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Residues in the eighth transmembrane domain of the proton-coupled folate transporter (SLC46A1) play an important role in defining the aqueous translocation pathway and in folate substrate binding

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Abstract

The proton-coupled folate transporter (PCFT-SLC46A1) is required for intestinal folate absorption and folate transport across the choroid plexus. This report addresses the structure/function of the 8th transmembrane helix. Based upon biotinylation of cysteine-substituted residues by MTSEA-biotin, 14 contiguous exofacial residues to Leu316 were accessible to the extracellular compartment of the 23 residues in this helix (Leu303–Leu325). Pemetrexed blocked biotinylation of six Cys-substituted residues deep within the helix implicating an important role for this region in folate binding. Accessibility decreased at 4°C vs RT. The influx K_t , K_i and V_{max} were markedly increased for the P314C mutant, similar to what was observed for Y315A and Y315P mutants. However, the K_t , alone, was increased for the P314Y mutant. To correlate these observations with PCFT structural changes during the transport cycle, homology models were built for PCFT based upon the recently reported structures of bovine and rodent GLUT5 fructose transporters in the inward-open and outward- open conformations, respectively. The models predict substantial structural alterations in the exofacial region of the eighth transmembrane helix as it cycles between its conformational states that can account for the extended and contiguous aqueous accessibility of this region of the helix. Further, a helix break in one of the two conformations can account for the critical roles Pro314 and Tyr315, located in this region, play in PCFT function. The data indicates

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that the 8th TMD of PCFT plays an important role in defining the aqueous channel and the folate binding pocket.

Graphical abstract

Fourteen contiguous residues of the 8th transmembrane domain (pink) are accessible to MTSEA-biotin. Accessibility of six of these residues (Blue type: _AA_HLP_L) is blocked by folate substrate. A homology model in the inward- and outward- open conformations suggests that flexibility in the exofacial region during carrier cycling can account for the extended accessibility.

Keywords

homology modeling; transporter; membrane transport; transmembrane domain; folate; Slc46A1

1. Introduction

Proton-coupled solute transporters are expressed in the proximal small intestine where they interact with their substrates within an acid microenvironment generated by sodium-proton exchangers (1). One of these transporters, the proton-coupled folate transporter (PCFT-SLC46A1), mediates transport across the apical membrane of the proximal small intestine, the initial step in the absorption of folates (2,3). PCFT is also required for the transport of folates across the blood:choroid plexus:cerebrospinal fluid barrier (3,4). When there is loss of PCFT function due to mutations in this gene, as occurs in the autosomal recessive disorder, hereditary folate malabsorption (OMIM #229050), there are defects in both processes resulting in severe systemic and cerebral folate deficiency (2,5,6).

This laboratory has explored the structure-function of PCFT using a variety of approaches to characterize the secondary structure, the aqueous translocation pathway and residues critical to function. The topology of the transporter has been defined as well as residues that play a role in proton-coupling, proton binding, the affinity of the carrier for its folate substrates and the rate of oscillation of the carrier among its conformational states (4,7–13). Recently, transmembrane helices were identified that participate in the formation of a gate at the external interface of the aqueous pathway (14). One apparently unusual property of this transporter is the increase in the influx K_t , K_i and V_{max} for its folate substrates when some residues are mutated to cysteine and other amino acids. Of note were three tyrosine residues located at the aqueous-lipid membrane interface, thought to play a role in anchoring the

transport protein to the membrane through cation- π interactions with the aromatic ring. The functional changes found when these residues were mutated were attributed to their role in the wild-type PCFT in maintaining the transporter in a high affinity configuration while constraining the rate at which the carrier alternates among its conformational states (15). However, a fourth - Tyr315, was located at a predicted breakpoint in the mid-region of the 8th transmembrane domain (TMD) (15).

To explore this phenomenon further, and to specifically evaluate the role of the helix that harbors the Tyr315 residue, the substituted cysteine-accessibility method was utilized to assess the accessibility of all residues in the 8th transmembrane domain (TMD) along with their role in the structure and function of PCFT. Fourteen contiguous Cys-substituted residues in the external portion of this helix (L303C–L316C), extending deep into the membrane, were found to be accessible to the aqueous compartment based upon biotinylation with MTSEA-biotin and inhibition of function by sulfhydryl-reacting reagents. Further, while none of the residues when mutated was absolutely required to sustain function, Pro314 like Tyr315 located at a predicted helix break-point resulted in a large increases in influx K_t , and K_i and V_{max} when mutated to cysteine or alanine. To understand the basis for these observations within the context of the three dimensional structure, homology models were built to simulate the structure of PCFT in both the inward-open and outward-open conformations based on the recently accomplished structural characterization of bovine and rodent Glut5 fructose transporters, the first structures obtained for a mammalian transporter in both conformations (16). The models predict substantial structural alterations in the carrier as the protein cycles between its two conformational states. These changes can account for the extended accessibility of the exofacial region of the helix and the critical roles Pro314 and Tyr315 play in PCFT function.

