Functional Symmetry of UhpT, the Sugar Phosphate Transporter of *Escherichia coli*

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UhpT, the sugar phosphate transporter of *Escherichia coli*, acts to exchange internal inorganic phosphate for external hexose 6-phosphate. Because of this operational asymmetry, we studied variants in which right-side-out (RSO) or inside-out (ISO) orientations could be analyzed independently to ask whether the inward- and outward-facing UhpT surfaces have different substrate specificities. To study the RSO orientation, we constructed a histidine-tagged derivative, His10K291C/K294N, in which the sole external tryptic cleavage site (Lys294) had been removed. Functional assay as well as immunoblot analysis showed that trypsin treatment of proteoliposomes containing His10K291C/K294N led to loss of about 50% of the original population, reflecting retention of only the RSO population. To study the ISO orientation, we used a His10V284C derivative, in which a newly inserted external cysteine (Cys284) conferred sensitivity to the thiol-reactive agent, 3-(N-maleimidylpropionyl)biocytin. In this case, 3-(N-maleimidylpropionyl)biocytin treatment of proteoliposomes containing His10V284C gave about a 60% loss of activity, and immunodetection of biotin showed parallel modification of an equivalent fraction of the original population. Together, such findings indicate that the UhpT RSO and ISO orientations are in about equal proportion in proteoliposomes and that a single population can be generated by exposure of these derivatives to the appropriate agent. This allowed us to study proteoliposomes with UhpT functioning in RSO orientation (His10K291C/K294N) or ISO orientation (His10V284C) with respect to the kinetics of glucose 6-phosphate transport by phosphate-loaded proteoliposomes and also the inhibitions found with 2-deoxy-glucose 6-phosphate, mannose 6-phosphate, galactose 6-phosphate, fructose 6-phosphate, and inorganic phosphate. We found no significant differences in the behavior of UhpT in its different orientations, indicating that the transporter possesses an overall functional symmetry.

In *Escherichia coli*, the *uhp* locus (for uptake of hexose phosphates) coordinates the expression of four proteins responsible for incorporation of external sugar phosphate. UhpA and UhpB together form a two-component regulatory system that activates expression of the transporter, UhpT, after extracellular G6P binds to the membrane receptor, UhpC (1). UhpT then acts to move sugar 6-phosphate inward in exchange for internal inorganic phosphate (2) in an electrically neutral antiport reaction (2, 3). Hydropathy analysis of the UhpT amino acid sequence (4), the properties of UhpT-PhoA fusions (5, 6), and comparisons with other members of the major facilitator superfamily (7, 8) all argue that UhpT has 12 transmembrane α-helices and that its N and C termini lie in the cytoplasm (see Fig. 1). Accordingly, the structure of UhpT (and related transporters) is strongly asymmetric along an axis perpendicular to the membrane surface. This fact, along with the biochemical asymmetry of the *in vivo* reaction, in which external sugar phosphate can exchanged for internal phosphate, raises the question of whether UhpT has an inherent bias in substrate preference at its extracellular and intracellular surfaces.

To address this issue, we used an in *vitro* preparation in which purified protein can be studied in proteoliposomes loaded with inorganic phosphate. Because proteoliposomes might have UhpT in either (or both) a right-side-out (RSO) or inside-out (ISO) orientation, we also designed UhpT derivatives in which one or the other of these orientations could be eliminated by exposure to an appropriate blocking agent. For example, in one case we reasoned that the high proportion of lysine and arginine residues at the UhpT inner surface would confer sensitivity to trypsin (see Fig. 1). To exploit any such side-specific trypsin sensitivity, we constructed a variant lacking the sole tryptic cleavage site at the UhpT extracellular surface (Lys294), so that only RSO molecules would survive exposure to protease. Alternatively, we inserted cysteine (Cys284) into the same extracellular loop (see Fig. 1), creating a mutant inhibitable by low concentrations of MPB, a thiol-reactive agent without effect on wild type UhpT (9); in this case, only ISO molecules would remain active after MPB exposure.

Study of these variants suggests that the RSO and ISO forms are present in about equal proportions in proteoliposomes. This finding, in turn, made it possible to ask whether the two orientations showed differential substrate specificity. For this purpose, we conducted a simple kinetic study of G6P transport by proteoliposomes containing UhpT in mixed, RSO, and ISO orientations and also evaluated the inhibitions exerted by alternative UhpT substrates. We found no substantial differences in the behavior of UhpT in its different orientations, indicating that the transporter possesses functional symmetry.

