0090-9556/09/3702-302–309$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 37, No. 2
24042/3429136
DMD 37:302–309, 2009
Printed in U.S.A.

Recombinant Zebrafish γ-Glutamyl Hydrolase Exhibits Properties and Catalytic Activities Comparable with Those of Mammalian Enzyme

Tseng-Ting Kao, Wen-Ni Chang, Hua-Lin Wu, Guey-Yueh Shi, and Tzu-Fun Fu

Department of Medical Laboratory Science and Biotechnology, College of Medicine (T.-T.K., W.-N.C., T.-F.F.), and Department of Biochemistry and Molecular Biology, College of Medicine, and Cardiovascular Research Center (H.-L.W., G.-Y.S.), National Cheng Kung University, Tainan, Taiwan

Received August 19, 2008; accepted November 7, 2008

ABSTRACT:

A cDNA encoding for zebrafish γ-glutamyl hydrolase (γGH) was cloned and inserted into a pET43.1a vector via SmaI and EcoRI sites and expressed in Rosetta (DE3) cells as a Nus-His-tag fusion enzyme (NH-zγGH). After induction with isopropyl thiogalactoside, the enzyme was purified with a Ni-Sepharose column, and approximately 8 mg of pure enzyme was obtained per liter of culture. The primary sequence of the recombinant zγGH was similar to mammalian γGH. Thrombin digestion of this NH-zγGH fusion protein resulted in zγGH with approximately 2-fold higher catalytic activity compared with the NH-zγGH fusion enzyme. This recombinant zγGH is active and exhibits comparable endopeptidase activity with folate substrate and antifolate drug methotrexate. Use of this recombinant zγGH significantly increased efficiency in folylpolyglutamate hydrolysis for folate analysis compared with current protocols.

Folate is an essential B vitamin and participates in the biosynthesis and metabolism of nucleic acids, proteins, several amino acids, methyl groups, many neurotransmitters, and some vitamins. Mammalian cells are unable to synthesize folates de novo and therefore depend on their food for the supply of folates. Naturally occurring folates are synthesized as poly-γ-glutamate forms (folylpolyglutamate) but are absorbed and transported most efficiently as folylmonoglutamates. The conversion of folylpolyglutamates in dietary food to folylmonoglutamates is catalyzed by carboxypeptidase II (EC 3.4.22.12) in mammals. In a recent study, γ-glutamyl hydrolase (γGH, EC 3.4.19.9), a lysosomal cysteine peptidase, was reported to be the enzyme responsible for hydrolyzing dietary folate in rat small intestine (Shafizadeh and Halsted, 2007). After entering cells, these folylmonoglutamates are elongated to folylpolyglutamates by folylpoly-γ-glutamate synthetase (EC 6.3.2.17) for more effective retention and cofactor activity. As part of a salvage pathway, γGH catalyzes the hydrolysis of Glu-γ-Glu bonds to form folylmonoglutamates, enabling folate cofactors to be exported from cells and enter circulation again (Suh et al., 2001). Therefore, folylpoly-γ-glutamate synthetase and γGH are crucial for the maintenance and regulation of intracellular folate homeostasis. Nevertheless, the mechanism of turnover and control of cellular folylpolyglutamate levels by γGH remain unclear. A better understanding of this enzyme and an efficient assay system is prerequisite to answer this question.

Consistent with this notion, the activity of γGH to hydrolyze the γ-glutamyl peptide bonds of folylpolyglutamates has rendered this enzyme a potential target of antifolate chemotherapy and, at the same time, a primary component in regulating the intracellular levels of some antifolate drugs. Antifolate drugs, such as methotrexate, owe much of their effectiveness to being substrates for both folylpoly-γ-glutamate synthetase and γGH. Removal of γ-linked glutamate residues decreases the retention and activity of these drugs. A polymorphism resulting in reduced catalytic activity of γGH was observed to be associated with greater accumulation of long-chain methotrexate polyglutamate forms (Cheng et al., 2004). In contrast, higher γGH activity has been connected to the development of drug resistance (Rhee et al., 1993). Therefore, alteration to γGH availability or activity seems to have functional and pharmacological consequences and even to be a potential tumor marker (Schneider and Ryan, 2006). Having pure and active γGH is essential to structural and kinetic studies of γGH and to additional investigation for its role in affecting antifolate drug efficacy.

The determination of individual folate derivatives in serum of patients receiving antifolate chemotherapy and in foods is an important current protocol. The first step in these determinations is converting folylpolyglutamates to folylmonoglutamates by γGH. Cur-

This work was supported by the National Science Council, Taiwan [Grant NSC 96-2320B-006-023-MY3]; and the Program for Promoting Academic Excellence of Universities, National Cheng Kung University [Grants D96-3500, D96-2200].

The amino acid numbering used for zγGH in this study is numbered starting from the first methionine in the full-length peptide with the signal peptide.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.108.024042.

ABBREVIATIONS: γGH, γ-glutamyl hydrolase; zγGH, zebrafish γGH; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; NH-zγGH, Nus-His-tag fusion γ-glutamyl hydrolase; IPTG, isopropyl-β-D-thiogalactopyranoside; THF, tetrahydrofolate; 5-CHO-THF-Glu_3, 5-formyltetrahydrofolate triglutamate; 5-CHO-THF-Glu_1, 5-formyltetrahydrofolate monoglutamate; PAGE, polyacrylamide gel electrophoresis; MTX, methotrexate.

rently, hog kidney, chicken pancreas, and rat serum/plasma are the main sources used to provide γGH activity (Quinlivan et al., 2006). However, large amounts of plasma or extract and long incubation times are often required for an acceptable degree of conversion, resulting in significant dilution, higher background, and loss of folate cofactors in samples. Having a pure and concentrated γGH will improve the accuracy of folate determinations.

An efficient animal model for drug safety and efficacy screening is indispensable, despite the historic progress in the past few decades in pharmaceutical manufacturing. Zebrafish, a vertebrate, is becoming a prominent animal model, providing a platform for fast drug screening. The advantages are its similarity to mammals in many biological pathways and pathogenesis, abundant offspring, rapid development, transparent embryo, and easy growth and breeding. Especially important for drug discovery is that zebrafish embryos are permeable to small molecules and drugs during organogenesis, providing easy access for drug administration and vital dye staining (Kari et al., 2007). In addition, the external fertilization and development of embryos prevent the often occurring misinterpretation of experimental results as a result of maternal supply of folates. Well established tools of molecular biology for gene manipulation have also significantly assisted progress with zebrafish. We have started an extensive study of folate-mediated one-carbon metabolism in zebrafish (Chang et al., 2006, 2007; Kao et al., 2008). In this study, we cloned the coding sequence of zebrafish γGH (zγGH) from a zebrafish cDNA library and developed an efficient protocol for expression and purification of the recombinant zγGH. To our knowledge, this is the first report for the cloning, expression, and purification of zγGH. We showed that this recombinant zγGH catalyzed the hydrolysis of natural folate substrates and an antifolate drug with similar efficiency. In addition, data showing improved efficiency of converting folate-polyglutamates to monoglutamates in a folate analytical protocol with high-performance liquid chromatography (HPLC) by replacing conventional rat serum with the recombinant zγGH is discussed.