2. EXPERIMENTAL PROCEDURES

2.1 Chemicals

[³H] MTX {[3,5,7-³H(N)]-MTX} and [³H]pemetrexed (generally labelled) were obtained from Moravек Biochemicals (Brea, CA). EZ-Link sulfo-NHS-LC biotin [sulfo-succinimidyl-6-(biotinamido) hexanoate] and streptavidin agarose beads were from Fisher Scientific (Pittsburgh, PA) and protease inhibitor cocktail from Roche Applied Science (Mannheim, Germany). *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin), methanethiosulfonate-ethyltrimethylammonium (MTSET⁺) and methanethiosulfonate-ethylsulfonate (MTSES⁻) were obtained from Biotium (Hayward, CA).

2.2 Cell lines, culture conditions, transfection procedure

The HeLa-R1-11 cell line, that lacks both PCFT and RFC expression, was used in these experiments as the transfection recipient (16–18). Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (Gemini Bio-Products, Irvine, CA) containing 2 μ M folic acid, 100 U/ml penicillin, 100 μ g streptomycin and grown under 5% CO₂. For transport studies, 3 $\times$ 10⁵ cells were seeded in 17-mm glass vials; for Western blots, 1 $\times$ 10⁶ cells were seeded per well in 6 well plates. After 48 h, cells for transport studies were transfected with the PCFT constructs (0.8 μ g DNA/vial); while cells for Western blots were transfected with

1.6 µg DNA/well after 24 h. Two percent lipofectamine 2000 (Invitrogen, Carlsbad, CA) was utilized in serum- and antibiotic- free RPMI 1640 medium.

2.3 Transport measurements

Transport measurements were made 48h post-transfection in cells grown in a monolayer at the bottom of the glass vials. In preparation for experiments, the medium was aspirated and the cells washed twice with HBS buffer (20 mM dextrose, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM dextrose, at pH 7.4). Then, 2 mL of HBS buffer was added, the cells incubated for 20 min at 37°C following which the buffer was aspirated and 0.5 mL of tritiated substrate in pre-warmed transport buffer was added. MBS buffer was used for studies at pH 5.5 (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM dextrose) and HBS buffer was used for transport studies at pH 7.4. After the desired uptake interval was achieved, ice-cold HBS buffer (pH 7.4) was added to stop the transport process following which cells were washed three times with HBS buffer at 0°C. After the washes, 0.5 mL of 0.2N NaOH was added to each vial followed by incubation at 65°C for 1 h. The digest (0.4 mL) was then assayed on a liquid scintillation spectrometer; 20 µL was used for measurement of protein by the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA).

2.4 Site-directed mutagenesis

All the residues in the 8th TMD were individually substituted with cysteine or other amino acids using the Quickchange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) in a pcDNA3.1+ vector. A modified PCFT scaffold (PCFT-DSL, “disulfide-less) was used as the template in all experiments. In this construct, serine was substituted for Cys66 and Cys298 and an HA (hemagglutinin) tag was added to the C-terminus. This template eliminates the disulfide bond and reaction with a sulfhydryl-reacting reagent should the procedure require pretreatment with dithiothreitol to remove a modifying molecule that interferes with biotinylation and function (13,14). This results in only a negligible decrease in function (13,14). The sequence of the open reading frame of all PCFT mutants was verified at the Albert Einstein Cancer Center Genomics Shared Resource.

2.5 PCFT expression and the substituted cysteine accessibility method

PCFT protein at the cell surface was determined by biotinylation of accessible lysine residues 48 h post-transfection. Cells were washed twice with HBS buffer (pH 7.4) and treated with EZ- Link Sulfo-NHS-LC-biotin in HBS buffer (pH 7.4) at room temperature for 30 min. The cells were then washed twice with HBS buffer (pH 7.4) and with 0.7 mL hypotonic buffer (0.5 mM NaHPO₄ and 0.1 mM EDTA at pH 7.0) and protease inhibitor cocktail was added. Unless otherwise indicated, all the steps were carried out at 4°C. Cells were scraped from the plates using cell lifters and centrifuged at 16,000 × g for 15 min. The membrane fraction was resuspended in 0.4 mL of lysis buffer (0.1% SDS, 1% triton X-100, 1 mM EDTA, and 150 mM NaCl, and 20 mM tris, pH 7.4); 50 µL of supernatant was stored as the crude membrane fraction. The remainder of the supernatant was rotated on a Rotamix for 1 h then centrifuged at 16,000 × g for 15 min. The supernatant obtained was mixed with 50 µL streptavidin-agarose beads (pre-washed in lysis buffer) and rotated overnight. After incubation, the beads were washed twice with 0.5 mL lysis buffer, then twice with 0.5 mL of

