Identification of Tyr residues that enhance folate substrate binding and constrain oscillation of the proton-coupled folate transporter (PCFT-SLC46A1)

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Submitted 8 July 2014; accepted in final form 20 January 2015

Visentin M, Unal ES, Najmi M, Fiser A, Zhao R, Goldman ID. Identification of Tyr residues that enhance folate substrate binding and constrain oscillation of the proton-coupled folate transporter (PCFT-SLC46A1). Am J Physiol Cell Physiol 308: C631–C641, 2015. First published January 22, 2015; doi:10.1152/ajpcell.00238.2014.—The proton-coupled folate transporter (PCFT) mediates intestinal folate absorption and transport of folates across the choroid plexus. This study focuses on the role of Tyr residues in PCFT function. The substituted Cys-accessibility method identified four Tyr residues (Y291, Y362, Y315, and Y414) that are accessible to the extracellular compartment; three of these (Y291, Y362, and Y315) are located within or near the folate binding pocket. When the Tyr residues were replaced with Cys or Ala, these mutants showed similar (up to 6-fold) increases in influx \( V_{\text{max}} \) and \( K_{\text{m}}/K_{\text{i}} \) for \([^{3}H]\)methotrexate and \([^{1}H]\)pemetrexed. When the Tyr residues were replaced with Phe, these changes were moderated or absent. When Y315A PCFT was used as representative of the mutants and \([^{1}H]\)pemetrexed as the transport substrate, this substitution did not increase the efflux rate constant. Furthermore, neither influx nor efflux mediated by Y315A PCFT was transstimulated by the presence of substrate in the opposite compartment; however, substantial bidirectional transstimulation of transport was mediated by wild-type PCFT. This resulted in a threefold greater efflux rate constant for cells that express wild-type PCFT than for cells that express Y315 PCFT under exchange conditions. These data suggest that these Tyr residues, possibly through their rigid side chains, secure the carrier in a high-affinity state for its folate substrates. However, this may be achieved at the expense of constraining the carrier’s mobility, thereby decreasing the rate at which the protein oscillates between its conformational states. The \( V_{\text{max}} \) generated by these Tyr mutants may be so rapid that further augmentation during transstimulation may not be possible.

hereditary folate malabsorption, HFM; methotrexate; proton-coupled folate transporter; PCFT, SLC46A1; pemetrexed; solute transporter

THE PROTON-COUPLED FOLATE transporter (PCFT) is a member of the superfamily of solute transporters (SLC46A1) and is the mechanism of transport of folates across the apical brush-border membrane of the proximal small intestine (37, 53). Loss-of-function mutations in this gene are the molecular basis for hereditary folate malabsorption, a disorder characterized by impaired intestinal folate absorption and impaired transport of folates across the choroid plexus (6, 37, 62). This transporter also contributes to the pharmacological activity of antifolates, in particular pemetrexed (61) and a class of new-generation glycaminide ribonucleotide formyltransferase inhibitors that have a high affinity for PCFT within the acidic microenvironment of solid tumors (4, 5, 33). In addition to PCFT, there are two other folate-specific transport systems: 1) the reduced folate carrier (RFC), an organic phosphate antiporter that delivers folates to systemic tissues optimally at pH 7.4 (29, 59), and 2) folate receptors expressed in hematopoietic tissues (9) and at epithelia that mediate transport via an endocytic mechanism (15, 19, 59).

Since the identification of PCFT, an understanding of its structure and function is emerging based on an analysis of the functional consequences of mutant forms of this carrier identified in subjects with hereditary folate malabsorption (6, 53) and on site-directed and random mutagenesis (42, 44, 51, 52, 64, 65). The current study addresses the role of Tyr residues in PCFT function and identifies four residues that are accessible to the extracellular compartment; three of these residues are located within or near the folate binding pocket. These residues have a unique impact on PCFT function, in that they enhance the affinity of the carrier for its substrates and decrease, in some cases markedly, the rate at which the carrier oscillates between its conformational states.

METHODS

Chemicals

\([3,5,7-^{3}H]\)methotrexate (MTX) and generally labeled \([^{1}H]\)pemetrexed were obtained from Moravek Biochemicals (Brea, CA). Purity was established and monitored by liquid chromatography, as described previously (58). Nonlabeled MTX was obtained from Sigma-Aldrich (St. Louis, MO), nonlabeled (6S)-formyltetrahydrofolate (5-formylTHF) from Schircks Laboratories (Jona, Switzerland), and nonlabeled pemetrexed from LC Laboratories (Woburn, MA). EZ-Link Sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido) hexanoate] was purchased from Pierce Biotechnology (Rockford, IL), streptavidin-agarose beads from Fischer Scientific (Pittsburgh, PA), and protease inhibitor cocktail from Roche Applied Science (Mannheim, Germany). The sulfhydryl-reactive reagent N-biotinylaminomethylmethanethiosulfonate (MTSEA-biotin) was purchased from Biotium (Hayward, CA). Methanethiosulfonate-ethyltrimethylammonium (MTSET+) and methanethiosulfonate-ethylsulfonate (MTSES−) were obtained from Affymetrix (Santa Clara, CA).