Materials and Methods

Materials. Polymerase chain reaction (PCR) primers were ordered from MDBio, Inc. (Taipei, Taiwan). The SMART RACE amplification kit was purchased from Clontech/Takara Bio Co. (Mountain View, CA). PCR Master Mix was purchased from ABgene (Epsom, Surrey, UK). Enzymes used for cloning were purchased from Invitrogen (Carlsbad, CA) and New England Biolabs (Ipswich, MA). The HPLC Aquasil C_{18} column and guard columns were purchased from Thermo Fisher Scientific (Waltham, MA). Nickel-Sepharose (Ni-Sepharose) resin slurry was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Rat serum was prepared as described previously (Wilson and Horne, 1982). All of the fully reduced monoglutamated folates were gifts from Dr. Moser (Merck Eprova AG, Schaffhausen, Switzerland). (6*S*)-N^5-CHO-tetrahydrofolate (Leucovorin) triglutamate was a gift from Dr. Schirch (Virginia Commonwealth University, Richmond, VA). Methotrexate pentaglutamate was purchased from Schircks Laboratories (Jona, Switzerland). All the other chemicals, including folic acid, dihydrofolate, buffers, and amino acids, were purchased from Sigma-Aldrich (St. Louis, MO).

Fish Care and Preparation of cDNA Libraries. Zebrafish (*Danio rerio*, AB strain) were bred and maintained in a 10-h/14-h light/dark diurnal cycle following the standard procedure described by Westerfield (1995). Embryos were staged according to Kimmel et al. (1995). Total RNA isolation and cDNA library construction from zebrafish embryos and tissues were prepared with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and the SMART-RACE cDNA Amplification Kit (Clontech) as described previously (Kao et al., 2008).

Bacterial Strains, Plasmids, and General Cloning Procedures. The *Escherichia coli* strain XL1 Blue (*rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17(r_K^-, m_K^+), *sup*E44, *rel*A1, *lac*$^-$) was used for the construction of clones. The *E. coli* strains Rosetta (DE3) (F^- recA r_{k12}^- m_{k12}^+) containing the T7 RNA polymerase gene was used for protein expression. The pET43.1a plasmid and all the *E. coli* strains for cloning and expression were obtained from Novagen (Madison, WI). The materials and methods for the general cloning procedures were as described previously (Kao et al., 2008).

Cloning of zγGH Coding Sequences. Primers were designed based on the zγGH cDNAs available in GenBank (GenBank accession number BC066746) to PCR amplify complete γGH coding sequence from zebrafish 5′-RACE-Ready cDNA libraries. The primer sequences were 5′-GGGGCATATGATTCACATCTTTCTTTTG-3′ (forward) and 5′-GGGGCTCGAGATTGAAAAAATATGTCTGTTC-3′ (reverse) with introduced NdeI and XhoI restriction enzyme sites (underlined) to simplify the cloning procedures. The PCR fragments close to 1 kilobase were cloned into the expression vector pET43.1a between NdeI and XhoI sites, generating zγGH/pET43.1a. For constructing Nus-His-fusion zγGH (NH-zγGH), PCR with another pair of primers, 5′-GACCCGGGGACCTTTAATAAAAACTAATGAAAG-3′ (forward) and 5′-GGGAATTCCTAATTGAAAAAATATGTCTG-3′ (reverse), was performed using the previously obtained zγGH/pET43.1a plasmid as template. The restriction enzyme sites for SmaI and EcoRI (underlined), respectively, were introduced for the convenience of subsequent cloning. The resulting 900-base pair fragment was cloned into the expression vector pET43.1a between SmaI and EcoRI, resulting in an open reading frame encompassing coding sequences of Nus-Tag, His-Tag, and Gly22 to Asn312 of zγGH. Successful cloning was confirmed by restriction enzyme digestion and DNA sequencing. The resulting constructs were transformed into Rosetta (DE3) cells for zγGH expression and purification.

Expression and Purification of Recombinant zγGH. *E. coli* containing the desired plasmid, either zγGH/pET43.1a or NH-zγGH/pET43.1a, was grown overnight at 37°C in 50 ml of enriched Luria broth (2-YT) containing 100 μg/ml ampicillin. This culture was used to inoculate 1 liter of the same broth and the inoculum continuously grown at 37°C. Recombinant zγGH was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM when the inoculum reached log phase. After 4-h incubation with vigorous shaking at 27°C, bacteria cultures were centrifuged, and cell pellets were subjected to protein purification.

For the purification of NH-zγGH, cells were resuspended in 20 ml of lysis buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 10% glycerol) and subjected to cell disruption with a French press. The cell lysate was treated with DNase I (30 μg/ml) at 25°C for 20 min and pelleted again by centrifugation at 4°C. The supernatant was mixed with an equal volume of binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 20 mM imidazole) before adding to 5 ml of Ni-Sepharose resin and incubating at 4°C for 2 h. The slurry of cell lysate and Ni-Sepharose resin was briefly centrifuged; the supernatant containing unbound protein was discarded; and the Ni-Sepharose resin removed to a column (1.5 × 5.0 cm). After a thorough wash with 5 to 10 resin volumes of binding buffer, NH-zγGH was eluted with binding buffer containing 200 mM imidazole. Fractions containing protein were combined, and NH-zγGH was precipitated by 50% ammonium sulfate, dialyzed in phosphate buffer containing 10% glycerol, and stored at −20 or −80°C.

To remove the N-terminal Nus-His tag, ammonium sulfate-precipitated NH-zγGH was subjected to a 2-h dialysis in 1 liter of thrombin cleavage buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% 2-mercaptoethanol). The desalted NH-zγGH was digested with thrombin in a 1:500 ratio at room temperature for 20 min followed by a further incubation at 4°C for 6 h. The digested sample was loaded on an equilibrated Ni-Sepharose column again as described previously. The zγGH in the fraction of flow-through was collected, and ammonium sulfate was precipitated and dialyzed. Both purified NH-zγGH and zγGH were stored at −80°C in the presence of 5 mM 2-mecaptolethanol, 20% ammonium sulfate, and 10% glycerol without significant loss of activity for at least 6 months.