lysis buffer containing 2% SDS following which the beads were centrifuged and pelleted at $21,000 \times g$ for 1 min at room temperature. Seventy five microliters of 2X Laemmli buffer (containing DTT) was added to the pelleted beads, heated at 95°C for 5 min, then centrifuged at $21,000 \times g$ for 3 min to remove the beads from the sample. An equal volume (50 μL) of Laemmli buffer (containing DTT) was added to the crude membrane fraction. Fifteen μL from the crude membrane fraction and the pull-down sample were then loaded onto 12% SDS-PAGE.

MTSEA-biotin was used to measure the accessibility of the cysteine residues substituted in the mutant PCFT constructs employing a similar procedure. MTSEA-biotin was dissolved in 2mg/100 μL DMSO then diluted 1:100 with HBS buffer (pH 7.4). Transient transfected cells were washed twice with HBS and incubated with 1 mL of MTSEA-biotin solution for 30 min at RT or 4°C following which the samples were processed as described for surface labelling. Some samples were treated first with 10 mM dithiothreitol (DTT) to assess whether the cysteine residue might be covalently linked to an endogenous sulfhydryl-reacting molecule that would prevent the reaction with MTSEA-biotin. For protection assays, cells were pre-treated with 10 mM DTT, washed and treated with 1 mM pemetrexed or HBS alone prior to MTSEA-biotin labeling.

2.6 Gel electrophoresis and Western blot analysis

Protein samples were resolved on 12% polyacrylamide gels (BioRad) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). After electroblotting, PVDF membranes were blocked with 10% nonfat dry milk in Tris-buffered saline (20 mM Tris base, 135 mM NaCl, 0.1% Tween-20 at pH 7.4). The membranes were then washed in 0.1% Tween-20 buffer, probed with anti-HA antibody (Cat. No. H3663, Sigma, St. Louis, MO) for 90 min at room temperature, and treated with HRP conjugated anti-IgG secondary antibody (Cat. No. 7074S, Cell Signaling Technology, Danvers, MA) under rocking conditions for 1 h. Blots were developed with ECL Plus reagent (Perkin Elmer, Shelton, CT). Actin was used as an internal loading control and probed with mouse anti-actin primary antibody (Cat. No. A5441, Cell Signaling Technology).

2.7 Statistical analysis

Statistical analyses were performed with a two-tailed paired t-test using GraphPad Prism 7.

3. RESULTS

3.1 The impact of Cys-substitutions on PCFT expression and function

Based upon the secondary structure of human PCFT, as indicated in Figure 1 (7,11–14), residues spanning from Leu303 to Leu325 are predicted to form the 8th TMD. To assess the accessibility of these residues to the extracellular compartment and their role in PCFT function, all the residues were replaced, individually, with cysteine in a PCFT scaffold that lacks two cysteine residues that form a disulfide bond between Cys66 and Cys298 within the 1st and 4th external loops (PCFT-DSL), respectively (7,14).

As indicated in Figure 2A, all Cys-substituted constructs trafficked to the cell membrane as assessed by biotinylation of lysine moieties at the cell surface. However, there was a modest decrease in the level of expression of the S318, L322C and K323C mutants in both the crude membrane preparation and at the cell surface.

Transport function, assessed by transfection of the constructs into RFC-/PCFT- null R1-11 Hela cells was determined at 0.5 μ M and 50 μ M [3 H]methotrexate (MTX), concentrations below and an order of magnitude above the influx K_t for MTX, respectively, at the optimal pH for this transporter of 5.5 (Figure 2B). Most of the Cys-substituted residues retained 75% of the function of PCFT-DSL at both concentrations. The most striking functional changes were the P314C and Y315C mutants for which there was a marked increase in transport at the high concentration consistent with a marked increase in influx V_{max} . For the former, transport at the low concentration was unchanged, for the latter it was modestly decreased. Since the V_{max} should increase transport rates by the same percentage irrespective of concentration, the low or unchanged rates at the low concentration are consistent with a large parallel increase in the influx K_t . Five other Cys-substituted residues (S308C, A309C, Q311C, K323C and L325C) showed a modest (~2-fold) increase in function at the high MTX concentration without a significant change at the low concentration, a pattern again consistent with an increase in both influx K_t and V_{max} .

