Transmembrane Segment 11 of UhpT, the Sugar Phosphate Carrier of Escherichia coli, Is an α-Helix That Carries Determinants of Substrate Selectivity*

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In Escherichia coli, transport of hexose 6-phosphates is mediated by the P_i-linked antiporter carrier, UhpT, a member of the major facilitator superfamily. We showed earlier that Lys391, a member of an intrahelical salt bridge (Asp388/Lys391) in the eleventh transmembrane segment (TM11) of this transporter, can function as a determinant of substrate selectivity (Hall, J. A., Fann, M.-C., and Maloney, P. C. (1999) J. Biol. Chem. 274, 6148–6153). Here, we examine in detail the role of TM11 in setting substrate preference. Derivatives having an uncompensated anionic charge at either position 388 or 391 (the D388C, D388V, or D388K/K391C variants) are gain-of-function mutants in which phosphoenolpyruvate, not sugar 6-phosphate, is the preferred organic substrate. By contrast, when an uncompensated anionic charge is placed at position 388 (K391C), we observed behavior consistent with an increased preference for monovalent rather than divalent sugar 6-phosphate. Because positions 388 and 391 lie deep within the UhpT hydrophobic sector, these findings suggested that an extended length of TM11 may be accessible to external substrates and probes. To explore this issue, we used a panel of TM11 single cysteine variants to examine the transport of glucose 6-phosphate in the presence and absence of the membrane-impermeant, thiol-reactive agent p-chloromercuribenzosulfonate (PCMBs). Accessibility to PCMBs, together with the pattern of substrate protection against PCMBs inhibition, leads us to conclude that TM11 spans the membrane as an α-helix, with approximately two-thirds of its surface lining a substrate translocation pathway. We suggest that this feature is a general property of carrier proteins in the major facilitator superfamily and that for this reason residues in TM11 will serve to carry determinants of substrate selectivity.

Secondary transport systems use the chemiosmotic energy generated by the movement of ions down their electrochemical gradients to facilitate the accumulation of small solutes (1–3). The best-studied secondary transporters of this sort belong to the MFS (4, 5), an evolutionarily related collection that accounts for a large fraction of known solute transporters (6). This taxonomic group is comprised of single-polypeptide carriers that show great diversity in both substrate specificity and kinetic mechanism. Despite this heterogeneity, most members of the MFS share a common structural theme, one characterized by the presence of 12 transmembrane segments believed to transverse the membrane in α-helical conformation. In select cases, a high preponderance of α-helix has been confirmed by circular dichroism or electron spin resonance spectroscopies (7–9). Similar tests suggest that an unrelated transporter, the Na⁺/H⁺ antiporter, NhaA, also has 12 transmembrane helices, and in this case two-dimensional crystallography has confirmed the inference (10). In no case, however, has the structure of a secondary transporter been solved to a resolution affording molecular analysis.

In the absence of detailed information concerning the structure of such transport proteins, helix relationships and helix function in members of the MFS have been analyzed by less direct genetic approaches, such as second-site suppressor, site-directed, and cysteine-scanning mutagenesis. Application of these techniques has clarified structural features for several important model systems. For example, second-site suppressor and site-directed mutagenesis has been used to identify intra- and interhelical salt bridges in the H⁺/lactose cotransporter LacY, the P_i:sugar 6-phosphate antiporter UhpT, and the H⁺:metal-tetracycline exchanger TetA(B), as well as in the unrelated Na⁺/melibiose carrier MelB (11–18). Cysteine-scanning mutagenesis, along with agents that exploit sulfhydryl chemistry, has provided a way to probe the functional and structural significance of specific amino acid residues in LacY, UhpT, and the oxalate:formate exchanger OxlT (12, 19–22). Such approaches have also given insight concerning residues likely to interact with substrate and helices that line the substrate translocation pathway (19, 20, 22). Taken together, this information has led to formulation of rational models for helix packing in the MFS (12, 14, 23, 24).