**EXPERIMENTAL PROCEDURES**

Cells, Plasmids, and Proteins—In His10UhpT, the N terminus of the wild type protein has a polyhistidine extension allowing metal affinity purification (10). Its histidine-tagged derivative, His10K291C/K294N, was generated by double stranded mutagenesis (Chameleon®, Stratagene), using an oligonucleotide specifying two mutations, K291C and K294N, as well as a silent *BstBI* site for purposes of identification; in

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1 The abbreviations used are: RSO, right-side-out; G6P, glucose 6-phosphate; ISO, inside-out; MOPS, N-[morpholino]propanesulfonic acid; MPB, 3-(N-maleimidylpropionyl)biocytin; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane α-helix.

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the His10V284C derivative, a single mutation (V284C) removing an AccI site was introduced. The nature of each mutant was verified by sequencing (Core Facility, Johns Hopkins University). As host for plasmids encoding His10UhpT and its derivatives, we used E. coli strain RK5000, which carries a deletion within the chromosomal uhp locus (4). Expression of His10UhpT and its derivatives was under the control trc promoter (10).

Purification and Reconstitution—To purify His10UhpT and its derivatives (10), membrane vesicles (20–40 mg of protein) (11) were solubilized at 4 °C by addition of 8 ml of buffer A (200 mM NaCl, 100 mM potassium phosphate, pH 8, 50 mM G6P, 10% (v/v) glycerol, 1.5% (w/v) n-dodecyl-b-maltoside, 0.2% (w/v) E. coli phospholipid, 5 mM 2-mercaptoethanol) and mixed with 0.4 mM of nickel-nitrilotriacetic acid-agarose equilibrated with buffer A. After overnight incubation, the mixture was placed in a Poly-PreP® column (Bio-Rad) and washed with 15 ml of buffer B (buffer A prepared at pH 7 containing 50 mM imidazole and 1.5% n-octyl-b-D-glucopyranoside in place of 1.5% n-dodecyl-b-maltoside). Before elution, the column was washed with 0.5 ml of buffer B in which n-dodecyl-b-maltoside replaced n-octyl-b-D-glucopyranoside; bound protein (0.3–0.5 mg) was eluted in 0.5 ml of buffer B with 200 mM imidazole added in place of 200 mM NaCl. Purified protein (5–10 µg) was reconstituted by detergent dilution into proteoliposomes loaded with 100 mM potassium phosphate (pH 7) as described (10), isolated and washed by centrifugation, and finally resuspended (4 °C) in 1 ml of assay buffer (100 mM potassium sulfate, 50 mM MOPS/KOH, pH 7). To study the UhpT RSO orientation, proteoliposomes with His10K291C/K294N were resuspended with or without 1 mg/ml trypsin and then incubated at 33 °C for 1 h before adding 0.3 mM phenylmethylsulfonyl fluoride to stop the reaction. To examine the ISO orientation, proteoliposomes with His10V284C were resuspended with or without 0.2 mM MPB before a quench with 20 mM 2-mercaptoethanol. After either treatment (trypsin or MPB), the suspension was expanded to 20 ml with an ice-cold assay buffer and centrifuged, and proteoliposomes were finally resuspended in 1 ml of assay buffer.

Transport Assay—To assay transport, washed proteoliposomes were diluted 10-fold with assay buffer and preincubated at 23 °C for 3 min before adding 50 µM [14C]G6P. At the indicated times, 100-μl aliquots were filtered on 0.22-μm GSTF Millipore filters and washed twice with 5 ml of assay buffer. To assess competition between G6P and alternate substrates, the test substrate was added together with 50 µM [14C]G6P for 2 min before filtration.

SDS-PAGE and Immunoblot Analysis—After SDS-PAGE (12), protein was visualized by systems directed to either the UhpT N or C terminus. In one case, we used horseradish peroxidase-conjugated nickel-nitrilotriacetic acid (Qagen) to monitor the N-terminal polyhistidine; alternatively, we used a rabbit polyclonal antibody directed against the UhpT extracellular surface (9). This is true for the parental molecule, His10UhpT, was otherwise unaffected by treatment with trypsin or MPB. Thus, with His10K291C/K294N we noted Kmax values of 67 and 39 mM, respectively; if it can be shown that trypsin and MPB act in the same extracellular loop (Fig. 1) to provide a target for the thiol-reactive agent, MPB (9); here, we expected MPB modification of Cys264 to inhibit the RSO but spare ISO proteins.