Three Cys-substituted PCFT constructs had decreased function at both MTX concentrations, I304C, S318C, and L322C. The decrease in function for the S318C mutant paralleled the decrease in protein expression. When Leu322 was substituted with serine, function was comparable to L322C; when substituted with valine, function was only modestly reduced at the low concentration (data not shown). When I304C was substituted with serine, activity was restored. Function was decreased for the G305C and G307C PCFT mutants at the low concentration alone consistent with an increase in the K_t . When the Gly305 and Gly307 residues were individually substituted with Ala, there was no change in function at the low concentration but the rate was increased ~2-fold for both mutants at the saturating concentration (Figure 2C). Hence the nature of the residue substituted is a determinant of the nature of the transport changes. The alanine substitution produced changes consistent with the high K_t and V_{max} phenotype.

3.2 Accessibility of the Cys-substituted mutants

Cys biotinylation with the membrane impermeant MTSEA-biotin reagent was employed to identify residues accessible to the extracellular compartment and the aqueous translocation pathway. As indicated in Figure 3A, illustrating an experiment conducted at room temperature, residues from L303C to L316C were biotinylated; residues from T317C-L325C were not. None of the latter residues became accessible when treated with DTT before exposure to MTSEA-biotin, excluding modification by an endogenous sulfhydryl-reacting molecule as previously described (13,14). Consistent with previous reports, none of the endogenous Cys residues were accessible nor was there a non-specific reaction of MTSEA biotin with another residue in the PCFT-DSL. To determine a potential role for the accessible residues in folate binding, these studies were repeated with MTSEA-biotin in the presence or absence of pemetrexed, an antifolate with the highest affinity for PCFT among

its folate and antifolate substrates. The first analysis (Figure 3B) was conducted at 0°C to assess whether conformational changes in the carrier would impact on accessibility and/or protection. This reduction in temperature eliminated the accessibility of the P314C and L316C residues, in contrast to the study at room temperature, and decreased the accessibility of the A310C and L313C residues. Pemetrexed inhibited biotinylation of the A309C, A310C and L313C PCFT mutants and to a lesser extent the H312C and Y315C mutants at 0°C but had no effect on accessibility of the other residues. To further evaluate the apparent temperature-related differences, accessibility and inhibition by pemetrexed was assessed at 0°C (Fig. 3C, lower left panel) and at room temperature (Fig. 3C, lower right panel) simultaneously. At room temperature A310C, L313C, P314C, Y315C and L316C were strongly biotinylated and, in the case of L313C, P314C and L316C, this was blocked by pemetrexed. Biotinylation of P314C and L316C was again lost at 0°C, but pemetrexed protection of biotinylation of A310C was greater at 0°C than at room temperature. Taken together A309C, A310C, H312C, L313C, P314C and L316C appear to play a role in pemetrexed binding by their location in or near to the folate binding pocket. A similar pattern was observed when MTX was used in the protection assay (data not shown).

3.3 Effect of MTSES/MTSET on transport activity of Cys-substituted mutants

To further evaluate the accessibility and function of residues in the 8th TMD, the impact of the membrane impermeant sulphydryl-reactive reagents, MTSES (negative charge) and MTSET (positive charge) was assessed. As indicated in Figure 4A, with two exceptions all the PCFT mutants that were biotinylated by MTSEA-biotin were inhibited by one or both of the reagents. MTSES, alone, inhibited H312C, Y315C and L316C transport activity; MTSET alone inhibited Y306C activity. Both MTSES and MTSET inhibited activity of I304C, G305C, G307C, A309C, A310C, Q311C, L313C, and P314C although the magnitude differed for ~half the mutants. Inhibition was not observed for the L303C and S308C mutants suggesting that the addition of a charged or bulky group at these sites did not interfere with function. The effect of MTSES/MTSET on residues inaccessible to MTSEA-biotin was assessed (Figure 3B); ~40% inhibition was observed for S318C with MTSET, L319C with MTSES and only a modest decrease for L323C with both the reagents. Hence, there was some discrepancy in accessibility to MTSEA-biotin versus the other reagents, likely related to the deeper penetration of the smaller sulphydryl reagents into the membrane.

3.4 Influx kinetic changes associated with the P314C mutation

Kinetic analyses previously demonstrated an increase in the pemetrexed influx K_t , K_i and V_{max} for the Y315A PCFT mutant, consistent with the data in Figure 2B (15). Pemetrexed is an excellent substrate for PCFT with a very low influx K_t allowing saturation of the carrier even when there is a substantial decrease in the affinity of the mutant for its folate substrates. As indicated in Figure 5, there was a 3.5- fold increase in the pemetrexed influx V_{max} along with a 4.7-fold increase in K_t for the P314C PCFT mutant as compared to PCFT-DSL. This was a similar pattern of change previously reported for the Y315A and Y315C mutants (15).