Determination of Stoichiometry for NH-zγGH and zγGH. Recombinant NH-zγGH and zγGH were chromatographed on a Superdex 200 size exclusion column (0.46 × 30.0 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, containing 100 mM NaCl and 5 mM 2-mercaptoethanol on an Agilent Technologies (Santa Clara, CA) 1100 HPLC. The retention times of analyzed proteins were compared with the protein standards of known molecular weight and molecular weights obtained by standard curve interpolation.

Measurements of γGH Activity. A modified reversed-phase HPLC method was used to detect γGH activity (Patring et al., 2005). γGH catalyzes

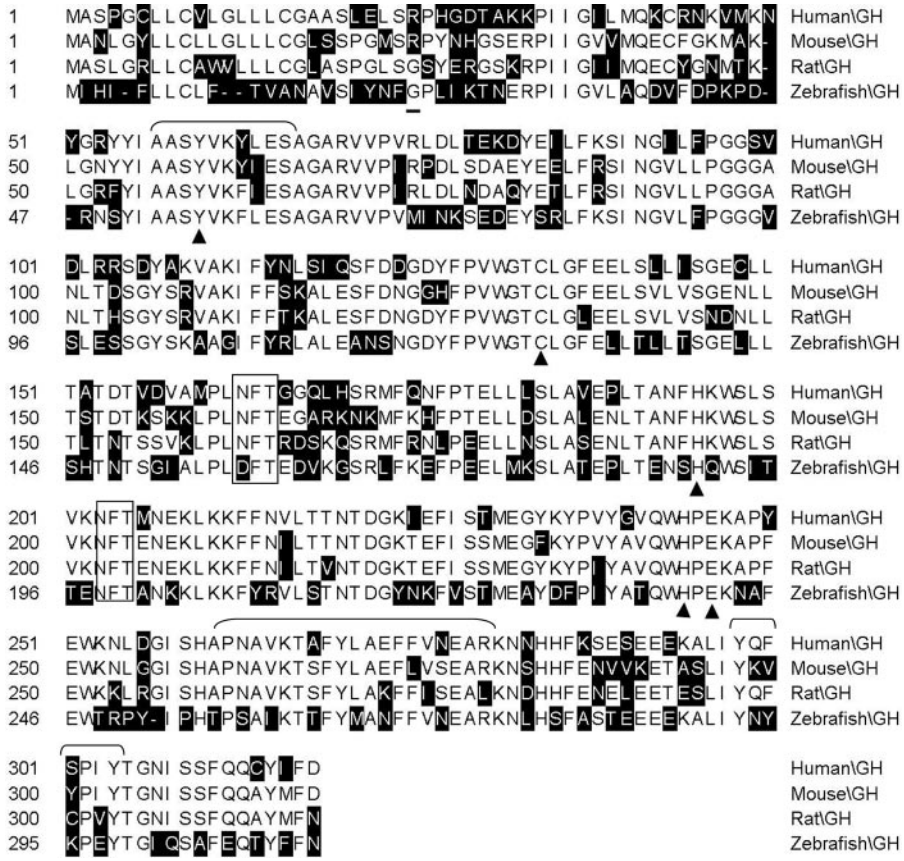


FIG. 1. Alignment of γGH peptide sequences. The shaded characters indicate amino acids that differ among compared species. Gaps, indicated by hyphens, are introduced for optimal alignment. The arrowheads indicate the conserved amino acid residues essential for enzyme activity. The brackets indicate the conserved sequences residing at the dimer interface. The boxes indicate the potential consensus residues for glycosylation. The sequences were aligned using the ClustalW method (Combet et al., 2000) with MegAlign/DNAstar sequence analysis software (DNASTAR, Madison, WI). The GenBank accession numbers of the aligned sequences are NP_003869 for human γGH, NP_034411 for mouse γGH, NP_037092 for rat γGH, and EU918170 for recombinant zγGH in the current report.

the hydrolysis of the γ-glutamate bond, resulting in folate-mono-Glu. The progression and rate of reaction were monitored by comparing the peak retention times and peak areas of 5-CHO-tetrahydrofolate (THF)-Glu_1 and -Glu_3 with those of pure corresponding folates of known concentrations. NH-zγGH or zγGH at less than 1% of substrate concentrations was used for the reaction rate measurement. In brief, 0.5 to 250 pmol of purified NH-zγGH (in the volume of 0.5 μl) or 5 to 20 μl of rat serum was added to 100 μl of reaction mixture containing 50 mM potassium phosphate, pH 6.0, 14 mM 2-mercaptoethanol, 2% ascorbic acid, and 2.5 μM 5-formyltetrahydrofolate triglutamate (5-CHO-THF-Glu_3) or methotrexate-Glu_5 in a 1.5-ml centrifuge tube. Tubes were flushed with nitrogen before capping and incubated at 37°C. After designated incubation times, tubes were boiled for 3 min and immediately chilled on ice to stop the reaction and precipitate protein simultaneously. The samples were centrifuged, and the supernatants were filtered and analyzed on an Aquasil C_{18} column, 150 × 4.6 mm, 3 μm (Thermo Fisher Scientific), using an HPLC system (Agilent 1100) with a fluorescence detector. The mobile phase used was acetonitrile/30 mM phosphate buffer, pH 2.3, under linear gradient elution conditions and the flow rate 0.4 ml/min. Retention times and peak areas of pure 5-formyltetrahydrofolate monoglutamate (5-CHO-THF-Glu_1) and 5-CHO-THF-Glu_3 of known concentrations were used for peak identification and standard curves construction. An estimation of the maximum rate was determined at the earliest time point when approximately 50% of 5-CHO-THF-Glu_3 had been converted to the monoglutamate. For kinetic studies, apparent K_m and k_{cat} were determined by performing reactions in the presence of 10 nM purified zγGH and 5-CHO-THF-Glu_3 ranging from 10 to 150 μM. The initial velocities were determined from the amount of 5-CHO-THF-Glu_1 generated within the first 30 s at each substrate concentration after the reaction was initiated. The kinetic constants were determined from double-reciprocal plots of initial velocity versus substrate concentration.