Initial experiments showed that each variant could be purified in high yield, and a simple kinetic study of [14C]G6P transport showed comparable behavior for both wild type and mutant proteins (Fig. 2); maximal velocities were within a 2-fold range, and in each case we recorded a Michaelis constant (Km) of about 50 µM. We confirmed that treatment of His10K291C/K294N proteoliposomes with trypsin and His10V284C proteoliposomes with MPB gave partial inhibitions (about 50% in each case) as expected if both RSO and ISO populations were present (data not shown; see below). In those cases, we also found that the Km for G6P transport was largely unaffected by treatment with trypsin or MPB. Thus, with His10K291C/K294N we noted Kmax values of 64 and 67 µM, respectively, for the control and trypsin-treated material, whereas with His10V284C, we found Km values of 32 and 39 µM for control and MPB-treated preparations. These findings would suggest that the RSO and ISO orientations have comparable kinetic responses to G6P (Km values of 67 and 39 µM, respectively), if it can be shown that trypsin and MPB act in the manner predicted. In the experiments described below, therefore, our first goal was to provide direct evidence that these probes function as expected.

RSO Molecules Remain after Exposure of His10K291C/K294N Proteoliposomes to Trypsin—The trypsin cleavage site at the UhpT extracellular surface is absent in His10K291C/K294N, leading one to expect that only the lysine/arginine-rich intracellular surface of ISO forms would be sensitive to external trypsin. To test this prediction we studied trypsin sensitiv-

Prior study concluded that Lys294 was the only trypsin-accessible residue at the UhpT extracellular surface (9). This is true for the conditions originally tested (9), but Lys293 also serves as a cleavage site at the high levels of trypsin (1 mg/ml) used in these experiments.
His10K291C/K294N is not a target for such agents (9). Proteoliposomes containing either His10UhpT or His10K291C/K294N (Fig. 3) both proteins showed partial sensitivity at low levels of trypsin (<10–100 μg/ml), whereas at higher levels (>100 μg/ml), although His10UhpT continued to display sensitivity, His10K291C/K294N never fell below about 55% of its initial activity (Fig. 3A). These findings are consistent with degradation of both RSO and ISO forms of His10UhpT but loss of only the ISO population of His10K291C/K294N, and direct tests fully support this interpretation. For example, immunoblots using a C-terminal antibody confirmed that trypsin had acted on the entire His10UhpT population but on only a subset of the His10K291C/K294N present (Fig. 3B, lanes 1–4). A similar conclusion came after examination of duplicate gels probed with the nickel-nitrioltriacetic acid-conjugated peroxidase designed to detect the polyhistidine N terminus (Fig. 3B, lanes 5–8). Thus, cleavage at Lys284 in the RSO population should generate an 18-kDa N-terminal fragment and a 33-kDa C-terminal peptide, and, in fact, both products are visualized in the appropriate blots (arrows in Fig. 3B, lanes 2 and 6). The 18-kDa fragment shows poor recovery, perhaps because small hydrophobic peptides are not well retained on nitrocellulose during transfer from the parent gel.) In this same experiment, we also monitored transport of [14C]G6P (Fig. 3C), and those data, too, supported the idea that trypsin targets both RSO and ISO forms of His10UhpT but only ISO molecules of His10K291C/K294N. Moreover, from the residual activity in His10K291C/K294N (55%), one would conclude that the RSO and ISO forms were present in equal proportion; as noted later, this reduction in activity is quantitatively matched by loss of immunoreactivity at 50 kDa (see below). Together, these data indicate that RSO and ISO orientations are present in about equal amounts after reconstitution of UhpT and that preparations containing only the RSO population can be generated by trypsin treatment of His10K291C/K294N.

**ISO Molecules Remain after Exposing His10V284C Proteoliposomes to MPB**—Work with His10V284C/K294N (Fig. 3) gave positive evidence for RSO molecules in proteoliposomes. To document the activity of the remaining ISO population, we next studied His10V284C, in which a V284C substitution confers high sensitivity to the thiol-reactive agent, MPB (9). (Cys291 in His10K291C/K294N is not a target for such agents (9).) Proteoliposomes containing His10UhpT were not responsive to MPB at concentrations as high as 200 μM, but we found a clear sensitivity on the part of His10V284C, whose activity was reduced by 60% (Fig. 4A), as anticipated if only the RSO population of His10V284C is inhibitable by the maleimide. With this assumption, the data indicate 50% inhibition of the RSO molecules at 10–15 μM MPB. Matos et al. (9) had found a considerably higher apparent sensitivity for this same mutation (50%...
inhibition at 1 μM MPB), but this may be expected because they exposed for both longer time (10 min versus 2 min) and at higher temperature (23 °C versus 4 °C). In this experiment (Fig. 4), we had also exposed MPB-treated and untreated proteoliposomes to the impermanent thiol-reactive probe, [2-(trimethylammonium)-ethyl]methane-thiosulfonate (13). In that case, inhibition of activity (by 55%) was found only for the preparation not previously exposed to MPB (data not shown), supporting the idea that MPB reactivity was confined to the RSO population.