Likewise, there was an 8.2 fold increase in the influx K_t for pemetrexed and a 20-fold increase in the influx K_t for MTX as compared to PCFT-DSL (Table 1). Hence,

perturbations at both the Pro314 or the Tyr315 residues result in a carrier with a much higher rate of conformational change but a much lower affinity for its folate substrates.

3.5 Further analyses of Pro314 and Tyr315 PCFT mutants

To further assess the structural requirements for the P314 residue, Ala and Gly substitutions were made and transport function assessed. As indicated in Figure 6A, Cys, Ala and Gly substitutions resulted in an increase in transport at 50 μ M MTX. There was a modest decrease in function for the P314Y mutant at both the low and high methotrexate concentrations; however, the Y315P mutant retained the high K_t and V_{max} phenotype. Hence, the phenotype was lost when there were two consecutive tyrosine residues but retained when there were two consecutive proline residues. When the residues were exchanged in the same construct, P314Y/Y315P, the influx V_{max} influx was comparable to PCFT-DSL but transport at the low MTX concentration was markedly reduced consistent with an increase in influx K_t alone. When both residues were substituted with Ala in the same construct, the V_{max} was markedly increased with transport at the low concentration comparable to PCFT-DSL, again consistent with a marked increase in influx K_t . The expression of all these residues at the cell surface and in the crude extract was comparable to that of the PCFT-DSL scaffold (Fig. 6B)

3.6 Analysis of functional changes that occur at proline residues located in other transmembrane helices

Studies were undertaken to determine whether the transport changes observed at the Pro314 residue, located at a break-point in the mid-region of the helix (see below), were unique to this location or might occur with mutations at proline residues in other TMDs. PCFT contains three other proline residues located in transmembrane helices. Two, Pro41 in the 1st, Pro373 in the 10th are located in predicted breakpoints, Pro436 is located in the central region of the 12th helix. As indicated in Figure 7, Cys-substitutions at these residues resulted in a decrease in expression and function for the P373C and P436C mutants although the pattern of functional changes at the low and high concentrations was different. Expression of the P41C mutant was intact, but there was a decrease in transport at 0.5 μ M, and a modest increase in function at the high concentration, suggestive of a transport phenotype qualitatively similar to that found with P314C. Hence, the functional changes associated with the P314 mutants, and the Y315 mutants reported earlier, were either unique qualitatively or greater in the magnitude of change as compared to prolines located in other transmembrane PCFT helices.

3.7 Generation of homology models for PCFT in the inside-open and outside-open conformations

To interpret the experimental observations within a three dimensional dynamic context, the recently solved bovine and rat GLUT5 fructose transporters, that share 88% sequence identity with each other, were used as templates (19) to build homology models of PCFT in its inward-open (Protein data Bank (PDB) code: 4YB9) and outward-open (PDB code: 4YBQ) conformations, respectively. This was the first time a mammalian transporter structure was solved in both conformations. Human PCFT and GLUT5 share 13% sequence identity, the same as the shared sequence identity to the E. Coli glycerol-6-phosphate

transporter (GlpT) previously utilized for homology modeling of PCFT (14). The optimal alignment between PCFT and its template structures 4YB9 and 4YBQ and the subsequent molecular model was obtained from MMM alignment optimization and comparative modeling (20–22). The alignment between PCFT and its templates that was used as input in homology modeling is provided in supplemental material (Supplemental Fig. 1). A variety of measurements were made to assess the quality of the models. Energetic evaluations were done using statistical pair potentials implemented in Prosa, resulting in Z-scores of -4.65 and -3.02 for the inward and outward open conformations, respectively (23). Additional quality measures included the confirmation of enriched occurrence of Trp and Tyr residues at the ends of TM helices on the membrane interface. An analysis of the distribution of positively charged amino acid residues, and corresponding electrostatic calculations, indicated an enriched occurrence of positive charges on the intracellular membrane interface in agreement with the “positive inside” rule of membrane proteins (24). The membrane topology was predicted with the HMMTOP program (25). Residue preferences were explored in the 8th TM region (positions 303–325). Sequence profile was prepared with Blastprofiler (20–22) and Seq2logo (<http://www.cbs.dtu.dk/biotools/Seq2Logo-2.0/>) was used for visualization. Optimal superposition of the models was obtained using the Align3D program of Modeller (26). The previous PCFT model was built on the crystal structure of GlpT (PDB code 1pw5) available only in the inward open configuration. The current model superposes with GlpT within 2.28 Å RMSD over 277 residue positions. The register of the 12 helices are in agreement between the two models.

