Altered Substrate Selectivity in a Mutant of an Intrahelical Salt Bridge in UhpT, the Sugar Phosphate Carrier of Escherichia coli*

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Site-directed and second site suppressor mutagenesis identify an intrahelical salt bridge in the eleventh transmembrane segment of UhpT, the sugar phosphate carrier of Escherichia coli. Glucose 6-phosphate (G6P) transport by UhpT is inactivated if cysteine replaces either Asp388 or Lys391 but not if both are replaced. This suggests that Asp388 and Lys391 are involved in an intrahelical salt bridge and that neither is required for normal UhpT function. This interpretation is strengthened by the finding that mutations at Lys391 (K391N, K391Q, and K391T) are recovered as revertants of the inactive D388C variant. Further work shows that although the D388C variant is null for G6P transport, movement of \( ^{32} \text{P} \) by homologous P/P exchange is unaffected. This raises the possibility that this derivative may have latent function, a possibility confirmed by showing that D388C is a gain-of-function mutation in which phospho-opyruvate (PEP) is the preferred substrate. Added study of the P/P exchange shows that in wild type UhpT this partial reaction is readily blocked by G6P but not PEP. By contrast, in the D388C variant, P/P exchange is unaffected by G6P but is inhibited by both PEP and 3-phosphoglycerate. These latter substrates are used by PgtP, a related P-linked antiporter, which lacks the Asp388-Lys391 salt bridge but has instead an uncompensated arginine at position 391. For this reason, we conclude that in both UhpT and PgtP position 391 can serve as a determinant of substrate selectivity by acting as a receptor for the anionic carboxyl brought into the translocation pathway by PEP.

In Escherichia coli, transport of hexose phosphates is mediated by the P-linked antiporter, UhpT (1–3). This well-characterized transporter is one of a class of secondary transport systems that together form the Major Facilitator Superfamily (4–6), the largest known collection of related secondary transporters. Although members of the Major Facilitator Superfamily show great diversity in their substrate specificity and kinetic mechanism, they all share a common structural and kinetic mechanism, they all share a common structural

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The abbreviations used are: TM, transmembrane segment; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PGA, phosphoglyceric acid; 2DG6P, 2-deoxyglucose 6-phosphate; MOPS, 4-morpholinepropanesulfonic acid.

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respectively.  cating anionic or cationic residues, shown as either circles or squares, indicating anionic or cationic residues, respectively.

**Immunoblot Analysis—**SDS-polyacylamide gel electrophoresis was performed using cell extracts without preheating in sample buffer (25). Protein was transferred to nitrocellulose and probed with a peptide-directed rabbit polyclonal antibody reactive to a UhpT C-terminal epitope (26, 27). Western blots were developed using chemiluminescence (Amersham Pharmacia Biotech), and after scanning the films, UhpT expression was quantitated by densitometry of digitized images, as described (27, 28).

Transport Assays—Cells grown overnight in LB broth plus antibiotic were diluted 200-fold in the same medium and grown at 37 °C to a density of 2–5 × 10⁶ cells/ml. Cells were harvested by centrifugation, washed twice, and then resuspended in Buffer A (50 mM MOPS/K, 100 mM potassium sulfate, 1 mM magnesium sulfate, pH 7.0) to achieve an OD₆₆₀ of 1.4, equivalent to about 2 × 10⁷ cells/ml. After equilibration at room temperature, G6P or PEP transport was initiated by adding a one-twentieth volume of labeled substrate (final concentration, 50 μM), after which aliquots were removed for filtration on Millipore filters (0.45-μm pore size) and washing (twice) with 5 ml of Buffer A lacking magnesium sulfate. Transport of Pᵢ was monitored in the same way, using cells pregrown in M63 minimal medium (as above) with 0.2% glucose as carbon source. The high Pᵢ content of M63 (24) ensured that Pi transport occurred via UhpT. To assay transport (and metabolism) of F6P or PEP in vivo, overnight broth cultures were diluted 1000-fold into M63 salts (above) containing 0.15% F6P or 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—Unlabeled substrates (G6P, F6P, 3-PGA, PEP, 2DG6P, and glycerol 3-phosphate), from Sigma, contained 0.5–1.0% inorganic phosphate (30), which was ignored in calculation of kinetic constants. [¹⁴C]G6P (56.6 μCi/μmol) and [³²P]KPi (1 Ci/mmol) were obtained from NEN Life Science Products. [¹⁴C]PEP (38.6 μCi/μmol) was from Amersham Pharmacia Biotech.