To show MPB modification directly, we used streptavidin to generate a His_{10}V284C-biotin-streptavidin complex whose formation is reflected by loss of immunoreactivity at 50 kDa and its partial recovery at 80–90 kDa (9) (Fig. 4B).3 We found no evidence of such a complex after MPB treatment of His_{10}UhpT proteoliposomes (Fig. 4B, lanes 1 and 2) unless endogenous cysteines had been exposed by a preincubation with SDS. By contrast, denaturation was not required for formation of the complex in parallel trials with His_{10}V284C (Fig. 4B, lanes 4 and 5). Assays of [14C]G6P transport in this same experiment confirmed that MPB modification was, in fact, associated with inhibition of function (Fig. 4C and see below).

**Distribution of RSO and ISO Orientations in Proteoliposomes**—In several experiments, we assessed the relative loss of sugar phosphate transport caused by treatment with trypsin or MPB and compared this with the fraction of protein that remained unmodified as monitored by immunoblots. Both measurements showed parallel change (Table I), and use of either data set leads one to conclude that proteoliposomes contain RSO and ISO orientations in roughly equal proportion.

### Table I

**Distribution of RSO and ISO orientations in proteoliposomes**

In experiments similar to those of Figs. 3 and 4, [14C]G6P transport remaining after treatment with trypsin or MPB was measured, and the fraction of protein unaffected by treatment was estimated by densitometric comparison of control and treated preparations after SDS-PAGE.

| Experiment          | Activity | Protein |
|---------------------|----------|---------|
| His_{10}K291C/K294N | Mean 0.53| 0.46    |
| I                   | 0.56     | 0.61    |
| II                  | 0.48     | 0.38    |
| III                 | 0.55     | 0.39    |
| Mean                | 0.53     | 0.46    |

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3 We noted here, as reported before (9), that reactivity of this complex to the anti-UhpT antibody was inefficient, likely because of steric hindrance caused by bound streptavidin. As a result, formation of the complex with streptavidin is judged by loss of immunoreactivity at 50 kDa.
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and presence of fructose 6-phosphate (top panels) or uniform orientation (open symbols) or RSO orientations using proteoliposomes containing UhpT of mixed orientation (bottom panels), these findings lead us to conclude that the two orientations of UhpT are functionally equivalent with respect to processing of fructose 6-phosphate, mannose 6-phosphate, 2-deoxyG6P, galactose 6-phosphate, and inorganic phosphate.

**DISCUSSION**

The orientation of proteins incorporated into proteoliposomes has been analyzed in several cases (15–21), and those findings suggest a variable result depending not only on the method of reconstitution but perhaps also on the protein in question and the detergent or phospholipid employed (reviewed in Ref. 20; see also Ref. 21). A simple argument developed by Rigaud (20) and others (21) suggests that if reconstitution proceeds by insertion of protein into preformed detergent-destabilized liposomes, one might anticipate a population of uniform orientation due to preferential insertion of the hydrophobic sector into the lipid bilayer. Because the cytoplasmic surface of most membrane proteins is considerably more hydrophilic than the extracellular face, this approach predicts recovery of proteins in a largely ISO orientation, as observed with the Ca$^{2+}$-ATPase (19), the sodium/proline symporter (22), and the chimeric LacS protein (21). By contrast, if reconstitution proceeds from mixed micelles, as in the method used here, the final population is more likely to be of mixed orientation; this latter expectation may also be favored if, as here, the use of high lipid/protein ratios avoids protein packing considerations by ensuring only a single transporter per proteoliposome (3). Despite such arguments, it is clear that direct tests must be used to evaluate each case.

In our work with UhpT, a simple detergent-dilution protocol was used for reconstitution at a high lipid-to-protein ratio (3, 23, 24), with the expectation that this antiporter might be recovered in mixed orientation in proteoliposomes. The availability of specifically engineered variants has allowed experimental verification of this prediction. Thus, use of His$_{10}$K291C/K294N, which lacks the sole extracellular tryptic cleavage site (9), enabled an unambiguous demonstration that about half the recovered activity arises from molecules of a RSO orientation (Fig. 3 and Table I). Access to the ISO population was offered by use of a second variant, His$_{10}$V284C, whose MPB-reactive cysteine allowed inhibition of the RSO orientation without effect on ISO molecules. It is also likely that such maneuvers, especially use of trypsin, are of general utility. Most membrane proteins are enriched for tryptic cleavage sites (lysine and arginine) on their cytoplasmic surfaces (25), so this surface should be targeted preferentially by trypsin. By contrast, a rarity of lysine and arginine at the extracellular surface suggests this surface will have few, if any, tryptic cleavage sites; those that are present may well be susceptible to mutagenesis.