Results

Sequence and Structural Analysis of Recombinant zγGH. The isolated full-length zγGH cDNA (EU918170) is 939 base pair and encodes a protein of 312 amino acids. Currently, γGH has been identified in several mammals, including humans. Substantial similarities of primary structure between zγGH and its mammalian counterparts reveal conservation during evolution of this enzyme. A high level of homology is observed in the primary sequences and more so for the amino acid residues essential for catalytic activity (Fig. 1). A 53.8% identity between zebrafish and human γGH primary sequences is observed. The residues crucial for catalytic activity—Tyr^{36}, His^{171}, His^{220}, and Glu^{222}—are conserved in the zebrafish enzyme, as well as Cys^{110}, the residue mediating nucleophilic attack at the active site (Schneider and Ryan, 2006). Higher peptide sequence variation is observed in the postulated signal sequence (from Ile^2 to Phe^{21}). This prediction is based on the comparison with γGH of other species with known signal peptide sequences (Yao et al., 1996a,b). The predicted sequence patches present in the homodimer interface of mammalian enzymes also show similarity in zγGH, especially the first patch (Ala^{54} to Ser^{60}). Two of the consensus Asn-linked glycosylation sites are also observed in zγGH (Yao et al., 1996b). The primary sequence of zγGH was subjected to on-line secondary structure prediction and compared with human γGH ("PredictProtein," http://www.predictprotein.org/newwebsite/submit.php; Rost et al., 1996). The predicted seven helices of zγGH strongly resemble those of human γGH, indicating structural similarity between zebrafish and human enzymes (data not shown).

Expression and Purification of zγGH. Initially, full-length zγGH, including the prospective signal peptide, was cloned into pET43.1a with its start codon adjacent right after the ribosomal binding site on the vector. However, no significant expression was observed, despite the completely accurate and in-frame coding sequence. All our attempts to solve this problem, including changing competent cells, culture medium, IPTG concentration, induction time, and temperatures and cell density, were not successful. No enriched

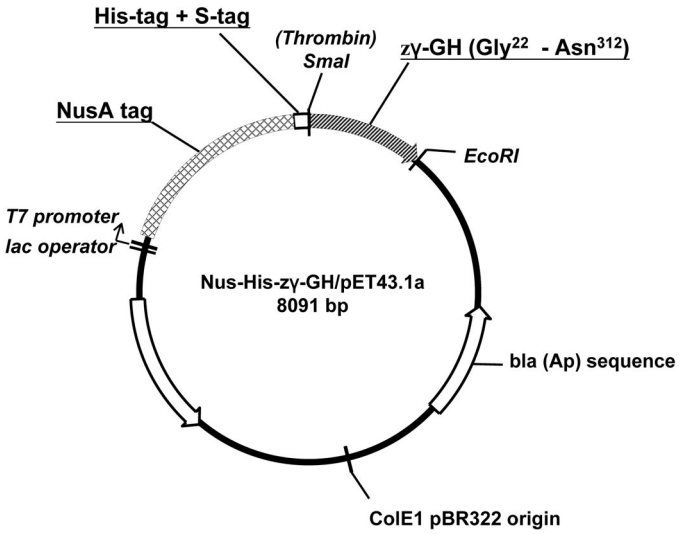


FIG. 2. Map of plasmid pET43.1a/NH-zγGH. The construction of this plasmid is described in detail under *Materials and Methods*. The crossed and shaded areas represent the Nus peptide and zγGH without the signal peptide, respectively.

protein band at the expected range of molecular weight was observed in the whole cell extract (data not shown). Significant expression of recombinant zγGH was accomplished only when the coding sequence of zγGH without the signal peptide region was subcloned downstream of the Nus coding sequence and expressed as a Nus-fusion protein (Fig. 2).

The recombinant NH-zγGH was induced by adding 0.4 mM IPTG at 27°C for 4 h. The majority of induced NH-zγGH remained in soluble fractions under this condition. The His-tag between Nus and zγGH allowed us to use Ni-Sepharose to greatly simplify the purification. The NH-zγGH eluted from Ni-Sepharose column was at least 95% pure, judged from SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Starting from the harvested cell pellet, we were able to obtain 8 mg of active NH-zγGH from 1 liter of culture cells in 5 h with good yield and purity.

To exclude the possible interference as a result of peptide fusion, we removed the N-terminal Nus peptide and His-tag by thrombin digestion of the NH-zγGH fusion protein combined with a second run of Ni-Sepharose column purification. The digestion efficiency and recovery rate were approximately 60% under the conditions used, judging from SDS-PAGE and the quantity of obtained thrombin

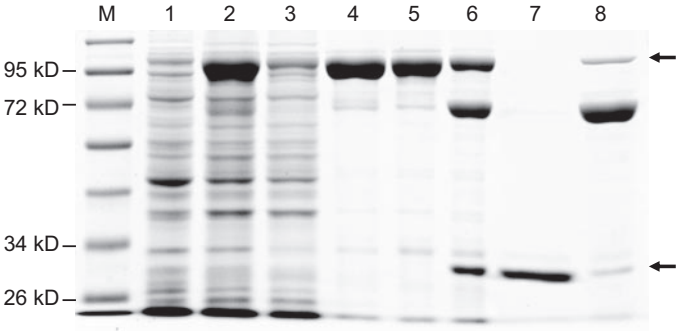


FIG. 3. SDS-PAGE of zγGH at each step of purification. Lane 1, uninduced cell lysate; lane 2, IPTG-induced cell lysate; lane 3, fraction flow through from Ni-Sepharose; lane 4, NH-zγGH eluted from Ni-Sepharose; lane 5, ammonium sulfate precipitated NH-zγGH after elution from Ni-Sepharose; lane 6, thrombin-digested NH-zγGH before fractionation on the second round of Ni-Sepharose; lane 7, Ni-Sepharose flow-through of thrombin digested NH-zγGH; lane 8, fraction eluted from the second round Ni-Sepharose; M, molecular weight marker. The upper and lower arrows indicate NH-zγGH and zγGH, respectively.

