_r of 84,000, the size expected for the fusion protein. This full-length species disappeared quickly, with the appearance of 48,000-M_r polypeptide that was stable during the chase period, as was

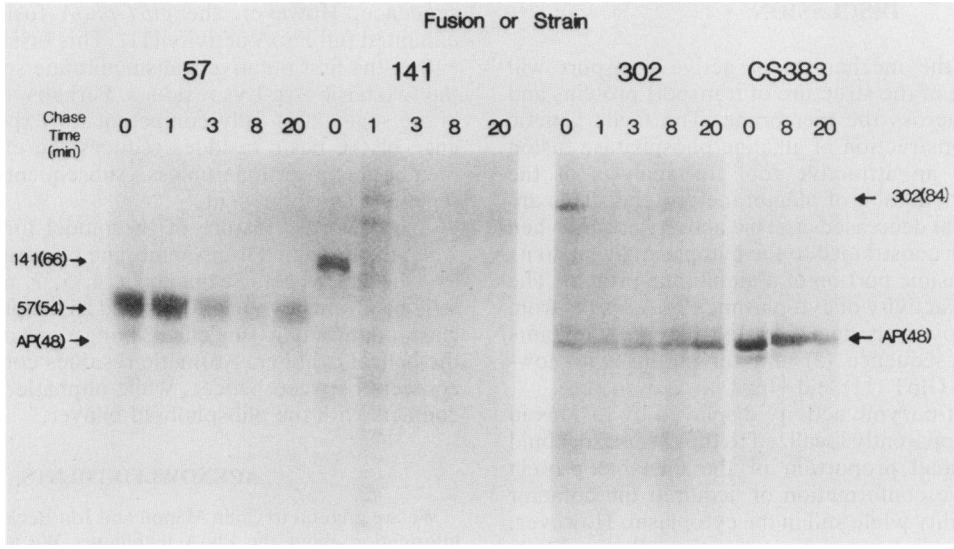

FIG. 2. Stability of UhpT-PhoA fusion proteins determined by immunoprecipitation. Cells of strain CC118 carrying the indicated *uhpT-phoA* fusion-bearing plasmid or of strain CS383 were labeled for 2 min with 0.15 mCi of [^{35}S]methionine. Aliquots were chased with nonradioactive methionine (1 mg/ml) for the indicated times. Proteins were extracted, and immunoprecipitates obtained with goat anti-PhoA antibody and Formalin-fixed staphylococcal A protein (Pansorbin) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Arrows indicate the positions of the intact UhpT-PhoA fusion proteins and intact alkaline phosphatase (AP), with their sizes in kilodaltons in parentheses.

authentic alkaline phosphatase produced by strain CS383. The strain with the low-activity fusion at residue 141 produced a 66,000-M_r polypeptide corresponding to the intact fusion protein. This polypeptide was rapidly lost during the chase period, and there was no substantial accumulation of the 48,000-M_r PhoA species. The strain carrying the intermediate-activity fusion at residue 57 produced a polypeptide corresponding to the intact fusion protein at an M_r of 54,000 and lower amounts of a 48,000-M_r PhoA species. Both polypeptides exhibited some turnover during the chase. Thus, the alkaline phosphatase activities expressed by UhpT-PhoA fusion protein-bearing strains were related to the stability of the PhoA moiety of the fusion protein.

Exposure of UhpT-PhoA proteins. It was expected that the unstable low-activity fusion proteins would be retained in the cytoplasm and rapidly degraded. To test this possibility, we subjected strains carrying UhpT-PhoA fusion proteins of different stabilities to osmotic shock and separated the soluble and membrane fractions of the shocked cells (Table 1). The ratio of alkaline phosphatase activities in the three strains was similar to that described in Fig. 1. The same proportion of alkaline phosphatase activity (81% of the total) was released from all three strains by osmotic shock treatment, indicating that the enzymatically active PhoA species in all three strains is located in the periplasmic space. As controls to show that osmotic shock did not result in extensive cell lysis, roughly 90% of the periplasmic β-lactamase activity, but less than 8% of the cytoplasmic glucose-6-phosphate dehydrogenase activity, was released from all strains.