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Site-Directed Mutagenesis

PCFT mutants were generated with the Quick Change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) using as template a pcDNA3.1(+) expression vector that encodes for the human PCFT tagged with hemagglutinin (HA) at the COOH terminus. Mutant constructs were verified by DNA sequencing at the Albert Einstein Cancer Center Genomics Shared Resource.

Cell Lines and Transient Transfection

HeLa R1-11 cells, which lack endogenous folate-specific transporters, were the recipient for transient transfections in this study. This cell line lacks RFC due to a genomic deletion; PCFT expression is silenced due to methylation of the promoter (7, 60). HeLa R1-11 cells were maintained in RPMI 1640 medium containing 2 mM folic acid and supplemented with 10% fetal bovine serum (Gemini Bio-Products, Irvine, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. For transport assays, 3.5 × 10^5 cells were seeded in 17-mm glass vials; for Western blot analyses, 5 × 10^5 cells/well were seeded in six-well plates. After 2 days, cells were transfected with PCFT constructs (0.8 μg/vial or 2 μg/well) with 3% Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum-free and antibiotic-free medium.

Transport Measurements

Transport assays were performed in cells grown in monolayer culture, attached at the bottom of glass vials, 2 days after transient transfection (41). In preparation for experiments, the medium was aspirated and the cells were washed twice in HBS buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 5 mM dextrose, adjusted to pH 7.4). Then 1 ml of HBS buffer was added, and the vials were incubated in a 37°C water bath for 20 min. The buffer was aspirated and the radiolabeled substrates, dissolved in the transport buffer, were added. HBS buffer was used for uptake at pH 7.4, and MBS buffer (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 5 mM dextrose, adjusted to pH 5.5) was used for uptake at pH 5.5. Uptake was stopped by injection of 10 volumes of ice-cold HBS buffer at pH 7.4; then the cells were washed three times in HBS buffer and digested with 0.2 N NaOH at 65°C for 45 min. A portion (400 μl) was assayed for tritium on a liquid scintillation spectrometer. Another portion (20 μl) was analyzed for protein content by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). Cellular antifolate is expressed as picomoles per milligram of protein; for determination of concentration in the intracellular water, this value is divided by 6 [cell water (μl)/cell protein (mg)], as described previously (55).

Transport measurements with [3H]MTX or [3H]pemetrexed were made at acidic and neutral pH. [3H]pemetrexed is a much better substrate for the transporter, particularly at neutral pH (61, 62). Influx was assessed over 1 min, and kinetic constants were determined by a nonlinear regression analysis of pemetrexed influx as a function of the extracellular pemetrexed concentration based on the Michaelis-Menten equation using Prism software (version 6.0 for Windows, GraphPad Software, San Diego, CA). Influx K_i values were determined from the degree of inhibition of [3H]pemetrexed influx (in the 40–60% range of inhibition) and the measured [3H]pemetrexed influx K_i. For efflux studies, cells were grown with 100 μM folic acid for 24 h before experiments were performed to suppress pemetrexed polyglutamation (see below). Cells were loaded with [3H]pemetrexed for 30 min at pH 7.4, washed twice in drug-free buffer, and suspended into a large volume of HBS buffer at pH 7.4. At specified intervals, portions were injected into 0°C HBS buffer and washed twice, and protein and radioactivity were analyzed as indicated above.

Sulfhydryl modifications by methane thiosulfonate (MTS) reagents were performed by incubation of HeLa R1-11 cells, transfected with PCFT expression vectors, with 3 mM MTSET^+ or MTSES^- in ice-cold HBS buffer at pH 7.4. After 30 min, the cells were washed twice with HBS buffer, and influx of radiolabeled substrate was measured as described above.

Biotinylation

Cell surface. At 2 days after transient transfection in six-well plates, HeLa R1-11 cells were washed twice with HBS buffer and treated with 1 mg/ml EZ-Link Sulfo-NHS-LC-biotin in HBS buffer on ice for 1 h. This nonpermeable reagent alkylates accessible Lys residues in wild-type and mutated PCFT. Cells were then washed twice, treated with hypotonic buffer (0.5 mM Na2HPO4 and 0.1 mM EDTA at pH 7.0) containing protease inhibitors, and kept on ice for 30 min. Cells were then scraped from the plates and centrifuged at 14,000 rpm for 15 min at 4°C. The membrane fraction was pelletted and resuspended in 400 μl of lysis buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, and 20 mM Tris, pH 7.4) containing protease inhibitors and rotated overnight at 4°C. A portion of the supernatant (25 μl) was mixed with 2× Laemmli buffer containing dithiothreitol for the crude membrane analysis. The remaining portion was spun down at 14,000 rpm at 4°C for 15 min and separated from the pellet and then mixed with streptavidin-agarose beads (50 μl/sample) overnight at 4°C. The beads were washed twice in lysis buffer (500 μl) and twice in lysis buffer supplemented with 2% SDS. After the final wash, bead-bound proteins were stripped by 5 min of heating at 95°C in 2× Laemmli buffer containing dithiothreitol and loaded directly onto polyacrylamide gels.