In work described here, we examined UhpT, the P_i-linked hexose phosphate antiporter carrier of Escherichia coli (25–27), with an emphasis on the study of TM11. We focused on this target because earlier work showed that one of its residues, Lys391, can play a direct role in determining substrate selectivity (15), suggesting that TM11 lines the substrate translocation pathway. In the present analysis, we used both cysteine-scanning and site-directed mutagenesis to broadly probe the role(s) of TM11 and to ask whether other residues in this segment of TM11 can span the membrane in an α-helical conformation.
segment might also influence substrate selectivity. Our analysis implicates positions 388 and 391 in TM11 as crucial determinants of substrate selectivity and suggests that TM11 is an α-helix, with roughly two-thirds of its surface facing a water-filled translocation pathway.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**Strain XL1-Blue (recA1 endA1 gyrA96 thiI hsdR17 supE44 relA1 lac (F' proAB lacY2 M15 Tn10) (Strategene Cloning Systems) was used for all cloning steps. Strain RK5000 (araD139 ΔargF-lacU169 relA1 rpsL150 thiH166 proAB lacI15 Tn5 502506) (28) served as host for tests of expression and function of plasmid-encoded UhpT. Plasmid pTrcHisC0S6 (Stratagene) was used for all cloning steps. Strain RK5000 (araD139 ΔargF-lacU169 relA1 rpsL150 thiH166 proAB lacI15 Tn5 502506) (28) served as host for tests of expression and function of plasmid-encoded UhpT. Plasmid pTrcHisC0S6 (Stratagene) was used for all cloning steps.

**Immunoblot Analysis—**SDS-polyacrylamide gel electrophoresis was performed using cell extracts without preheating in sample buffer, as described (31, 32). Protein was transferred to nitrocellulose and probed with a peptide-directed rabbit antibody reactive to a UhpT C-terminal epitope (31, 32). Western blots were developed using chemiluminescent HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech), or by the Chemosil and Sequencing Facility of Johns Hopkins Medical School.

**RESULTS**

**TM11 as a Determinant of Substrate Specificity—**In previous work, we had identified Asp 388 and Lys 391 of UhpT TM11 as participants in an intrahelical salt bridge (15). Further study of this ion pair showed that if the anionic partner is replaced by Lys, the substrate specificity is biased toward compounds, such as PEP, that carry an additional positive charge. Such findings confirm that an uncompensated positive charge (lysine) at position 391 alters selectivity so as to favor substrates with an increased anionic character. With this in mind, we next asked whether the location of such charge is also a critical determinant of specificity. To do this, we constructed the D388C/K391C variant to remove both partners in the native salt bridge. This variant, like its parent, is able to transport sugar 6-phosphates but not PEP (Fig. 1). We then scanned this derivative with lysine placed at positions in a registration (i, i ± 3 or 4) that would span four turns of an α-helix. In most

| UhpT derivative | P_i, transport | K_i | 2-DG6P | F6P |
|-----------------|---------------|-----|--------|-----|
| Cysteine-less UhpT (parent) | 46 ± 0.2 | 840 ± 220 | 52 ± 16 | 28 ± 5.4 |
| D388C | 29 ± 0.8 | 580 ± 180 | 1200 ± 39 | nd* |
| D388V | 34 ± 7.5 | 710 ± 260 | 1200 ± 20 | nd |
| D388K/K391C | 8.3 ± 1.1 | 79 ± 26 | 510 ± 18 | 33 |
| K391C | 22 ± 4.5 | 270 ± 75 | 81 ± 28 | 160 ± 44 |

* Rates of P_i self-exchange were measured as described under “Experimental Procedures” using the method of Hofstee (36).

**Kinetic constants** are means ± S.E. for three independent experiments.

**Inhibition constants** (K_i) were determined for each substrate as an inhibitor of P_i, self-exchange using linear Dixon plots (37), with the assumption of competitive inhibition between P_i and the test substrate. The concentration range of inhibitor used was 2.5 μM to 5 μM.

**Growth** was monitored by measuring the optical density at 660 nm.

**The maximal velocity (V_max) and Michaelis constant (K_m) of P_i transport were estimated using the method of Hofstee (36). The inhibition constants (K_i) for 2-DG6P, F6P, or PEP as inhibitors of P_i transport were determined using linear Dixon plots (37), with the assumption of competitive inhibition between P_i and the test substrate.** In these latter assays, the unlabeled test substrates (2-DG6P, F6P, PEP) contained 0.5–1.0% P_i (68), and because this was ignored in calculation of kinetic constants, the derived K_i values should be considered minimal estimates.