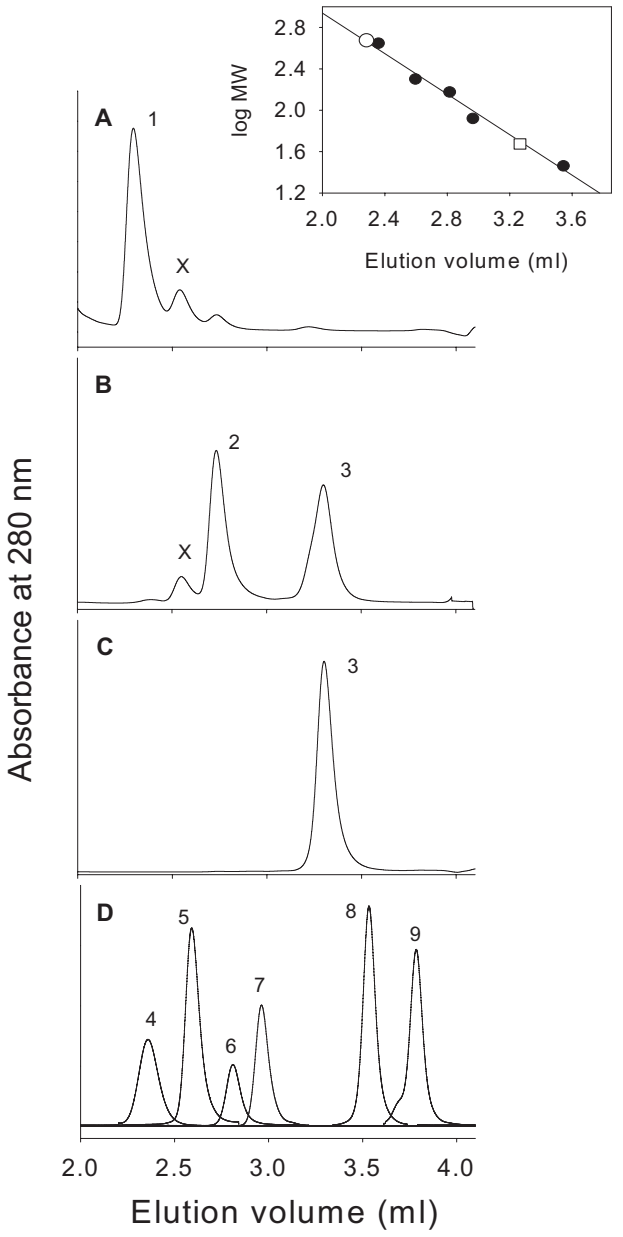
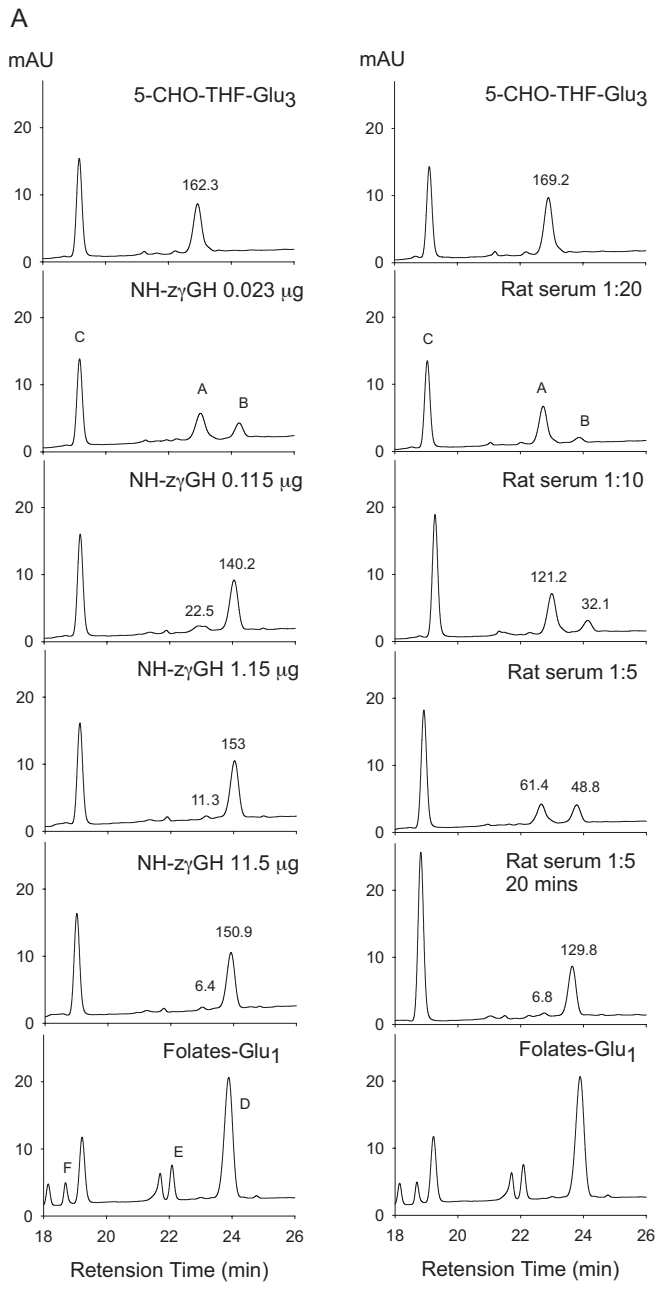
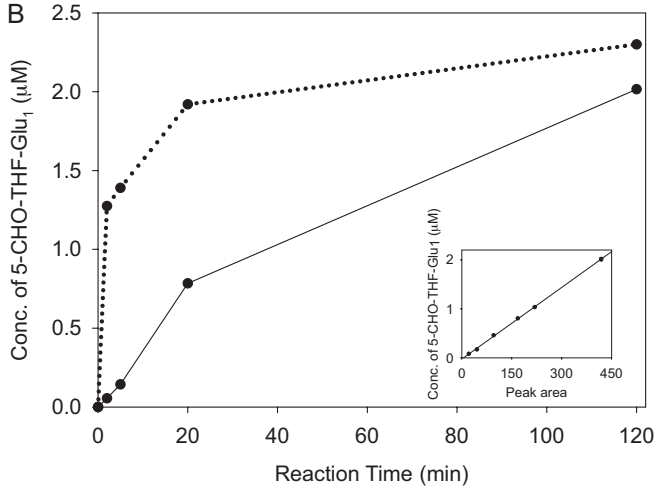


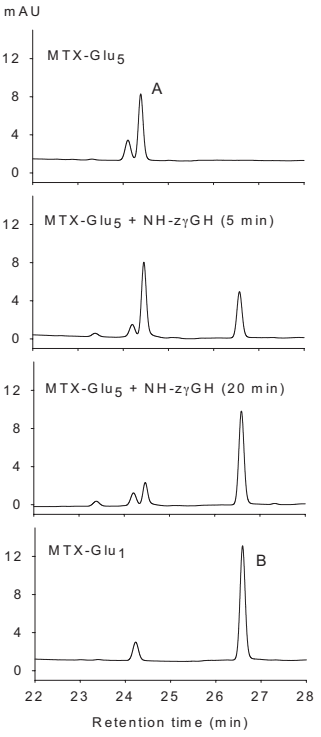
FIG. 4. Size-exclusion chromatographic profiles showing quaternary structures of recombinant zγGH. Approximately 50 μg of recombinant zebrafish NH-zγGH (A), NH-zγGH thrombin digestion mixture (B), and zγGH cleaved from NH-zγGH and purified by a second Ni-Sepharose chromatography (C) were analyzed on Superdex 200 (0.46 × 30.0 cm) and compared with molecular mass standard markers (D). Peak 1, NH-zγGH; peak 2, Nus-His-tag; peak 3, zγGH; peak 4, apo-ferritin (443 kDa); peak 5, β-amylase (200 kDa); peak 6, alcohol dehydrogenase (150 kDa); peak 7, albumin (66 kDa); peak 8, carbonic anhydrase (29 kDa); peak 9, ribonuclease A (14 kDa). X, molecule with unknown identity. The inset shows the plot of log M (molecular mass of standard markers) versus elution volume (closed circle) and from which the molecular mass of NH- zγGH (open circle) and zγGH (open square) was estimated.