**RESULTS**

**Identification of an Intrahelical Salt Bridge—**Our main objective was to identify possible inter- or intrahelical salt bridges that might exist among or within UhpT transmembrane helices. The topological map of this transporter (Fig. 1), as well as results from earlier studies (28), led us to choose four cationic residues (Lys⁸₂, Arg⁷⁷, Lys⁴⁰⁴, and Lys⁴⁹⁴), and four anionic residues (Asp²⁷⁹, Glu³⁰⁵, Asp³⁸⁸, and Asp³⁹⁴) as candidate participants in salt bridges. Our experimental approach to this question then proceeded in two phases. Initially, we replaced each target with cysteine to determine whether normal function was disrupted. For those cases in which a significant deficit was recorded, we then asked whether function was preserved if a residue of like charge was present (Table I). In this screen, only the K404C variant retained partial function, indicating that most of our target residues were essential for G6P transport by UhpT. In most of these latter cases, it appeared that this requirement was specific, because only for position 279 and 388 was it possible to substitute a residue of like charge and recover significant function (Table I).

The second step in our analysis was based on the fact that three pairs of these residues were in a registration (i, i+3) or (i, i+4) consistent with their participation in a salt bridge in an α-helix. Members of the remaining pair, Lys⁸₂ and Glu³⁰⁵, were at the center of their respective helices, where they too might interact (7). Accordingly, we generated double mutants in which cysteine residues replaced each target pair (Table II). This test provided clear positive evidence that Asp³⁸⁸ and Lys³⁹⁴ might normally interact as an ion pair, because sugar phosphate transport in the D388C/K391C double mutant was about half that found in the wild type protein (Table II); when normalized to UhpT expression, the specific activity of this double mutant was about 75% that of the wild type.² In no other case, however, did the specific activity in the double mutants rise above about 1% of the parental protein. Thus, this approach did not provide evidence of interaction for our other targets.

**Isolation of Second Site Suppressor Mutants—**To further test the idea of an interaction between Asp³⁸⁸ and Lys³⁹¹, we isolated revertants of the inactive D388C mutation by screening for growth on F6P as a carbon source. This genetic screen was initiated with the expectation that if Asp³⁸⁸ and Lys³⁹¹ interact so as to eliminate an uncompensated charge, at least some revertants of D388C might map to position 391. We performed this screen in an otherwise cysteine-less background rather than with the wild type protein, because the partially reduced activity of the cysteine-less protein (compare Tables I and III) allowed for a denser background lawn on minimal plates,²

![FIG. 1. UhpT topology. Shown is the topology of UhpT as derived from the analysis of hydrophathy (30), the results of reporter gene fusions in UhpT and GlpT (31, 32), and the accessibility of residues in UhpT to impermeant probes (27). Targets for mutagenesis in this study are shown as either circles or squares, indicating anionic or cationic residues, respectively.](Image)

**TABLE I**

| UhpT derivative | G6P transport |
|-----------------|--------------|
| Wild type       | 82.1         |
| K82C            | 0.7          |
| R275C           | 1.9          |
| D279C           | 0.2          |
| E305C           | 3.6          |
| D388C           | 1.0          |
| K391C           | 0.2          |
| D400C           | 2.8          |
| K404C           | 23.8         |
| K82R            | not recovered|
| R275K           | 0.3          |
| D279K           | 18.4         |
| E305D           | 1.9          |
| D388E           | 20.7         |
| K391R           | 1.1          |
| D400E           | 0.2          |

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increasing the frequency of revertants. In addition, this cysteine-less parental protein had an N-terminal polyhistidine tag (20) to facilitate later biochemical work.

As noted under “Experimental Procedures,” we were able to eliminate revertants arising from mutations outside the UhpT structural gene. Those that remained contained the original D388C mutation along with a second site suppressor at position 391 in which Lys (\textsuperscript{391}) was replaced by one of three uncharged residues, threonine, glutamine, or asparagine.

We characterized these revertants in several ways. For example, phenotypic growth tests in liquid medium confirmed that each of these double mutants utilized F6P (Fig. 2). Indeed, they grew slightly more rapidly than did the parental, cysteine-less protein (Fig. 2). By contrast, the host strain, RK5000 (\textsuperscript{100\%}), and the D388C mutant showed greatly delayed responses; eventual growth of these strains presumably reflects the appearance of adaptive revertants arising after slow growth under strong selective pressure (33, 34). We also examined the three suppressor mutants with respect to their expression of UhpT and their capacity to transport G6P (Table III). In the wild type protein, the D388C mutation had reduced UhpT expression nearly 2-fold (not shown), and a similarly reduced expression was found when the D388C mutation was placed in the cysteine-less background (Table III). In both cases, however, G6P transport was reduced to near background levels (Tables I and III). By contrast, in all suppressor mutations, both UhpT expression and G6P transport were elevated to levels resembling that found in the parental protein. It is evident, then, that neither Asp (\textsuperscript{388}) nor Lys (\textsuperscript{391}) are required for normal UhpT function. Equally clear, the presence of one without the other is detrimental. This behavior strongly argues that Asp (\textsuperscript{388}) and Lys (\textsuperscript{391}) form an ion pair in TM11.