Cys-mutant PCFTs. MTSEA-biotin was used to evaluate the accessibility of Cys mutants to the aqueous compartment. MTSEA-biotin was dissolved in DMSO and diluted with HBS buffer at a ratio of 1:100 (0.5 mM final concentration). After two washes with HBS buffer, the cells were treated with MTSEA-biotin for 30 min on ice. Then the solution was aspirated, and the cells were washed twice with HBS buffer. Cells were then processed as described above.

Gel Electrophoresis and Western Blot Analysis

Protein samples were resolved on 12% polyacrylamide minigels and electrobotted onto polyvinylidene difluoride membranes (GE HealthCare, Piscataway, NJ). The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline (20 mM Tris base and 135 mM NaCl) + 0.1% Tween 20 (TBST) buffer (pH 7.4), washed in TBST buffer, and incubated for 1 h at room temperature with anti-HA antibody (Sigma, St. Louis, MO) and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) were added. Blots were developed with the Amersham ECL Plus reagent (GE HealthCare). As the loading control, the crude membrane sample blots were first probed with rabbit anti-actin antibody (Cell Signaling Technology).

Analysis of [3H]Pemetrexed Accumulated Within Cells

HeLa R1-11 cells, transiently transfected with wild-type or Y315A PCFT, were grown with 100 μM folic acid for 24 h to accumulate folypolyglutamates to minimize, as much as possible, the subsequent formation of pemetrexed polyglutamate derivatives, as previously described (54). Cells were then incubated for 30 min with 200 μM [3H]pemetrexed in transport buffer at pH 7.4 and washed twice with ice-cold HBS buffer. Protein was precipitated by addition of 0.5 ml of 10% trichloroacetic acid for 10 min, and the supernatant was neutralized by addition of 0.1 ml of 1 M NaH2PO4 and 0.05 ml of 6 M NaOH. A portion of the supernatant was assessed for total radioactivity. Nonlabeled pemetrexed was added to the other portion of the supernatant, which was analyzed by liquid chromatography on a reverse-phase column (Waters Spherisorb, 5 mm ODS2, 4.6 × 250 mm; see above). The column was eluted with 0.1 M sodium acetate, pH 5.5, for 5 min followed
by two linear gradients of 0–30% and 30–50% acetonitrile in 0.1 M sodium acetate over 35 and 20 min, respectively, and then by 100% acetonitrile for 11 min. The flow rate was 1 ml/min; 1-ml fractions were collected in 13-ml vials, and radioactivity was measured on a liquid scintillation spectrometer. Optical density was monitored at 280 nm; the pemetrexed monoglutamate peak was detected at 67 min.

Molecular Modeling

A homology model for PCFT has been generated for the interpretation of experimental observations within a three-dimensional context. A variety of homology models that differed in their target-to-template input alignments were built; accuracy of target-to-template input alignment largely determined the quality of resulting models. Enenergetically, the most stable model [according to Prosa energy score evaluation (46)] was obtained using input from the HHpred method (16), a hidden Markov model-based fold-recognition and alignment method. The best-scoring template remained the crystal structure of the glycerol-3-phosphate transporter from Escherichia coli (Protein Data Bank code 1pw54), as in other studies (23, 44, 45, 51, 65). The optimal alignment between PCFT and 1pw54 served as input for the comparative protein structure modeling program MMM (10, 11, 38, 39) implementing Modeller (12, 40). Additional model quality verification was conducted by predicting the location of transmembrane segments using HMMTOP (50) and comparing it with the location of transmembrane helices in the three-dimensional model. Alternative models were obtained by other alignment techniques, such as Muscle (8), ClustalW (3), MMM (38, 39), and Align2D (26, 27). The corresponding models resulted in a largely conserved core of transmembrane segments, but there were substantial variations in the modeling of the extra- and intracellular loop regions.

Statistical Analysis

Statistical comparisons were performed with the two-tailed Student’s paired t-test using GraphPad Prism (version 6.0 for Windows).

RESULTS

Expression and Function of Cys-Substituted Conserved PCFT Tyr Residues

Human PCFT contains 14 Tyr residues: 9 are fully conserved, 3 are semiconserved, and 2 are not conserved among species (Fig. 1). The accessibility of each of these 12 conserved residues was established by replacement of each Tyr, one at a time, with Cys, and the functional properties were determined by measurement of influx of 0.5 μM [3H]MTX at pH 5.5, which is optimal for the wild-type carrier. As indicated in Fig. 2A, none of the Tyr residues were absolutely essential for PCFT function. However, Y46C, Y315C, and Y414C retained only 20–30% of activity under these conditions; in all the other mutants, ≥50% of wild-type activity was preserved. Furthermore, transport activity in the HeLa R1-11 cell line, which was the transfection recipient, was trivial, consistent with the lack of endogenous expression of RFC and PCFT.