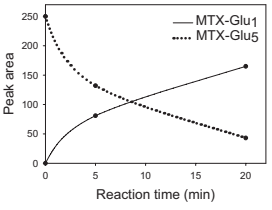
digested zγGH. Approximately 1.5 mg of thrombin-digested zγGH was obtained from 8 mg of purified NH-zγGH protein.

Structure and Activity of zγGH. Recombinant NH-zγGH and zγGH appeared on SDS-PAGE in the ranges of their estimated size of 92 and 33 kDa, respectively (Fig. 3). Unexpectedly, the retention volumes during chromatography on a Superdex 200 column showed that NH-zγGH and zγGH had Stokes radii close to globular proteins of 400 and 60 kDa, respectively (Fig. 4). These results suggest that zγGH exists as a homodimer, as observed for the human enzyme,


A
mAU
5-CHO-THF-Glu3
162.3
NH-zγGH 0.023 μg
C
A
B
NH-zγGH 0.115 μg
140.2
22.5
NH-zγGH 1.15 μg
153
11.3
NH-zγGH 11.5 μg
150.9
6.4
Folates-Glu1
D
E
F
Retension Time (min)
mAU
5-CHO-THF-Glu3
169.2
Rat serum 1:20
Rat serum 1:10
121.2
32.1
Rat serum 1:5
61.4
48.8
Rat serum 1:5
20 mins
129.8
6.8
Folates-Glu1
Retension Time (min)


B
Conc. of 5-CHO-THF-Glu1 (μM)
Reaction Time (min)
Conc. of 5-CHO-THF-Glu1 (μM)
Peak area


C
mAU
MTX-Glu5
A
MTX-Glu5 + NH-zγGH (5 min)
MTX-Glu5 + NH-zγGH (20 min)
MTX-Glu1
B
Retention time (min)


MTX-Glu1
MTX-Glu5
Peak area
Reaction time (min)

whereas NH-zγGH is a tetramer (Eisele et al., 2006). It seems that the presence of the N-terminal Nus and His-tag interferes with the quaternary structure of zγGH.

zγGH converts folate-polyglutamates to folate-monoglutamates. This activity was monitored by the conversion of 5-CHO-THF-Glu_3 to 5-CHO-THF-Glu_1 with HPLC. Shown in Fig. 5A are the chromatograms of 5-CHO-THF-Glu_3 and 5-CHO-THF-Glu_1 present in reaction mixtures after incubation for 5 min on adding various amounts of NH-zγGH or rat serum to initiate the reactions. Judging from the peak areas corresponding to substrate and product, we found that NH-zγGH, 0.1 μg (equivalent to 10 nM) was sufficient to convert more than 60% of 5-CHO-THF-Glu_3 (2.5 μM) to 5-CHO-THF-Glu_1 at 37°C in 5 min. The better efficiency of deconjugation mediated by NH-zγGH than by rat serum was also observed in the experiment performed in a time-dependent manner (Fig. 5B). The rate of product formation catalyzed by recombinant NH-zγGH in 2 min was 65 nmol/nmol protein/min. Conversion of all the substrate reached approximately 80% at 20 min and was completed in 2 h using NH-zγGH. On the other hand, less than 2 and 30% of 5-CHO-THF-Glu_3 was deconjugated when rat serum was added in the volume ratio of 1:10 (serum/sample) in 5 and 20 min, respectively. More than 2 h was required to convert 80% of substrate with rat serum. No significant loss of product was observed (2.4 μM) at the end of 2-h incubation with NH-zγGH, whereas only 80% of the product was detected in the rat serum sample. Methotrexate-Glu_5, an antifolate drug commonly used in regimens for chemotherapy, was also a substrate of NH-zγGH and zγGH with comparable efficiency to 5-CHO-THF-Glu_3 (Fig. 5C). The presence of only methotrexate-Glu_5 and methotrexate-Glu_1 during hydrolysis suggests that zγGH is most likely an endopeptidase, as observed with rat γGH (Yao et al., 1996b).

An approximately 2-fold increase was observed for the catalytic activity of zγGH on the removal of the N-terminal Nus peptide and His-tag compared with NH-zγGH. The 5-CHO-THF-Glu_1 generated was almost doubled when the same molecular concentration of NH-zγGH and zγGH were used (Fig. 6). The rates of product formation in the presence of 1.6 μM 5-CHO-THF-Glu_3 and 3 nM enzymes in 5 min were 20 and 38 min^{-1} for NH-zγGH and zγGH with approximately 55 and 95% of product formed, respectively. These results suggest interference in enzyme activity as a result of the presence of the N-terminal Nus peptide and His-tag. No significant difference in the activities was observed when the catalytic reactions were performed at either pH 4 or 6, although slightly higher activity was observed in acidic condition. Catalytic efficiencies of both NH-zγGH and zγGH were lowered to 65% when the reaction mixtures were performed at pH 12 (data not shown). The estimated apparent k_{cat} and K_m of zγGH for 5-CHO-THF-Glu_3, obtained from the average of three independent repeats, are 87.6 min^{-1} and 57.0 μM, respectively (Table 1).

Discussion

In this study, we report the cloning, characterization, and significance of zγGH and NH-zγGH fusion proteins. The purposes for obtaining pure and active zγGH are 2-fold. First, it enables detailed studies for the properties of zγGH and its role in regulating the intracellular availability of folates and some antifolate drug. This will help not only to advance our knowledge in antifolate drug mechanisms and development but also to evaluate the feasibility of using zebrafish as a model for antifolate drug discovery. Second, it improves the efficiency of the critical step of folylpolyglutamate hydrolysis for folate measurements.