**Altered Substrate Specificity of the D388C Mutant—UhpT**

and its relatives comprise a coherent family of transporters and receptors (4, 6), each having high specificity for some organic phosphate ester and low affinity toward inorganic phosphate (1). This family has twelve bacterial members, seven of which have been functionally characterized (28). A multiple alignment of the amino acid sequences within this group (28) suggests that some residues within TM11 are highly conserved. This is not true, however, for residues assigned to the intrahelical salt bridge in UhpT, Asp (\textsuperscript{388}) and Lys (\textsuperscript{391}) (Fig. 3). Instead, most family members have either an aliphatic (Ala or Val) or polar (Ser, Thr, or Asn) residue at these positions. However, in one case, the phosphoenolpyruvate transporter of *Salmonella typhimurium*, we noted the presence of Val (\textsuperscript{388}) and Arg (\textsuperscript{391}). This was of interest for two reasons. First, the presence of an apparently uncompensated positive charge in the PgtP TM11 contrasted sharply with evidence that this was detrimental to UhpT. Second, because of their acidic carboxyl group, substrates of PgtP (PEP and PGAs) (35, 36) carry one more negative charge than do substrates of UhpT. For this reason, we considered it feasible that Arg (\textsuperscript{391}) in PgtP might serve as an internal receptor for the anionic carboxyl on PEP and PGA. And, if so, we reasoned that the D388C variant in UhpT, which no longer transports PEP or G6P, might show an unexpected bias toward transport of PgtP substrates due to the remaining Lys (\textsuperscript{391}).

To test this idea, we exploited the fact that UhpT and PgtP (and other members of this family) each display a F\textsubscript{1}, F\textsubscript{2} self-exchange reaction (3, 37, 38). Thus, if the UhpT D388C derivative...
not respond to sugar 6-phosphates (Table III). Accordingly, for both wild type and mutant proteins, we ranked putative substrates with respect to their capacity to inhibit Pi self-exchange. As expected from earlier work (29), this reaction was strongly inhibited by G6P or the nonmetabolizable 2DG6P in wild type UhpT, whereas PEP and 3-PGA were ineffective inhibitors (Fig. 5A). These findings contrasted sharply with results obtained with the D388C variant, which was strongly inhibited by PEP and 3-PGA but not by G6P and 2DG6P (Fig. 5B). Neither protein responded to glycerol 3-phosphate, a substrate of the related GlpT antiporter (37, 38). To quantitate these observations, we calculated for each substrate an inhibition constant ($K_i$) from linear Dixon plots (40) (Fig. 5C and Table IV). This showed that the D388C mutation causes a 50–100-fold decrease in the $K_i$ value for normal UhpT substrates while at the same time increasing by 20-fold the apparent affinity for PEP or 3-PGA.

These findings strongly suggested that D388C is a gain-of-function mutation in UhpT, and to test this directly we examined this protein with respect to both growth on and transport of PEP. The first test clearly documented that the D388C variant is expressed at only 30% identity to UhpT. Residues conserved in at least five family members are highlighted with shading; charged residues are shown in bold type. The alignment is modified from Fann et al. (28) by addition of GlpT from Rickettsia prowazekii (52).

The study of membrane transport proteins reveals several instances in which a functional or structural role can be assigned to one or more charged residues in a transmembrane helix. The former category is well represented by several cases in E. coli, including two charged residues (Glu$^{126}$ and Arg$^{144}$) thought necessary for substrate binding in the lactose permease, LacY (8), a pair of cationic residues (Arg$^{46}$ and Arg$^{275}$) that may function as substrate recognition elements in UhpT (28), and acidic residues (Asp$^{51}$ and Asp$^{120}$) that help set co-ion specificity for MelB, the Na$^+$/melibiose cotransporter (41). Such residues may also act as stabilizing factors by virtue of their interaction in a salt bridge. Thus, in bacteriorhodopsin, Arg$^{227}$ and Asp$^{396}$ form an interhelical salt bridge, whereas Arg$^{392}$ and Asp$^{355}$ are involved in an intrahelical ion pair (14). Interhelical salt bridges in LacY have also been recognized (9, 17); indeed, these have been indispensable to development of current models of helix packing in this symporter (9, 17). Similarly, it has been proposed that Arg$^{399}$ and Glu$^{503}$ form an ion pair in the Na$^+$/glucose cotransporter, SGLT1 (42). In the present study, we used site-directed and second site suppressor mutagenesis as tools to ask if charged residues in the UhpT hydrophobic sector might take part in salt bridges, basing our interpretation on the original analysis of salt bridges in LacY (43). This approach highlighted two residues in TM11, Asp$^{398}$ and Lys$^{391}$, as interacting in an intrahelical ion
pair. Thus, sugar phosphate transport was not found after individual replacement of either Asp388 or Lys391, but function was retained when uncharged residues replaced both of them (Tables II and III).