To establish the extent to which the level of transport activity correlated with PCFT protein or trafficking to the cell membrane, Western blot analyses of the crude membrane preparation and cell surface labeling by biotinylation were assessed. As shown in Fig. 2B and on the basis of densitometry from three experiments, expression of the Y46, Y57, Y208, and Y225 Cys mutants in the crude membrane preparation and at the cell surface was lower than that of the wild-type PCFT (P ≤ 0.05). Total and membrane expression of the Y270 Cys mutant was decreased, but the difference was not significant. There was no significant change in expression of the other mutants. The correspondence between total protein and surface expression was consistent with a reduced level of PCFT synthesis and/or stability. However, as indicated in subsequent experiments, these studies at 0.5 μM MTX were not representative of the functional potential of the constructs because of the nature of the kinetic basis for the changes in influx.

Fig. 1. Localization of the Tyr residues based on the confirmed proton-coupled folate transporter (PCFT) topology (66). Black circles, 9 fully conserved Tyr residues; gray circles, 3 semiconserved Tyr residues.
Sulfhydryl Modification by MTS Reagents

Cys-reactive MTS reagents were used to assess the accessibility of the Tyr residues to the extracellular compartment and their location relative to the folate substrate binding pocket. The substituted Cys residues were first modified with MTSEA-biotin, a large water-soluble reagent. Figure 3A indicates that 8 of 12 Cys-substituted residues were biotinylated (Y46C, Y57C, Y208C, Y291C, Y306C, Y315C, Y362C, and Y414C). As previously reported, biotinylation of the wild-type PCFT did not occur, consistent with the lack of accessibility of MTSEA-biotin to any of the endogenous Cys residues or nonspecific reactions with other residues (66). To assess which residues are in proximity to the folate binding pocket, the ability of pemetrexed to protect the protein from biotinylation was assessed. Pemetrexed was chosen, because it has the highest affinity for PCFT among the folates and antifolates at neutral pH. As indicated in Fig. 3A, pemetrexed pretreatment abolished biotinylation of the Y315C and Y362C mutants and markedly decreased biotinylation of the Y291C mutant but had no effect on biotinylation of any of the other Cys mutants.

Further studies were performed to assess the impact of sulfhydryl modification by MTS reagents on PCFT function. The 12 Cys mutants were treated with 3 mM MTSET+ (Fig. 3B) or 3 mM MTSES− (Fig. 3C). The Y414C mutant was more sensitive to MTSET modification, with a ~70% reduction of [3H]MTX influx compared with the control (29.81 ± 1.67 vs. 9.44 ± 1.01 pmol·mg protein−1·min−1, P = 0.013).
Residual transport mediated by the Y315C PCFT was also inhibited (60%) by MTSES$^-$ (14.84 ± 0.75 vs. 5.93 ± 0.36 pmol-mg protein$^{-1}$·min$^{-1}$, $P = 0.01$), but not by MTSET$^+$. 

Comparison of the Impact of Ala, Phe and Cys Substitutions on PCFT Activity

On the basis of results of the various assays directed to the Cys substitutions, four Tyr residues (291, 315, 362, and 414) appeared to be located in proximity to the folate binding site. All four residues were accessible to MTSEA-biotin and/or inhibition by a sulfhydryl reagent; residues 291, 315, and 362 were protected by pemetrexed. To further explore the role of these four Tyr residues in the PCFT function, each was replaced with Ala or the more conservative Phe.

The impact of the mutations on protein stability and/or trafficking was assessed. As indicated in Fig. 4A, expression of the Y291F and Y362A mutants was reduced ($P = 0.002$ and 0.03, respectively) at the cell surface relative to wild-type PCFT (based on densitometry from 4 replicate experiments), consistent with levels of expression in the crude membrane preparation. Other small differences did not reach statistical significance, nor were the small differences in actin expression significant. In particular, there was no difference in the surface expression of the Y315A mutant.

$[^{3}H]$MTX influx was assessed at low (0.5 μM) or high (50 μM) concentrations, and the data are plotted as percentage of wild-type activity. As indicated in Fig. 4B, there was a good deal of variability in the functional consequences of these substitutions. At the high concentration, $[^{3}H]$MTX influx mediated by the Cys-substituted mutants was higher than that mediated by the wild-type PCFT for all Tyr residues except Y315C. The Ala substitutions led to significant increases in all the mutants, but the largest increases were observed in the Y315A and Y291A mutants. The Phe substitutions resulted in increases in only the Y414F and Y362F mutants. In contrast, the changes at the lower MTX concentration were much more restricted. There were decreases in influx for all the Y315 substituted forms; the most prominent was for the Y315C mutant. There were modest decreases for the Y414C and Y315A mutants. Rates for the Y291F and Y362A mutants are underestimated due their reduced level of expression (Fig. 4A).