To monitor in vivo function of UhpT and its derivatives, cells from an overnight broth culture were diluted 1000-fold into M63 containing 0.15% F6P or 0.15% PEP as carbon source. Cultures were placed at 37°C with continuous shaking, and growth was monitored by changes in optical density at 660 nm.

**Chemicals—** [3H]G6P (52.8 μCi/μmol) and [3H]PEP (30.0 μCi/μmol) were obtained from PerkinElmer Life Sciences. [14C]F6P (300 Ci/mmol) and [14C]PEP (29.0 Ci/mmol) were from American Radiolabeled Chemicals, Inc. and Amersham Pharmacia Biotech, respectively. Unlabeled organophosphate substrates (2-DG6P, F6P, PEP) were obtained from Sigma.

**Immunoblot Analysis—**SDS-polyacrylamide gel electrophoresis was performed using cell extracts without preheating in sample buffer, as described (30). Protein was transferred to nitrocellulose and probed with a peptide-directed rabbit antibody reactive to a UhpT C-terminal epitope (31, 32). Western blots were developed using chemiluminescent HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech), or by the Chemosil and Sequencing Facility of Johns Hopkins Medical School.
cases (positions 384, 387, 395, and 398), the introduction of
lysine into the D388C/K391C background yielded a non-functional
protein (data not shown). It was evident, however, that
placement of Lys at either position 388 or 391 altered UhpT
substrate selectivity so as to favor PEP. Thus, the D388K/
K391C variant showed a 10-fold decrease in affinity for 2-dG6P
substrate specificity.

Our next tests addressed the idea that the uncompensated
negative charge at position 388 biased selectivity toward sub-
strates carrying one negative charge rather than two (i.e.
HG6P$^{-}$ rather than G6P$^{2-}$). If so, one might expect enhanced
transport and growth under more acidic conditions where
monovalent sugar phosphate is enriched. To test this possibil-
ity, we measured transport and growth at external pH values
from pH 5.5 to 8.25, a range that spans the pK$_m$ of sugar
phosphate (pK$_m$ = 6.1). In such work, we found that maximal
transport by the mutant (tested at 50 $\mu$M substrate) occurred at
a pH approximately 0.5 pH units more acidic than found for the
parent protein (pH 6.3 and 6.9, respectively) (Fig. 2).
We also found that the K391C variant, unlike its parent, showed a
marked decrease in function above pH 7. Both observations are
consistent with the idea that the mutant works best when
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marked decrease in function above pH 7. Both observations are
consistent with the idea that the mutant works best when
monovalent sugar phosphate is the predominant species. This
idea was further supported by kinetic work showing that the
K$_m$ for sugar 6-phosphate transport by the mutant increased
4–5-fold at alkaline pH (125–640 $\mu$M), whereas that of the
parental protein remained constant throughout the entire pH
range studied (Fig. 3). In similar fashion, growth of the K391C
variant was pH-dependent. Thus, although the parent pro-
tein supports growth equally well between pH 5.5 and 7.0, the
K391C mutant grows at progressively slower rates as the en-
vironment becomes more alkaline, with complete growth stasis
at pH 7 (Fig. 2, A and B). This finding, coupled with the kinetic
observations (Fig. 3), leads us to suggest that in this variant
the capacity to handle divalent sugar phosphate is com-
promised. This interpretation further emphasizes the region in
and around these positions as essential determinants in sub-
strate specificity.

UhpT TM11 Lies on the Substrate Transport Pathway—
Because the topology of UhpT shows the Lys$_{391}$-Asp$_{388}$ salt
bride to be deep within the hydrophobic sector (15, 19, 39, 40),
its seemed plausible that a water-filled pathway would extend