For these reasons, trypsin may serve as a simple diagnostic tool for determining the orientation of reconstituted protein and perhaps also as the basis of a method for generating a preparation of uniformly RSO orientation as illustrated here and in unpublished work in the OxIT, the oxatate/formate antiporter of oxalobacter (26).

Use of His$_{10}$K291C/K294N or His$_{10}$V284C, along with appropriate treatments with trypsin or MPB, made it possible to characterize the UhpT RSO and ISO orientations in a direct way. Our key finding has been that the RSO and ISO forms are functionally equivalent in the handling of substrates of physiological relevance (Table II). On the one hand, as noted earlier, the absence of a $K_m$ shift for G6P after conversion of a population of mixed to single orientations argues that the two UhpT surfaces have roughly comparable kinetic features. We also complemented this observation with a more extended survey

![Figure 5. Apparent $K_i$ values for fructose 6-phosphate and inorganic phosphate. ($^{14}$C)G6P transport was measured in the absence and presence of fructose 6-phosphate (top panels) or inorganic phosphate (bottom panels) using proteoliposomes containing UhpT of mixed orientation (open symbols) or uniform orientation (closed symbols). The protein used to obtain RSO or ISO orientations is indicated on the individual graphs. Dotted lines intersect the abscissa at the apparent $K_i$ value. Given a purely competitive interaction with G6P, for these assay conditions the apparent $K_i$ should be about 80% of the true $K_i$.](http://example.com/figure5)

**TABLE II**

Apparent $K_i$ values of UhpT substrates measured with proteoliposomes containing protein of mixed, RSO, or ISO orientation

As in Fig. 5, $K_i$ values (where specified, ± S.E. from three separate experiments) were determined for the indicated compounds, using His$_{10}$UhpT, His$_{10}$K291C/K294N, and His$_{10}$V284C along with appropriate treatments (± trypsin or ± MPB) to generate RSO or ISO orientations.

| Test substrate   | $K_i$ (μM) |
|------------------|------------|
|                  | RSO + ISO  | RSO + ISO  | RSO         | RSO + ISO  | ISO         |
| 2-DeoxyG6P       | 60 ± 20    | 120 ± 20   | 150 ± 50    | 60 ± 10    | 80 ± 10    |
| Mannose 6-phosphate | 90        | 120 ± 20   | 100 ± 30    | 90 ± 10    | 150 ± 20   |
| Fructose 6-phosphate | 110       | 200 ± 30   | 240 ± 60    | 190 ± 10   | 240 ± 10   |
| Galactose 6-phosphate | 3600 ± 500 | 3800       | 3600        | 3200 ± 100 | 3900 ± 700 |
| Phosphate        | 1300 ± 500 | 4800 ± 100 | 5100 ± 400  | 1900 ± 300 | 2500 ± 300 |
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The finding that UhpT has functional symmetry is compatible with the suggestion of a single substrate binding site alternately accessible from either membrane surface (28, 29), as well as the proposal of a simple Ping Pong kinetic mechanism (28) in which rate-limiting events are not imposed by the pathways substrates use to move into and out of the binding site (29). This last point is of special interest. Current evidence suggests that the pathway is lined by several residues at the center of TM7 (29, 30), and by two required arginine residues, Arg$^{46}$ and Arg$^{275}$, located in equivalent positions with respect to the N- and C-terminal halves of the molecule (8). Because arginine is commonly found in binding sites that accept phosphate or organic phosphates (cf. Ref. 8), it is presumed that Arg$^{46}$ and Arg$^{275}$ contribute to the anionic selectivity mechanism that ensures an overall electroneutral exchange (14, 28). If so, one might expect these residues to lie at the midpoint of the translocation pathway, yet present models of topology (5, 6) place them at its external border (8). This, along with our present finding of functional symmetry of the RSO and ISO forms, suggests that the main rate-limiting events in anion exchange must occur after substrates interact with the binding site and/or selectivity mechanism; such events are presumably associated with changes of protein conformation required to close the pathway after substrate enters from one surface, trapping substrate in an occluded state and then opening the pathway to the other surface, enabling transmembrane passage (29).

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