To investigate the extent to which these changes were substrate-specific, $[^{3}H]$pemetrexed influx was assessed under the same conditions (Fig. 4C). Transport of pemetrexed was unchanged at the low (0.5 μM) concentration for all the mutants. However, at the high concentration, pemetrexed influx increased three- to fourfold for the Y291C, Y315C, and Y362C mutants and the Y315A mutant and to a lesser extent for the Y291A mutant. Significant changes were not detected for Phe mutants. Hence, the impact of these substitutions was predominantly at high concentrations of the antifolates, with some variability in the changes detected based on the transport substrate. Because of the concentration dependence of the functional changes at these Tyr residues, influx was assessed at 50 μM pemetrexed for all 12 Tyr mutants. Only Y291C, Y315C, Y362C, and Y315A showed an increase in influx relative to cells that expressed wild-type PCFT.

Impact of Ala Substitution on Pemetrexed Influx Kinetics

The high levels of MTX or pemetrexed influx for the PCFT mutants, relative to wild-type PCFT, at the high concentration were consistent with substantial increases in influx $V_{\text{max}}$ relative to transport mediated by wild-type PCFT. The decreased or unchanged influx at the low concentration, within the context of an increase in $V_{\text{max}}$, suggested a substantial increase in influx $K_{\text{i}}$ for the Tyr mutants. Accordingly, the kinetic basis for these changes in influx was assessed. Pemetrexed was a particularly good substrate for these measurements, because the influx $K_{\text{i}}$ mediated by wild-type PCFT is low, so that
saturation occurs at relatively low concentrations, allowing accurate estimation of influx $K_i$, even when there is a substantial increase in this parameter. The Y315A mutant was particularly suitable for these studies because of its high level of activity, and level of expression equivalent to that of wild-type PCFT. As indicated in Fig. 5 and Table 1, on the basis of a nonlinear regression analysis, the pemetrexed influx $K_i$ for Y315A PCFT was 3.62 μM and influx $V_{\text{max}}$ was 930 pmol·mg protein$^{-1}$·min$^{-1}$. These values were 4.9- and 4.7-fold higher, respectively, than the pemetrexed influx $K_i$ and $V_{\text{max}}$ for wild-type PCFT of 0.74 μM and 197 pmol·mg protein$^{-1}$·min$^{-1}$. Hence, there was a comparable increase in influx $K_i$ and $V_{\text{max}}$ for Y315A PCFT. The pemetrexed influx $K_i$ and $V_{\text{max}}$ for the Y291A mutant were increased four- and fivefold, respectively; these parameters increased twofold for the Y362A and Y414A mutants, while the increase (30%) for Y315F PCFT was modest (data not shown).

Preliminary experiments indicated that the MTX influx $K_i$ mediated by Y315A PCFT was $\geq$50 μM, complicating accurate measurements at saturating concentrations because of the emergence of diffusional and/or low-affinity facilitated route(s). Rather, the MTX influx $K_i$ was determined on the basis of inhibition of $[^{3}\text{H}]$pemetrexed influx and compared with the pemetrexed $K_i$ determined in the same way. These measurements were especially important to establish that the changes in $K_i$ were due to an alteration in the affinity of the mutated transporter for its folate substrate. As indicated in Table 1, for wild-type PCFT, the pemetrexed influx $K_i$ (0.44 μM) was slightly less than the influx $K_i$; the MTX influx $K_i$ (3.8 μM) was fourfold higher, as observed previously (45).

The influx $K_i$ was increased 7- and 13-fold for pemetrexed and MTX, respectively, for the Y315A mutant. The increase in the influx $K_i$ and $K_i$ for pemetrexed indicates that the change in the former parameter reflects a true decrease in the affinity of Y315A PCFT for this substrate.

**pH Dependence of Transport Mediated by Y315A PCFT**

The relationship between $[^{3}\text{H}]$pemetrexed transport and pH was assessed for the wild-type and Y315A PCFT over a pH range of 4.5–7.0 at an extracellular concentration of 0.5 μM. At this concentration, influx mediated by the Y315A mutant was comparable to that of the wild-type at pH 4.5, as indicated in Fig. 6. The decline in pemetrexed influx as pH was increased in cells that express the mutant PCFT was slightly less than that observed in cells that express wild-type PCFT to pH 6 ($P = 0.001$). This modest difference was due to a relative increase in influx $V_{\text{max}}$ for the mutant over this pH range (not shown). Hence, under conditions in which there was a strong proton gradient, influx mediated by Y315A PCFT was favored. Therefore, the major decline in transport activity to pH 7.0 was not different.