To our knowledge, this is the first report for the cloning and purification of zγGH. This recombinant zγGH expressed and purified from *E. coli* was active, suggesting that glycosylation is not essential for zγGH catalytic activity. This is in agreement with a previous observation with human enzyme (Yao et al., 1996b). However, we could not exclude the possibility that glycosylation is important for the regulation of enzyme stability or function. The K_m of recombinant zγGH is comparable with that of human enzyme. However, the V_{max} is approximately 35-fold higher than that of human GH (Chave et al., 1999). Several factors might have contributed to the difference. First, to avoid potential product inhibition, the initial rate of reaction catalyzed by zγGH was determined for the first 30 s after adding enzyme to initialize the reaction. That was when less than 2% of substrate was converted to product. Human enzyme was measured when less than 15% of substrate was converted to product. Second, different substrates used in the assays for zebrafish and human enzymes might also affect the catalytic efficiency of the enzymes. What we used in the assay was 5-CHO-THF-Glu_3, whereas it was 4-NH_2-10-CH_3 PteGlu_2 for human enzyme. Nevertheless, we could not exclude the possible fundamental differences reside in these enzymes as a result of different species, although we think it is less likely.

It was unexpected that our initial attempt to overexpress zγGH without the Nus-expression partner was not successful. We had carefully examined the coding sequence for accuracy and codon usage and found several rarely used codons in *E. coli*. However, the use of the Rosetta strain for expression should have overcome this potential problem (Chang et al., 2007). We also tested for inclusion bodies and culture broth with SDS-PAGE for the possibility of aggregation or export of expressed zγGH because secretion of this protein had been reported in mammalian tumor cell lines (Gourdon et al., 2008). However, no enriched protein band was found in these fractions. Although we still have no confirmed answer for this result, several possibilities might help explain this observation. It is possible that the change of enzyme structure caused by the presence of Nus-His-Tag expression partner had altered the quaternary structure and the overall conformation, rendering this recombinant NH-zγGH less susceptible to protease degradation. This hypothesized change in protein conformation might also contribute to the observed decreased activity. An additional possibility is the lack of an endothelial reticulum system in *E. coli.* The presence of a leader sequence on the N-terminal of zγGH implies an endothelial reticulum-associated expression and/or organelle location for this enzyme. It has been suggested that the signal sequence must be removed for efficient expression in an *E. coli* system (Gourdon et al., 2008).

FIG. 5. HPLC chromatograms showing the concentration and time dependence of 5-CHO-THF-Glu_3 and methotrexate-Glu_5 hydrolysis. A, 5-CHO-THF-Glu_1 generated in hydrolysis reactions catalyzed by recombinant NH-zγGH (left) and rat serum (right) was analyzed by HPLC on a C_{18} reversed-phase column. The purified NH-zγGH or rat serum of indicated amounts was incubated with 2.5 μM 5-CHO-THF-Glu_3 at 37°C for 5 min before stopping the reaction and loading on column. The number on the top of the peak represents the peak area. Peak A, 5-CHO-THF-Glu_3; peak B, 5-CHO-THF-Glu_1; peak C, 2-mercaptoethanol; peak D, 5-CHO-THF-Glu_1 standard; peak E, 10-CHO-THF-Glu_1; peak F, THF-Glu_1. B, the concentrations of 5-CHO-THF-Glu_1 generated in the hydrolysis reactions were plotted against the reaction time. 5-CHO-THF-Glu_3 (2.5 μM) was incubated with 0.1 μg of NH-zγGH (dotted line) or rat serum of 1:10 ratio (solid line) at 37°C for indicated time. The concentrations of 5-CHO-THF-Glu_1 in the reaction mixtures were calculated by a linear interpolation of the peak areas of 5-CHO-THF-Glu_1 in the HPLC chromatograms from a standard curve constructed with 5-CHO-THF-Glu_1 of known concentrations (inset). C, methotrexate-Glu_5, instead of 5-CHO-THF-Glu_3, was used to examine the catalytic activity of NH-zγGH with HPLC using the condition as described for 5-CHO-THF-Glu_3. Peak A, methotrexate (MTX)-Glu_5; peak B, MTX-Glu_1. The concentrations of MTX-Glu_5 (dotted line) and MTX-Glu_1 (solid line) generated in the hydrolysis reactions were plotted against the reaction time (inset).

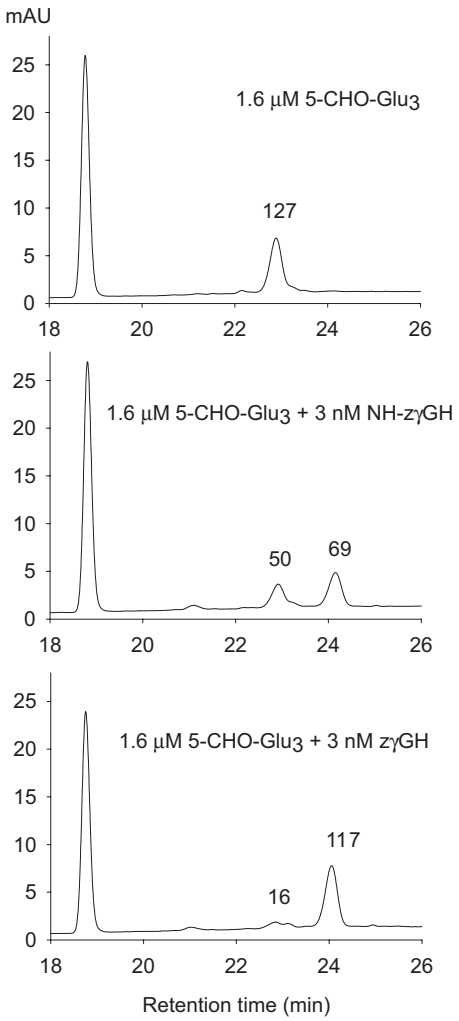


FIG. 6. HPLC chromatograms showing the higher catalytic activity observed with thrombin-digested zγGH than with NH-zγGH fusion enzyme for 5-CHO-THF-Glu_3 hydrolysis. 5-CHO-THF-Glu_3 (1.6 μM) was incubated with buffer only (top), 3 nM NH-zγGH (middle), and 3 nM thrombin-digested zγGH (bottom) at 37°C for 5 min before being analyzed with HPLC. The number on the top of each peak represents the area under the corresponding peak.

TABLE 1

Comparison of kinetic parameters for recombinant zebrafish and human γGH

Species	K_m[a]	k_{cat}	V_{max}	Reference
	μM	min^{-1}	*nmol/min · μg*	
Zebrafish	57.0 ± 9.8	2890 ± 215	87.6 ± 6.5	Present report
Human	49.0 ± 14.8	N.A.	2.6 ± 1.0	Chave et al. (1999)

[a] The substrate used in the assays for zebrafish enzyme was 5-CHO-THF-Glu_3, whereas the substrate used for human enzyme was 4-NH_2-10-CH_3 $PteGlu_2$. N.A., not available.