| Derivative | UhpT expression$^a$ | Specific activity$^b$ (G6P transport) |
|------------|---------------------|--------------------------------------|
| Parent     | 100                 | 100 $\pm$ 6.9                       |
| A383C      | 70                  | 87 $\pm$ 0.8                        |
| E384C      | 63                  | 170 $\pm$ 12                        |
| G385C      | 51                  | 65 $\pm$ 8.1                        |
| A386C      | 98                  | 91 $\pm$ 9.8                        |
| A387C      | 33                  | 260 $\pm$ 16                       |
| D388C      | 84                  | 4.5 $\pm$ 1.2                       |
| G389C      | 91                  | 0.3 $\pm$ 0.3                       |
| I390C      | 27                  | 270 $\pm$ 45                       |
| K391C      | 110                 | 59 $\pm$ 8.7                        |
| G392C      | 55                  | 12 $\pm$ 9.2                        |
| T393C      | 19                  | 390 $\pm$ 14                       |
| F394C      | 63                  | 150 $\pm$ 13                       |
| A395C      | 68                  | 35 $\pm$ 10                        |
| Y396C      | 73                  | 11 $\pm$ 6.9                       |
| L397C      | 66                  | 180 $\pm$ 14                       |
| I398C      | 93                  | 120 $\pm$ 9.2                       |
| G399C      | 31                  | 0.4 $\pm$ 0.6                       |
| D400C      | 63                  | 2.0 $\pm$ 1.2                       |
| S401C      | 50                  | 49 $\pm$ 1.7                       |
| F402C      | 100                 | 56 $\pm$ 14                        |
| A403C      | nr                  |                                     |
| K404C      | 32                  | 47 $\pm$ 4.0                       |

$^a$ Values are means of three independent experiments.

$^b$ Values are means $\pm$ S.E. of three independent experiments.

nr, not recovered.

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| K391C      | 110                 | 59 $\pm$ 8.7                        |
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| A395C      | 68                  | 35 $\pm$ 10                        |
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| L397C      | 66                  | 180 $\pm$ 14                       |
| I398C      | 93                  | 120 $\pm$ 9.2                       |
| G399C      | 31                  | 0.4 $\pm$ 0.6                       |
| D400C      | 63                  | 2.0 $\pm$ 1.2                       |
| S401C      | 50                  | 49 $\pm$ 1.7                       |
| F402C      | 100                 | 56 $\pm$ 14                        |
| A403C      | nr                  |                                     |
| K404C      | 32                  | 47 $\pm$ 4.0                       |

$^a$ Values are means of three independent experiments.

$^b$ Values are means $\pm$ S.E. of three independent experiments.

nr, not recovered.
inward from the periplasm to at least position 388. If so, we reasoned that one might identify the part of TM11 bordering this pathway by use of a suitable probe. Accordingly, we next analyzed a library of single-cysteine variants encompassing the whole of TM11 (residues 383–404), so that individual positions along TM11 could be probed by PCMBs, an impermeant, thiol-directed agent with the same molecular volume and charge as the normal substrates of UhpT (20).

An initial screen of TM11 single-cysteine variants showed that most were expressed at a level sufficient for functional analysis (usually 30%) (Table II). In the preceding work (Table I, Fig. 1; Ref. 15), we found that gain-of-function variants in the parental cysteine-less UhpT (15) and its K391C derivative (15). Assays were performed as described under “Experimental Procedures” except that assay and wash buffers used MES/MOPS rather than MOPS. Kinetic constants are means ± S.E. for three independent experiments.

For a number of membrane transport proteins, use of site-directed and scanning mutagenesis has identified charged residues that contribute to a substrate-binding domain. For example, within the MFS two charged residues (Glu126 and Arg144) coordinate substrate binding by LacY (24), several cationic residues (Arg170, Arg275, and Lys93) function as recognition elements in UhpT (15, 33), and a lysine (Lys355) facilitates substrate binding by OxlT, the oxalate-formate antiporter of Oxalobacter formigenes (22, 41). The MFS also contains examples in which one or more transmembrane helices have been associated with the permeation pathway. Thus, TM5 of the GLUT1 glucose uniporter and TM7 of UhpT are known to line the sugar/sugar phosphate transport pathway (20, 42), as do residues on TM11 of OxlT (41). Similarly, several helices in LacY have been shown to line the pathway taken by lactose (12).