**Impact of the Y315A Mutation on the Unidirectional Efflux of Pemetrexed**

To assess the extent to which alterations in influx kinetics were associated with changes in pemetrexed efflux, cells were grown with 100 μM folic acid for 24 h before experiments were performed to suppress polyglutamation of pemetrexed. Cells were incubated for 30 min with 200 μM $[^{3}\text{H}]$pemetrexed in transport buffer at pH 7.4 to achieve high intracellular levels. These studies cannot be performed at acidic pH, because these cells acidify within a few minutes. The cells were then washed at 0°C and resuspended into a large volume of drug-free 37°C buffer at pH 7.4, and the decline in intracellular $[^{3}\text{H}]$pemetrexed was monitored. As indicated in Fig. 7A, all drug rapidly exited the cells that expressed wild-type PCFT or the Y315A mutant. Hence, all intracellular drug was exchangeable. Figure 7B shows a semilogarithmic plot of the percentage of drug remaining in the cells as a function of time, with the y-axis set to 2, the $\log_{10}$ of 100%, at time 0. Efflux of $\geq$75% of drug from both cell types can be described by a single

![Fig. 5](image_url)

**Fig. 5.** Impact of Y315A PCFT substitution on the kinetics of pemetrexed influx. Influx was assessed over 1 min at pH 5.5 at 0–20 μM $[^{3}\text{H}]$pemetrexed in cells transfected with wild-type PCFT or the Y315A mutant from which influx $K_i$ and $V_{\text{max}}$ were computed. Line is best fit to Michaelis-Menten equation $V = V_{\text{max}}[S]/(K_i + [S])$, where [S] is substrate extracellular concentration.

![Fig. 6](image_url)

**Fig. 6.** Effect of pH on $[^{3}\text{H}]$pemetrexed influx. Influx of 0.5 μM $[^{3}\text{H}]$pemetrexed was assessed over 1 min at pH 4.5–7.0 in HeLa R1-11 cells expressing wild-type or Y315A PCFT. Values are means ± SE from 3 independent experiments.

### Table 1. Influx kinetics

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<th>Pemetrexed</th>
<th>MTX</th>
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<tr>
<td>$K_i$</td>
<td>0.74 ± 0.34</td>
<td>0.44 ± 0.03</td>
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<tr>
<td>$V_{\text{max}}$</td>
<td>197 ± 22</td>
<td>930 ± 95</td>
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<tr>
<td>$K_i/\text{WT}$</td>
<td>4.9</td>
<td>4.7</td>
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Values are means ± SE from 3 independent experiments. Influx $K_i$ and $V_{\text{max}}$ for pemetrexed transport mediated by wild-type (WT) and Y315A mutant PCFT were computed from the Michaelis-Menten equation: $V = V_{\text{max}}[S]/(K_i + [S])$. $K_i$ (μmol/l) represents extracellular concentration at which pemetrexed influx is one-half of maximum; $V_{\text{max}}$ is in pmol·mg protein$^{-1}$·min$^{-1}$. Inhibition constant ($K_i$) was determined from the following formula: $V = V_{\text{max}}[S]/(K_i + [S] + K_i [1 + ([I]/K_i)]/[V_{\text{max}}][S]/K_i)$, where $V_i$ is influx in the presence of inhibitor (i), $V_0$ is influx in absence of inhibitor, and $K_i$ and $V_{\text{max}}$ were derived from the measured $[^{3}\text{H}]$pemetrexed (S) influx kinetics. *$P = 0.01$ vs. pemetrexed $K_i$.
neutral pH in the absence of a pH gradient, reaching a factor of 6 at 100 μM pemetrexed. However, irrespective of pemetrexed concentration or pH, no transstimulation of influx could be detected in cells that express the Y315A mutant.

To assess transstimulation of [3H]pemetrexed efflux, cells incubated with 200 μM [3H]pemetrexed for 30 min at pH 7.4 were washed at 0°C and then resuspended into [3H]pemetrexed-free buffer at pH 7.4 in the presence or absence of 200 μM nonlabeled pemetrexed. Figure 8B shows that [3H]pemetrexed efflux was increased threefold (–0.114 ± 0.018 vs. –0.294 ± 0.012 min⁻¹, P = 0.015) in the presence of extracellular nonlabeled pemetrexed in cells that express wild-type PCFT. However, as indicated in Fig. 8C, there was only a negligible increase in efflux from cells that express the Y315A mutant (–0.072 ± 0.006 vs. –0.102 ± 0.006 min⁻¹, P = 0.03). Hence, when pemetrexed was present in the intracellular compartments, transstimulation of the bidirectional fluxes was substantial in cells that express wild-type PCFT. However, transstimulation was negligible or absent in cells that express Y315A PCFT under these conditions.

**DISCUSSION**

Aromatic residues, particularly Trp and Tyr, play an important structural role in solute transporters by anchoring transmembrane proteins to the lipid bilayer via cation–π interactions between the flat rigid structure of the residue side chain and the lipid headgroup region (1, 14, 35). Hence, these residues are prevalent in the membrane-water interfaces of the exterior and cytoplasmic regions of the transmembrane α-helices. Consistent with this, the majority of conserved PCFT Tyr residues (Y46, Y208, Y291, Y306, Y362, and Y414) are located in these regions based on the predicted secondary structure (Fig. 1) and a homology model of this carrier. Two Tyr residues (Y225 and Y315) are located deep within transmembrane domains 6 and 8, respectively. However, the side chain of the former appears to point away from, while the latter appears to point into, the aqueous translocation pathway (Fig. 9), consistent with the observation that only the latter residue is accessible and plays an important role in PCFT function.