Adding purified NH-zγGH to a deconjugation reaction has increased the hydrolysis efficiency significantly. A 1:5 to 1:10 ratio between mammalian serum and biological sample is commonly used for folate deconjugation in most of the folate measurement assays used currently. Incubation at 37°C for at least 4 h is usually required for complete hydrolysis. However, this long incubation time has been reported to be detrimental to folates and therefore decreases accuracy significantly (Quinlivan et al., 2006). The incubation time required to reach 80% conversion with purified NH-zγGH was less than 15% of that when rat serum was used. Besides, the dilution effect caused by the volume of added enzyme (0.5 μl) was negligible. We even needed to dilute the purified enzyme before adding to the reaction mixture. This high efficiency allows a wide range for the amount of enzyme added without affecting folate concentrations in the samples. For example, a second boost with more purified enzyme after the first 5-min incubation might significantly reduce the time needed for complete conversion. In addition, we used NH-zγGH in most of our assays because small amounts of this fusion enzyme had provided sufficient activity for our experiments. Nevertheless, using thrombin-digested zγGH is expected to improve the hydrolysis efficiency and also decrease the incubation time.

There are still unanswered questions remaining about zγGH. It is important to investigate whether γGH is the enzyme responsible for hydrolyzing folylpolyglutamates in zebrafish intestines because that will affect the absorbance of potential antifolate drugs to be tested in zebrafish. In addition, comparable catalytic efficiencies of zγGH at both neutral and acidic environments raise the question whether this enzyme resides only in lysosomes. The enzyme purified in this study can be used to make an antibody that will help answer the above questions. Using the zebrafish model for drug discovery is foreseen to significantly speed up the translation of drug or treatments from bench research to bedside reality by revolutionizing the cell-based assays to embryo-based screening (Kari et al., 2007). In previous studies, we have shown that zebrafish folate enzymes are comparable with their human and mammalian counterparts for their catalytic and structural properties, as well as susceptibility to antifolate compounds (Chang et al., 2006, 2007; Kao et al., 2008). This purified recombinant zγGH will allow for further investigation to unravel the role of this key enzyme in regulating the intracellular availability of folates and antifolate drugs and to use zebrafish as an in vivo model for folate-related studies and antifolate drug discovery.

Acknowledgments. We thank Dr. Verne Schirch at the Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, for generous advice and assistance on the manuscript.

References

Chang WN, Tsai JN, Chen BH, and Fu TF (2006) Cloning, expression, purification, and characterization of zebrafish cytosolic serine hydroxymethyltransferase. *Protein Expr Purif* **46:**212–220.

Chang WN, Tsai JN, Chen BH, Huang HS, and Fu TF (2007) Serine hydroxymethyltransferase isoforms are differentially inhibited by leucovorin: characterization and comparison of recombinant zebrafish serine hydroxymethyltransferases. *Drug Metab Dispos* **35:**2127–2137.

Chave KJ, Galivan J, and Ryan TJ (1999) Site-directed mutagenesis establishes cysteine-110 as essential for enzyme activity in human gamma-glutamyl hydrolase. *Biochem J* **343 (Pt 3):**551–555.

Cheng Q, Wu B, Kager L, Panetta JC, Zheng J, Pui CH, Relling MV, and Evans WE (2004) A substrate specific functional polymorphism of human gamma-glutamyl hydrolase alters catalytic activity and methotrexate polyglutamate accumulation in acute lymphoblastic leukaemia cells. *Pharmacogenetics* **14:**557–567.

Combet C, Blanchet C, Geourjon C, and Deléage G (2000) NPS@: network protein sequence analysis. *Trends Biochem Sci* **25:**147–150.

Eisele LE, Chave KJ, Lehning AC, and Ryan TJ (2006) Characterization of human gamma-glutamyl hydrolase in solution demonstrates that the enzyme is a non-dissociating homodimer. *Biochim Biophys Acta* **1764:**1479–1486.

Gourdon P, Alfredsson A, Pedersen A, Malmerberg E, Nyblom M, Widell M, Berntsson R, Pinhassi J, Braiman M, Hansson O, et al. (2008) Optimized in vitro and in vivo expression of proteorhodopsin: a seven-transmembrane proton pump. *Protein Expr Purif* **58:**103–113.

Kao TT, Wang KC, Chang WN, Lin CY, Chen BH, Wu HL, Shi GY, Tsai JN, and Fu TF (2008) Characterization and comparative studies of zebrafish and human recombinant dihydrofolate reductases–inhibition by folic acid and polyphenols. *Drug Metab Dispos* **36:**508–516.

Kari G, Rodeck U, and Dicker AP (2007) Zebrafish: an emerging model system for human disease and drug discovery. *Clin Pharmacol Ther* **82:**70–80.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, and Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* **203:**253–310.

Patring JD, Jastrebova JA, Hjortmo SB, Andlid TA, and Jägerstad IM (2005) Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection. *J Agric Food Chem* **53:**2406–2411.

Quinlivan EP, Hanson AD, and Gregory JF (2006) The analysis of folate and its metabolic precursors in biological samples. *Anal Biochem* **348:**163–184.

Rhee MS, Wang Y, Nair MG, and Galivan J (1993) Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. *Cancer Res* **53:**2227–2230.

Rost B, Fariselli P, and Casadio R (1996) Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci* **5:**1704–1718.

Schneider E and Ryan TJ (2006) Gamma-glutamyl hydrolase and drug resistance. *Clin Chim Acta* **374:**25–32.
Shafizadeh TB and Halsted CH (2007) γ-Glutamyl hydrolase, not glutamate carboxypeptidase II, hydrolyzes dietary folate in rat small intestine. *J Nutr* **137:**1149–1153.
Suh JR, Herbig AK, and Stover PJ (2001) New perspectives on folate catabolism. *Annu Rev Nutr* **21:**255–282.
Westerfield M (1995) *The Zebrafish Book: Guide for the Laboratory Use of Zebrafish (Danio rerio).* University of Oregon Press, Eugene.
Wilson SD and Horne DW (1982) Use of glycerol-cryoprotected Lactobacillus casei for microbiological assay of folic acid. *Clin Chem* **28:**1198–1200.
Yao R, Nimec Z, Ryan TJ, and Galivan J (1996a) Identification, cloning, and sequencing of a cDNA coding for rat gamma-glutamyl hydrolase. *J Biol Chem* **271:**8525–8528.
Yao R, Schneider E, Ryan TJ, and Galivan J (1996b) Human gamma-glutamyl hydrolase: cloning and characterization of the enzyme expressed in vitro. *Proc Natl Acad Sci U S A* **93:**10134–10138.

Address correspondence to: Tzu-Fun Fu, Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, No. 1, University Road, Tainan 701, Taiwan. E-mail: tffu@mail.ncku.edu.tw