The GLUT5 structures indicate a substantial difference in the inward-open and outward-open conformations of the 8th TMD of PCFT as illustrated in Figure 8. In the outward-open conformation the helix is broken while it remains straight along its entire length in the inward open conformation (upper panels). The exofacial segment (301–315) is tilted away in the outward-open conformation, while the endofacial portion of the 8th TMD, (residues 316–329), is aligned and in a similar position in both conformations. Pro314 is located at the helix break point (yellow residue-lower panel) and appears to point away from the aqueous pathway while Tyr315 (green residue) appears to point into the aqueous pathway. Nonetheless, the P314C mutant is fully accessible at both 0°C and RT/37°C. The models suggest the probability that during the PCFT transport cycle, the 301–315 segment dynamically switches between two conformations allowing access of all the residues to the molecular probes. The localization of Pro314 and Tyr315 at and adjacent to the helix breakpoint, respectively, is consistent with the large changes in function when these residues are mutated that impact on folate binding and the rate of oscillation of the protein between its conformational states.

4. DISCUSSION

PCFT is one of several proton-coupled transporters that import their substrates within the acid microenvironment at the apical membrane of the proximal small intestine (1). Unlike most of these transporters, PCFT has a high affinity, and a high degree of substrate specificity, for its transport substrates (1,2,4,27). This transporter is critical to the intestinal absorption of folates and the transport of folates across the choroid plexus into the cerebrospinal fluid (4,28). PCFT also transports antifolates into cancer cells, in particular,

the antifolate pemetrexed, primarily a thymidylate synthase inhibitor. While the influx K_t increases, and the V_{max} decreases, substantially for most folates and antifolates as the pH is increased into the neutral range, changes in these parameters for pemetrexed are only modest (29). A family of antifolate inhibitors of purine synthesis are being developed that have a high affinity for PCFT but are very poor substrates for the reduced folate carrier. The objective is to achieve selective delivering of these agents into cancer cells mediated by PCFT within the acidic microenvironment of tumors while minimizing their effects on the most susceptible host tissues of the GI mucosa and bone marrow, mediated by the reduced folate carrier (30,31).

The substituted cysteine accessibility method, SCAM, is routinely used to define the topology of transporters, to identify residues accessible to the extracellular and intracellular compartments and to define the aqueous translocation pathway. Accessibility to water-soluble Cys-reacting reagents is usually restricted to those residues that are extracellular or point into the aqueous pathway with each turn of the helix or every 3–4 residues. The pattern for the 8th TMD of PCFT was considerably different. Of the 23 residues in the 8th TMD, 14 contiguous residues in the exofacial portion of the helix were accessible to MTSEA-biotin, encompassing residues that extend deep into the membrane. The pattern of MTSET/MTSES inhibition suggests that accessibility to these smaller, water soluble, molecules could be even deeper. In the 4th TMD of PCFT accessibility was sporadic but involved some residues within the membrane (32). Only five consecutive Cys-substituted residues in the exofacial region of the 12th TMD of the Glut1 transporter, of all the 12 TMDs, were accessible to the membrane impermeant, p-chloromercuribenzoic acid (33–36). Subsequent acquisition of the crystal structure for this protein indicated that this TMD is outside the central cavity. These residues were considered “solvent accessible” to the aqueous compartment but not in the aqueous pathway (37). Of all the 12 transmembrane helices of the reduced folate carrier, four consecutive exofacial Cys-substituted mutants in the 7th TMD were accessible based upon inhibition of transport by MTSET (38,39). Hence, the degree of accessibility of PCFT’s 8th transmembrane helix appears to be quite unusual.

The development of a homology model of PCFT based upon the inward-open and outward-open structures of the bovine and between rodent GLUT5 (SLC1) fructose transporters provides an explanation for the high degree of accessibility of the exofacial residues of the 8th TMD to the aqueous probe. Hence, there is a substantial change in the orientation of the exofacial residues that likely results in their accessibility to the aqueous pathway as the carrier shifts its orientation between its two conformations. Further evidence that this motion of the carrier is likely critical to the accessibility pattern is indicated by the observation that four of the Cys-substituted residues A310C, L313C, P314C and L316C were not, or were minimally, accessible at 0°C but were accessible at room temperature or 37°C. The data implicates an important role for the 8th TMD in defining the aqueous translocation pathway and also suggests that this region, deep within the membrane, plays an important role in folate binding to the protein in that pemetrexed inhibited biotinylation of each of these residues as well as A310C and L313C. Additional evidence for the importance of this region in folate binding comes from the functional changes that occur when Tyr315 and Pro314 are mutated. This results in a large decrease in the affinity of the carrier for its folate substrates, as reflected in the increase in influx K_i and K_t , associated with comparable large increases in

influx $V_{\max}$, the latter likely related to relaxation of the protein, allowing a higher rate of cycling.