Biotinylation of three of the Cys-substituted Tyr residues (Y291, Y315, and Y362) was blocked by pemetrexed, suggesting a location in or near the folate binding pocket. The observation that transport mediated by Y414C PCFT was markedly inhibited by sulfhydryl reagents and that the transport phenotype was similar to that of the other three accessible residues implies an important role for Y414 as well in PCFT binding and function, despite its apparent extracellular orientation (Fig. 9). Initial functional analyses of the Cys mutants determined at low substrate concentrations indicated impaired or unchanged function. However, subsequent studies at high substrate concentrations indicated that the kinetic basis for the alterations in function was complex and due to increases in both influx $K_t$ and $V_{\text{max}}$, resulting in reduced, or unchanged, influx at low concentrations but increased rates at saturating concentrations when Tyr residues were replaced by the nonaromatic amino acids Ala and Cys. The differences in baseline

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**Fig. 7. Analysis of efflux of [3H]pemetrexed. HeLa R1-11 cells that express wild-type PCFT or the Y315A mutant were grown with 100 μM folic acid for 24 h to suppress pemetrexed polyglutamation and then incubated for 30 min with 200 μM [3H]pemetrexed. After intracellular [3H]pemetrexed was assessed, cells were washed at 0°C and then resuspended into drug-free buffer at pH 7.4 and 37°C, and the decline in intracellular [3H]pemetrexed was monitored. Ordinate is linear in A and logarithmic (base 10) in B. Values are means ± SE from 3 independent experiments.**

**Impact of the Y315A PCFT Mutation on Transstimulation of the Unidirectional Fluxes of PCFT**

The increased pemetrexed influx $V_{\text{max}}$ in cells that express the Y315A mutant was consistent with an augmented rate of oscillation of the carrier between its conformational states due, presumably, to an increase in the rate-limiting step of the carrier cycle. To determine the impact of the Y315A mutation on the ability to transstimulate PCFT influx, studies were performed in cells loaded in the presence of 200 μM 5-formyl-THF for 20 min at pH 7.4. After the extracellular 5-formyl-THF was removed, influx of [3H]pemetrexed was measured at pH 5.5 and 7.4. As indicated in Fig. 8A, transstimulation of [3H]pemetrexed influx for wild-type PCFT as the concentration was increased; this was much more marked at
wild-type MTX and pemetrexed influx \( K_t/K_i \) values account, at least in part, for the different impact of the increase in \( K_t/K_i \) that occurs with the mutant PCFT forms. These observations indicate how assessment of function of a mutant can provide incomplete information when transport is measured at concentrations far from the influx \( K_t \). Many of the studies were performed with pemetrexed, because of its low influx \( K_t \) mediated by wild-type PCFT, which makes partial saturation possible at neutral pH, where its influx \( K_t \) is \( \approx 15 \) \( \mu \)M (61, 63), or full saturation possible at pH 5.5, even when the \( K_t \) of the mutant was substantially increased.

Of particular interest is that increases in \( V_{\text{max}} \) were observed for multiple Tyr residues in different parts of this protein. Hence, these residues appear to play a similar role in PCFT function, because of its low influx \( K_t \) mediated by wild-type PCFT, which makes partial saturation possible at neutral pH, where its influx \( K_t \) is \( \approx 15 \) \( \mu \)M (61, 63), or full saturation possible at pH 5.5, even when the \( K_t \) of the mutant was substantially increased.

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Lack of flexibility is a property associated with high affinity and specificity of binding, because only a small loss of conformational entropy is encountered when a side chain is immobilized in the binding interface under these conditions (20). This association between protein rigidity and binding affinity is particularly important to the formation of antibodies, where very high affinity and specificity are required (22, 25, 49). The C154G mutant of the lactose permease of \( E. \ coli \) LacY appears to constrain the carrier in an outward-facing conformation that is associated with an affinity for its substrate so great that there is insufficient energy to overcome this constraint to achieve mobility and transport (31, 34, 47). On the other hand, the binding pocket of the ATP-binding cassette transporters is...
highly flexible, with a low level of selectively and affinity for their substrates, so that they can accommodate and export a variety of diverse, structurally unrelated, compounds (30, 56).

Since the identification of PCFT (37), a variety of residues have been established as important for folate binding and rates at which the carrier undergoes changes in its conformational state. It is of interest that influx mediated by the Y315A mutant relative to wild-type PCFT is favored under conditions in which there is a pH gradient. This is the opposite of observations with the H281 and E185 mutations, which appeared to decrease proton binding and coupling, respectively (28, 43, 45, 51, 52). Furthermore, while decreases in $V_{\text{max}}$ have accompanied increases and decreases in influx $K_{i}$ and $K_{i}^t$ (28, 45), for the first time, modification of PCFT residues has resulted in an increase in influx $V_{\text{max}}$ that accompanies the increase in $K_{i}/K_{t}$. Substitutions of residues in other solute transporters that result in an increase in the maximum transport rate are unusual but can occur in association with a decrease in influx $K_{i}$. However, a similar phenotype was observed for the serotonin transporter (SERT) F263C mutant, with a fourfold increase in influx $K_{i}$ and $V_{\text{max}}$ (21).