In the present study, we exploited site-directed and cysteine-
scanning mutagenesis, together with the use of thiol-directed probes, as tools to analyze TM11 of UhpT. This focus was based on early work showing that Lys30, normally part of an intrahelical TM11 salt bridge, acts as a determinant of substrate specificity when present without its normal partner, Asp388 (15). This finding showed that at least one residue on TM11 must interact with the UhpT transport pathway. New evidence presented here both reinforces this idea and extends the argument to implicate a substantial portion of TM11 as lining the pathway. Thus, it is now clear that positive charge placed at the boundary of this surface. We also note that this region lies at the substrate C6 (or C5) position. Crystallography of enzyme and receptors that interact with Pi or organophosphates at the polyol ring of its ligands, but crystallography of carbohydrate-protein complexes suggests that this may occur via aromatic amino acid stacking interactions with the furanose/pyranose ring and by hydrogen bonding with at least some of the offshooting hydroxyls (51–53). These interactions are presumed to occur within the hydrophobic core of UhpT, because the arginines required for recognition of the phosphoryl group are located near the periphery. Certainly, the finding that an anionic phosphate pointed toward the arginines required for recognition of the phosphoryl group (44–48; summarized in Ref. 33). (A prominent exception, triose phosphate isomerase, uses lysine rather than arginine (49)). We believe that in UhpT, this role is played by two arginines (Arg46 and Arg275) at the periplasmic poles of TM1 and TM7, about 30 Å from positions 388 and 391. To understand this phenomenon, it is helpful to recall the elements believed to contribute to maintenance of substrate selectivity by the wild type protein. Because UhpT transports sugar phosphates but not sugars, there must be a mechanism that identifies the presence of phosphate at the substrate C6 (or C5) position. Crystallography of enzymes and receptors that interact with Pₐ or organophosphates shows that arginine residues almost always take part in the recognition of the anionic phosphoryl group (44–48; summarized in Ref. 33). (A prominent exception, triose phosphate isomerase, uses lysine rather than arginine (49)). We believe that in UhpT, this role is played by two arginines (Arg46 and Arg275) at the periplasmic poles of TM1 and TM7, about 30 residues prior to the internal duplication characteristic of all members of the MFS (33, 50); only these arginines are both essential and conserved throughout the UhpT family (33). Much less is known about the interaction between UhpT and the polyol ring of its ligands, but crystallography of carbohydrate-protein complexes suggests that this may occur via aromatic amino acid stacking interactions with the furanose/pyranose ring and by hydrogen bonding with at least some of the offshooting hydroxyls (51–53). These interactions are presumed to occur within the hydrophobic core of UhpT, because the arginines required for recognition of the phosphoryl group are located near the periphery. Certainly, the finding that an un-compensated positive charge at position 388/391 allows UhpT to process substrates carrying an additional anionic charge (e.g. PEP) is consistent with the idea that a substrate orients within UhpT with its anionic phosphate pointed toward the periplasm and the C1 position toward the cytoplasm (15). What determines whether substrates on the translocation pathway will be transported? Early work indicated that the exchange reactions mediated by UhpT are electrically neutral in nature (27, 54), and the abrupt alteration in selectivity that accompanies lysine insertion at positions 388 or 391 reinforces

### Table III

| Variant | G6P transport* (200 μM) | PCMBs *K₀.₅ Substrate protection | G6P transport* (+G6P) |
|---------|------------------------|----------------------------------|----------------------|
|         | PCMBs K₀.₅ % | PCMBs | -G6P | +G6P |
| Parent  | 89 ± 5.3 | 2 | 65 |
| A383C   | 110 ± 5.6 | 5 | 70 ± 2.2 |
| I384C   | 84 ± 5.0 | 2 | 56 ± 0.6 |
| G385C   | 140 ± 8.1 | 40 | 4.5 ± 0.6 |
| A386C   | 98 ± 11 | 50 | 22 ± 7.1 |
| A387C   | 67 ± 3.5 | 8.1 | 97 ± 2.4 |
| D388C   | 11 ± 4.2 | 8.1 | 8.1 |
| G389C   | nd | 0 | 0.075 |
| I390C   | 100 ± 13 | 3.0 | 0.1 |
| K391C   | 3.7 ± 1.0 | 5 | 1 ± 0.1 |
| G392C   | 88 ± 4.5 | 4.5 | 47 ± 8.1 |
| T393C   | 26 ± 1.5 | 22 | 100 ± 7.5 |
| F394C   | 73 ± 8.1 | 5 | 65 ± 2.2 |
| A395C   | 0 | 0.1 | 100 ± 2.4 |
| Y396C   | 82 ± 8.1 | 0.6 | 28 ± 5.7 |
| L397C   | 0.8 ± 0.5 | 2.5 | 45 ± 12 |
| I398C   | 0.2 ± 0.2 | 3.4 | 28 ± 5.7 |
| G399C   | nd | 5 | 45 ± 12 |
| D400C   | nd | 14 | 12 |
| S401C   | 3.8 ± 0.9 | 1.7 | 2.4 |
| P402C   | 1.5 ± 0.8 | 1.6 | 2.4 |
| K404C   | 2.4 ± 2.4 | 22 | 2.4 |

* Values are the means ± S.E. for three independent experiments.