Both Pro314 and Tyr315 are located at, or adjacent to, respectively, the predicted breakpoint of the 8th transmembrane helix. It is not surprising that proline, a residue that destabilizes the helix, is located at this site and that substitutions have a profound impact on function. Unexpected is that the same phenotype was preserved when the adjacent tyrosine was mutated, an amino acid with substantially different properties, resulting in two contiguous proline residues which would complement the disruption of the helix at this site. On the other hand, the transport phenotype was lost with the P314Y mutation, resulting in adjacent Try residues, a condition in which the break might be minimized or absent. Helix breakpoints due to prolines are associated with hinge regions that are sites of motion as the carrier alternates its conformation during the transport cycle (40,41). This is consistent with the homology models that predict large shift in conformation at this region as the carrier oscillates. While there are three other proline residues located in PCFT helices, two in predicted break-points, changes at these sites were either smaller in magnitude or qualitatively different from what was observed for the proline in the 8th TMD. There are numerous examples in which mutation of prolines in transmembrane helices do not produce a similar transport phenotype. For instance, mutation of prolines in helices of the sulfate and purine-cytosine transporters decreased transport activity (42,43). Mutation of Pro272 or Pro528 in transmembrane helices of the dopamine transporter (SLC6A3) resulted in an increase in the K_t but this was accompanied by a decrease in the $V_{\max}$ (44).

It is of interest that five additional Cys-mutants in the 8th TMD, three at least two residues internal to Pro314, and two at the cytoplasmic end of the helix, showed a modest increase in transport at the high MTX concentration consistent with an increase in $V_{\max}$. Hence, taken together with earlier observations (15), the data indicates that this transport phenotype is common with mutations in this transporter at residues with diverse properties and locations within the protein. Whether this is unique to this carrier is unclear. When residues in all 12 transmembrane helices of Glut1 were substituted with Cys, it was rare to find a mutant with an increase in function. However, in these and other studies function was usually assessed at low substrate concentrations, often an order of magnitude below the influx K_t . Glut1 has an influx K_t of 1 mM, but the transport studies that characterized the accessibility of Cys-substituted residues in this transporter were performed at a substrate concentration of 50 μ M (36). Similarly, in studies with the reduced folate carrier, transport was assessed at 0.5 μ M MTX, while the influx K_t is ~5 μ M (38,39) and assays with the glutamate transporter were performed at a substrate concentration of 0.8 μ M (45) while the influx K_t is ~15 μ M (46). Hence, if there was an increase in $V_{\max}$ equal to or exceeding the increase in K_t , a functional change at low substrate concentrations might be masked as observed in the current study. The results reported here suggest that for initial screening of site-directed mutants for functional alterations, in general, studies should include concentrations both below and at saturating concentrations far above the influx K_t for the wild-type transporter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PCFT	proton coupled folate transporter
TMD	transmembrane domain
GLUT5	fructose transporter
RFC	reduced folate carrier
MTX	methotrexate
DTT	dithiothreitol

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Highlights

- Contiguous exofacial residues (14) deep into helix are accessible to aqueous channel
- Novel homology models were developed in the inward- and outward- open conformations
- Models attribute accessibility to exofacial helix flexing during carrier cycling
- Multiple residues within this helix impact on folate substrate binding
- Residues at predicted conformation-dependent helix-break determine carrier cycling

FIGURE 1.

A topological model of PCFT. The predicted secondary structure of PCFT is based upon topological analyses using the substituted-cysteine accessibility method (7,11,12). Residues that define the 8th TMD and were individually Cys-substituted span from L303 to L325. Residues accessible to MTSEA-biotin are indicated in pink. Residues inaccessible are indicated in blue. Pro314 and Tyr315 are highlighted. Filled circles indicate prolines located in TMDs, those located in the 1st and 10th TMDs are in predicted breakpoints.

FIGURE 2.

The effect of cysteine substitutions on PCFT expression and function. (A) Expression of Cys-substituted PCFT mutants in the crude membrane and at the cell membrane, the latter as assessed by biotinylation of lysine residues. β -actin is the loading control. The data are representative of three separate blots. The vertical line indicates two separate gels, run at same time. (B) Influx of was assessed over 1 min at 0.5 μM and 50 μM $[^3\text{H}]$ MTX at pH 5.5 and 37°C. (C) The effect of amino acid substitutions, I304S, G305A, and G307A on influx assessed over 1 min at 0.5 μM and 50 μM $[^3\text{H}]$ MTX at pH 5.5 and 37°C. Data indicated are percentage $\pm$ SEM of PCFT-DSL activity based upon three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

FIGURE 3.