Recently, the structural basis for the molecular recognition of folic acid by folate receptor-$\alpha$ was solved and showed the importance of the Tyr and Trp residues that stabilize, by hydrophobic interactions, the folic acid aminobenzoate within the binding pocket (2, 57). The affinity of folic acid for the receptor was decreased when these residues were mutated, but it is unclear whether this was associated with increased flexibility of the binding pocket (2). Perhaps related to this observation, while molecules with a high affinity for PCFT and a very low affinity for RFC have been developed, no molecule with a high affinity for PCFT and a low affinity for a folate receptor has been synthesized as yet, suggesting some commonality in the binding pocket (4).

The marked increase in the influx $V_{\text{max}}$ mediated by Y315A PCFT was not accompanied by a comparable increase in the unidirectional efflux rate constant. Nor was there an alteration in the unidirectional fluxes mediated by the mutant when there was substrate in the opposite compartment, in contrast to wild-type PCFT, so that the efflux rate constant mediated by the mutant carrier was one-third of that mediated by wild-type PCFT under conditions of net transport. Hence, wild-type carrier cycling is increased during exchange, but cycling of Y315A PCFT is not, perhaps because this is already occurring at a near maximum rate.

Transstimulation of pemetrexed influx mediated by wild-type PCFT was increased as the transport substrate concentration increased, reflecting an increase in influx $K_i$ and $V_{\text{max}}$. For most solute carriers, including RFC, transstimulation is characterized by an increase in $V_{\text{max}}$ alone, presumably due solely to an increase in the reorientation of the carrier to its outward-facing conformation in the absence of accompanying substrate, the rate-limiting step. In that case, the degree of influx stimulation was constant, irrespective of the substrate concentration (13, 17, 55). The degree of transstimulation for wild-type PCFT was most prominent in the absence of a proton gradient across the cell membrane, suggesting that additional factor(s) may limit the rate of oscillation of the carrier under these conditions. The increase in influx $K_i$ during transstimulation suggests that when the magnitude of the $V_{\text{max}}$ increase is

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Fig. 9. Location of some Tyr mutants based on a homology model of PCFT. A: aqueous channel viewed from the extracellular space. Tyr residues 414 and 291 are situated close to the extracellular end of the channel and predicted to be accessible. Y362 points toward the membrane bilayer; however, conformation of this residue is unusual, making a sharp turn after the Cα position. This could be an artifact of the model building, where, due to the lack of proper packing, the residue is squeezed out toward the membrane bilayer, although its take-off position points into the channel. B: side-view presentation in which the bottom of the molecule is adjacent to the intracellular space of PCFT, while the top of the molecule is adjacent to the extracellular space. In the cartoon representation, cylinders represent helices, and NH2 and COOH termini are colored red and blue, respectively. Two Tyr residues are shown in space-filling model, with atom type-specific coloring (green = carbon, red = oxygen, and blue = nitrogen). Y315 residue is located at a helix break point, and the side chain points inward into the transport channel. Y225 is located in an external helix and points into the membrane bilayer.
sufficiently high, the rate of reorientation of the carrier may be sufficiently high to be a significant component of the $K_i$ term, which is not the case under usual conditions. In contrast, the increases in influx $K_i$ associated with the increases in influx $V_{max}$ as a result of the Tyr mutations, were due to actual decreases in the affinity of the mutated carriers for their substrates. Mutations at the E185 residue that eliminated proton coupling were associated with a decrease in influx $V_{max}$ alone, due presumably to impaired release of protons into the cytoplasm, making this the rate-limiting step under these conditions (52).

PCFT-mediated transport of pemetrexed at neutral pH is relevant to conditions in which the drug is administered and delivered to tumor and peripheral tissues clinically: the maximal plasma concentration for pemetrexed at the current dose of 500 mg/m$^2$ is $\sim 200 \mu M$ (32). Similarly, MTX blood levels reach into the millimolar range with high-dose regimens (18, 36). Hence, despite the pH optimum of this transporter, residual transport can contribute to drug activity, particularly in the mildly acidic pH microenvironment of solid tumors (48).

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**GRANTS**

This study was supported by National Cancer Institute Grant CA-82621.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

M.V. and I.D.G. developed the concept and designed the research; M.V., E.S.U., and M.N. performed the experiments; M.V. and A.F. analyzed the data; M.V., E.S.U., R.Z., and I.D.G. interpreted the results of the experiments; M.V. and A.F. prepared the figures; M.V. drafted the manuscript; M.V., R.Z., and I.D.G. edited and revised the manuscript; M.V., E.S.U., M.N., A.F., R.Z., and I.D.G. approved the final version of the manuscript.

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