* For PEP transport.

** nd, not determined.

* PCMBS inhibition and substrate protection were assayed at a cell density of 2.5 × 10⁶ cells/ml. Coincubation of cells with glucose did not afford substrate protection, ruling out the possibility that this protection, due to the low K₀.₅ of this mutant, reflected increased excretion of reducing compound(s) caused by an amidolyl metabolizable substrate.
the idea that maintenance of electrostatic neutrality is an essential criterion. We also believe that this same view can help interpret the behavior of the K391C variant, which shows a distinct acid shift in the pH optimum for growth and transport (Figs. 2 and 3). We speculate that in this mutant the uncompensated electronegative center, Asp$^{388}$, acts as a resident fixed anion, so that preservation of an electrostatic neutrality would require a selectivity biased toward monovalent sugar phosphate. A consequence of this bias would be that, as observed, the pH optimum for sugar phosphate transport and growth would shift in the acid direction. However, at the sugar phosphate concentrations used for growth studies, the levels of monovalent substrate would exceed the $K_m$ for transport, even at pH 7. Therefore, we believe that the growth phenotype is best explained by changes in the protonation state of Asp$^{388}$. At more alkaline pH, the anionic charge associated with this position would preclude transport of divalent sugar phosphate, because electrostatic neutrality could not be achieved. Protocination of the resident anion would be favored as external pH is lowered, thereby restoring the parental character of the translocation pathway. This interpretation would be unrealistic if the $pK_a$ of Asp$^{388}$ is as low as that found for aspartate in model compounds ($pK_a$ of 4) (55). On the other hand, it is known that the $pK_a$ of aspartate may take on a significantly higher value, depending on the nature of the local environment. It remains feasible, then, that the $pK_a$ of Asp$^{388}$ falls within the range of pH values we have tested (Figs. 2 and 3).

The assumption that substrate preference is influenced by the protonation state of Asp$^{388}$ also allows us to reconcile the contradictory findings that at pH 7 the K391C variant transports but does not grow on sugar phosphate (Fig. 2). UhpT, as do other P$_i$-linked antiporters, carries out both heterologous (P$_i$:sugar phosphate) and homologous (P$_i$:P$_i$, sugar phosphate: sugar phosphate) exchanges (25, 27, 38, 56). One might conclude that heterologous exchange is the preferred reaction in vivo, because homologous exchanges do not usually lead to net substrate fluxes. However, the affinity of UhpT for sugar phosphate is 10–40-fold higher than for P$_i$, suggesting that homologous exchange must be preferred over heterologous reaction (56). Furthermore, because E. coli maintains a slightly alkaline cytoplasmic pH over a wide range of external pH values (57), one expects a reaction in which net sugar phosphate accumulation arises from the electroneutral exchange of two monovalent sugar phosphates (external) for a single divalent species (internal) (25). We suggest that such an asymmetric exchange is not possible for the K391C mutant placed at relatively alkaline pH, because the deprotonated state of Asp$^{388}$ would not permit use of the divalent anionic substrate. Instead, this mutant would only be able to carry out exchange using monovalent sugar phosphate. This might then appear as near normal levels of transport, but the overall 1-for-1 stoichiometry of such a reaction would not support growth.

Our observations also indicate that a large tract of TM11 lines the UhpT translocation pathway. That UhpT TM7 also lies on the translocation pathway (20) is in agreement with the general helix-packaging model proposed by Goswitz and Brooker (23), which suggests that both of these helices are within the core of potential pathway-lining segments in MFS transporters. This model, as well as mutagenesis studies in LacY and the H$^+$/sucrose symporter CscB (17, 58, 59), suggests that TM7 and TM11 may be neighbors in UhpT, an idea that can be tested by disulfide cross-linking using the single-cysteine libraries currently available.

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