FIGURE 3. Accessibility of Cys-substituted residues in the 8th TMD. (A) Accessibility of the Cys-substituted mutants was assessed with MTSEA-biotin. Cells were treated with 0.5 mM MTSEA-Biotin in HBS for 30 min at room temperature (upper band). Protein expression in the crude membrane fraction is indicated in the lower band. The Western blots shown are representative of 3 independent experiments. (B) Analysis of the accessibility of Cys-substituted residues to MTSEA-biotin at 0°C along with protection by pemetrexed. Cells were treated 0.5 mM MTSEA-biotin in the presence and absence of 1 mM pemetrexed at pH 7.4. (C) A comparison of accessibility and protection by pemetrexed at 0°C and RT assessed simultaneously. The Western blots shown are representative of 3 independent experiments. The vertical line indicates 2 separate gels, run at the same time.

FIGURE 4.

Cells were treated with 3 mM MTSEA or 3 mM MTSES for 30 min at pH 7.4 and RT following which influx of 0.5 μ M [3 H]MTX over 1 min was assessed. (A) Cys-substituted mutants accessible to MTSEA-biotin — from Figure 3A. (B) Cys-substituted mutants not accessible to MTSEA-biotin — From Figure 3A. Data are indicated as percentage of control rates in the absence of inhibitor $\pm$ SEM, from 3 independent experiments. ** P < 0.01, *** P < 0.001, **** P < 0.0001

FIGURE 5.

Pemetrexed influx kinetics. Influx of 0.2-20 μM [³H]pemetrexed was assessed at pH 5.5 over one min in the PCFT-DSL and the P314C mutant transfectants. The data are the best fit to the Michaelis-Menten equation $V = V_{\max}[S]/(K_t + [S])$, where [S] is extracellular substrate concentration. Data indicated are the mean $\pm$ SEM from three independent experiments.

FIGURE 6.

The impact of cysteine and other substitutions at Pro314 and Tyr315 on PCFT activity. (A) Influx of 0.5 μ M and 50 μ M [3 H]MTX was assessed at pH 5.5 for 1 min at 37°. Data are expressed as percentage of PCFT-DSL activity $\pm$ SEM from 3 independent experiments. * P <0.01 ** P <0.0001. (B) Western blot of the expression of PCFT mutants biotinylated at cell surface lysine residues (upper band) and in the crude membrane preparation (middle band). β -actin is the loading control (lower band). The Western blots shown are representative of 3 independent experiments. The vertical line indicates bands from different lanes in the same gel.

FIGURE 7.

Impact of cysteine substitutions on the activities of proline residues located in TMDs: P41C-1st TMD, P314C-8th TMD, P373C-10th TMD and P436C-12th TMD. (A) Influx of 0.5 μM and 50 μM [^3H]MTX was assessed at pH 5.5 for 1 min at 37°C. Data are indicated as percentage of PCFT-DSL activity $\pm$ SEM from 3 independent experiments. * P <0.05, ** P <0.0001. (B) Expression of the proline mutants in the crude membrane preparation and biotinylated at cell surface lysine residues. β -actin is the loading control. The Western blot shown is representative of 3 independent experiments. The vertical line indicates bands from different lanes in the same gel.

FIGURE 8.

Homology models of PCFT based upon the mammalian Glut5 structures. The upper structures are different perspectives of the protein about the vertical axis with the 8th TMD highlighted in the outward-open (4YBQ-blue) and inward-open (4yB9-green) conformations. The lower structures illustrate the positions of the Pro314 and Tyr315 residues with the transporter in the outward-open conformation. Only the 8th TMD (blue) is highlighted. The left structure is a horizontal view, the right structure is a view looking into the aqueous pathway from the extracellular compartment. Pro314 is indicated in yellow, Tyr315 in green.

Table 1
Influx kinetic parameters for the P314C PCFT mutant

[³H]pemetrexed influx was assessed over a concentration range of 0.2 – 20 μM. The inhibition constant (K_i) for MTX was based upon inhibition of [³H]pemetrexed influx as determined from: $V_1/V_2 = V_{max}S/S + K_t [1 + (i/K_i)] / (V_{max}S/S + K_t)$, where V_1 is influx in the presence of inhibitor, V_2 is influx in absence of inhibitor (i), S is the substrate concentration. Values are mean ± SEM from 3 independent experiments.

	Pemetrexed			K_i (MTX)
	K_t	V_{max}	K_i	
PCFT DSL	0.50 ± 0.09	253 ± 10	0.62 ± 0.02	3.04 ± 0.03
P314C	2.37 ± 0.35	890 ± 37	5.11 ± 0.04	61.59 ± 0.04
P314C/DSL	4.7	3.5	8.2	20.2