TABLE 1. Distribution of enzyme activities in cell fractions

Enzyme	Plasmid[a]	Enzyme activity (change in the absorbance/min per ml) in the following fraction:			
		Total[b]	Osmotic shock[c]	Cytoplasm[c]	Membrane[c]
Alkaline phosphatase	pUhpT-PhoA57	29.1	23.0 (79)	5.6 (19)	0.5 (2)
	pUhpT-PhoA141	12.1	9.9 (82)	1.7 (14)	0.5 (4)
	pUhpT-PhoA302	191.6	155.8 (81)	28.8 (15)	7.0 (4)
	pRJK10	0.5	0.1	0.3	0.1
β-Lactamase	pUhpT-PhoA57	102.0	91.2 (89)	9.7 (10)	1.1 (1)
	pUhpT-PhoA141	257.8	238.2 (92)	16.8 (7)	2.8 (1)
	pUhpT-PhoA302	144.5	133.1 (92)	8.8 (6)	2.6 (2)
	pRJK10	158.9	145.1 (91)	11.5 (7)	2.3 (1)
Glucose-6-phosphate dehydrogenase	pUhpT-PhoA57	34.7	1.2 (3)	33.2 (96)	0.3 (1)
	pUhpT-PhoA141	32.9	1.8 (6)	30.8 (94)	0.3 (1)
	pUhpT-PhoA302	26.0	2.4 (9)	23.2 (89)	0.4 (2)
	pRJK10	33.3	2.4 (7)	30.6 (92)	0.3 (1)

[a] The number (e.g., 57) indicates the location of the fusion junction in UhpT.
[b] Sum of the values for the three cell fractions.
[c] The percentage of the total activity that was present in each fraction is indicated in parentheses.

DISCUSSION

Understanding the mechanism of active transport will require knowledge of the structure of transport proteins and their orientation across the membrane. The facile genetic method for the construction of alkaline phosphatase fusion proteins provides an attractive tool for analysis of the transmembrane orientation of membrane proteins. This approach assumes that decreased enzyme activity occurs when the PhoA moiety is constrained to the cytoplasm owing to its fusion to a cytoplasmic portion of a membrane protein. The decreased enzyme activity of cytoplasmic PhoA results from its increased turnover, as demonstrated for *phoA* mutants lacking its signal sequence (2) and for strains with low-activity fusions to GlpT (11) and UhpT.

The low level of enzyme activity displayed by fusions in which PhoA was apparently localized to the cytoplasm could indicate that a small proportion of the unstable protein assumed the native conformation or acquired the cofactor necessary for stability while still in the cytoplasm. However, we found that the same proportion of active alkaline phosphatase was released by osmotic shock from *uhpT-phoA* strains with high, intermediate, or low activity. This result indicates that a small proportion (<10%) of the alkaline phosphatase produced by low-activity fusions was exported across the cytoplasmic membrane in violation of the transmembrane orientation of the region of the target protein at which the fusion occurred. It appears that the improperly exported PhoA moiety in the periplasm is stable and enzymatically active, whereas properly oriented hybrid molecules in the cytoplasm are completely degraded by the turnover process. The small amount of the active species could preclude its detection in labeling studies.

High-activity fusions were obtained in three of the six periplasmic loops predicted by the structural model with the amino and carboxyl termini in the cytoplasm. Fusions to the highly homologous GlpT protein defined a fourth periplasmic loop (11). Low-activity fusions were obtained in two of the seven predicted cytoplasmic segments. The predicted topologies of UhpT and GlpT are similar to those proposed for the *E. coli* lactose permease LacY and the erythrocyte glucose transporter, both of which may possess 12 transmembrane segments with their carboxyl termini accessible to specific antibodies only from the cytoplasmic face (4, 12). No structural reason for the inability to obtain fusions which defined all of the potential extramembranous loops in the *glpT* or *uhpT* systems was obvious, although the growth rates of strains with longer fusions were substantially reduced.

Fusions of *phoA* to any desired location can be constructed by in vitro techniques. However, several of the *uhpT* fusions obtained with Tn*phoA* occurred within predicted membrane-spanning regions. In these cases, high-activity fusions were in inwardly directed transmembrane segments, while low-activity fusions were in outwardly directed segments. In both events, the fusion could prevent the transmembrane region from completing its passage across the membrane and thus would be expected to be located in the compartment determined by the previous extramembranous loop. This possibility reduces the value of *phoA* fusions as predictors of the location of extramembranous domains.

The fusion at amino acid 57 was anomalous in having intermediate activity and stability. It was possible that the first transmembrance segment, which was of moderate hydrophobic character and contained or ended in positively charged residues, was unable to serve as an effective signal sequence. However, the *glpT-phoA* fusion at residue 44 exhibited full PhoA activity (11). This fusion occurred at the end of the first putative transmembrane segment but before the two basic Arg-Lys residues. Perhaps the first transmembrane segment is fully competent for export, but the presence of the basic residues reduces the efficiency of transmembrane insertion unless subsequent transmembrane regions are also present.

A noteworthy feature of the model for UhpT was seen when the predicted transmembrane segments were displayed as a helical wheel. Segments 1, 4, 5, 7, and 10 revealed a striking aromatic moment, defined as the presence of all of at least four aromatic side chains lying along one side (180°C) of the helical cylinder. Aromatic residues could be involved in contacts between helices, while aliphatic side chains make contacts with the phospholipid bilayer.

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