Initially, the null phenotype arising when Asp388 and Lys391 were removed individually led us to believe that UhpT function was disrupted due to inappropriate helix packing, but this view did not easily explain why a related antiporter, PgtP (32% identity), contained an apparently uncompensated arginine at position 391. We therefore considered the possibility that lack of function in some of our derivatives was related to altered substrate specificity rather than a change in structure. This seemed more likely when we found that Pi self-exchange was unaffected by the D388C mutation (the K391C mutant has not been tested). Subsequently, by using this partial reaction as a screening tool, we showed that D388C is a gain-of-function mutation that biases substrate preference toward PEP and 3-PGA and away from G6P and F6P (Figs. 4–6 and Table IV).

Two questions are raised by our findings. 1) What is the molecular basis of the new substrate specificity in the D388C variant? 2) Why is there at the same time a discrimination against normal UhpT substrates? No definitive answers can be offered to either question, but a single perspective does address both issues. An early model (44) specified that two electropositive centers in UhpT associate with a pair of negative charges brought into the active site by the anionic substrates, Pi and G6P; indeed, we recently identified two required arginines (Arg46 and Arg275) that may fulfill this function (28). If the configuration that accepts sugar phosphate must achieve an

![FIG. 5. Substrate specificity of UhpT and its D388C derivative.](image)

**TABLE IV**

Inhibition constants for UhpT substrates

Inhibition constants ($K_i$) were determined for each test substrate as an inhibitor of $P_i$ self-exchange, using linear Dixon plots (40) as reported earlier (see Fig. 5), with the assumption of competitive interaction between $P_i$ and the test substrate. G3P, glycerol 3-phosphate.

| Test substrate | $K_i$ (μM) Wild type UhpT | $K_i$ (μM) D388C UhpT |
|---------------|--------------------------|----------------------|
| G6P           | 22 ± 5                   | 1100 ± 480           |
| 2DG6P         | 17 ± 4                   | 1500 ± 440           |
| PEP           | 1200 ± 150               | 98 ± 36              |
| 3-PGA         | 2400 ± 290               | 160 ± 48             |
| G3P           | 4900                     | 5900                 |

![FIG. 6. Growth on and transport of PEP.](image)
electroneutrality, one might expect that substrates such as PEP and PGA, which carry an additional negative charge relative to G6P, might require the presence of a third positive center before productive binding can occur. The second question is answered in much the same way. That is, when the D388C variant is presented with G6P, failure to achieve an electrostatic balance at the sugar phosphate binding site would be associated with an abortive carrier-substrate complex.

Although such arguments account for our findings related to transport of PEP or G6P, this reasoning does not directly explain why the P\(_e\) self-exchange is unimpaired in the D388C mutant. At the least one must conclude that TM11 is not involved in binding of P\(_e\). This might be expected if the P\(_e\) self-exchange exploits a different configuration of the protein (44), possibly relying predominantly on factors related to either or both of the two essential arginine residues, one of which lies in TM1 (Arg\(^{46}\)), the other in TM7 (Arg\(^{275}\)). The absence of either of these inactivates G6P transport (28), but in light of the work reported here, it could be instructive to examine the P\(_e\) self-exchange reaction as these arginines are removed individually.

The finding of PEP transport by the D388C mutant also leads one to question the stoichiometry and electrical character of the reaction. The kinetic parameters of P\(_e\) self-exchange by D388C deviate little from its parent (Fig. 4), implying that the D388C variant is presented with G6P, failure to achieve an electrostatic balance at the sugar phosphate binding site would be associated with an abortive carrier-substrate complex.

Fig. 4. Kinetics of P\(_e\) self-exchange by wild type UhpT and the D388C variant. The wild type and the mutant are compared with respect to G6P and PEP in the reaction. The effect of removing individual arginines in the TM helix on the kinetic parameters for the P\(_e\) self-exchange reaction is illustrated in the figure. The removal of Arg\(^{304}\) leads one to question the stoichiometry and electrical character of the reaction as these arginines are removed individually.

Fig. 5. Alignment of arginine residues associated with the electrostatic balance at the sugar phosphate binding site. The arginines are arranged with respect to the helical axis and are shown in the open (G6P) and close (PEP) conformation of the sugar phosphate moiety. The electrostatic balance at the sugar phosphate binding site would be associated with the abortive carrier-substrate complex.

Fig. 6. Position of the arginine residues associated with the electrostatic balance at the sugar phosphate binding site. The arginines are arranged with respect to the helical axis and are shown in the open (G6P) and close (PEP) conformation of the sugar phosphate moiety. The electrostatic balance at the sugar phosphate binding site would be associated with the abortive carrier-substrate complex.