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Enzymatic and Structural Characterization of rTSγ Provides Insights into the Function of rTSβ

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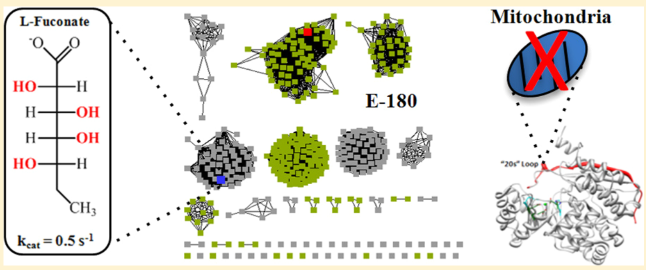
Supporting Information

ABSTRACT: In humans, the gene encoding a reverse thymidylate synthase (*rTS*) is transcribed in the reverse direction of the gene encoding thymidylate synthase (*TS*) that is involved in DNA biosynthesis. Three isoforms are found: α, β, and γ, with the transcript of the α-isoform overlapping with that of *TS*. rTSβ has been of interest since the discovery of its overexpression in methotrexate and 5-fluorouracil resistant cell lines. Despite more than 20 years of study, none of the rTS isoforms have been biochemically or structurally characterized. In this study, we identified rTSγ as an L-fuconate dehydratase and determined its high-resolution crystal structure. Our data provide an explanation for the observed difference in enzymatic activities between rTSβ and rTSγ, enabling more informed proposals for the possible function of rTSβ in chemotherapeutic resistance.



■ INTRODUCTION

In establishing a quantitative PCR assay for human thymidylate synthase (*TS*), a partially overlapping gene at the chromosome location 18p11.32, designated *reverse thymidylate synthase* (*rTS*), was found to be transcribed in the reverse direction with marginal overlap between the 3′-untranslated region of *rTS* and the last intron of *TS*.[1] Since then, two other isoforms have been identified from alternatively spliced mRNA, yielding a total of three isoforms: rTSα (Uniprot ID G2MQH2), rTSβ (G2MQH3), and rTSγ (Q7L5Y1).[2,3] All three proteins share the same 341 C-terminal amino acid sequence but differ in their N-terminal sequences and lengths. rTSα appends an additional 20 residues to the N-terminal of the shared sequence making it a 361 residue polypeptide. rTSβ has a different N-terminal sequence of 75 residues making it a 416 residue polypeptide, while rTSγ appends an additional 27 amino acids to the N-terminus of rTSβ to form a 443 residue polypeptide (Supporting Information Figure S1). The *rTS* genes have been of clinical interest in the cancer research community for over 20 years, yet their functions remain largely unknown. The main interest lies in the correlation of cell lines resistant to the common chemotherapeutics methotrexate and 5-fluorouracil, with rTSβ overexpression.[2−7] Methotrexate is a competitive inhibitor of dihydrofolate reductase (DHFR), and 5-fluorouracil is an irreversible inhibitor of TS. Both DHFR and TS play central roles in the synthesis of dTMP, a precursor for DNA synthesis. In clinical studies, rTSβ was found to be expressed in breast cancer tissue but not the surrounding tissues.[8] Furthermore, a statistically significant correlation was found between the level of rTSβ expression and a decrease in the five year survival rate of colon cancer patients.[5] The 27 residue longer N-terminus of rTSγ, compared to rTSβ, is proposed to constitute a mitochondrial signaling sequence,[9] suggesting that rTSβ and rTSγ serve similar enzymatic functions. The function of rTSα, the shortest of the three proteins, remains to be postulated.

Based on sequence homology and inspection of key catalytic residues, the three rTS isozymes are members of the mandelate racemase (MR) subgroup of the enolase superfamily (ENS) that is known to catalyze the racemization of mandelate as well as dehydration of various acid sugars.[10] All ENS members catalyze the abstraction of a proton alpha to a carboxylate group of the substrate to form a Mg^{2+} stabilized enediolate anion, although the overall reaction is not conserved.[11] Furthermore, members of the ENS share common structural motifs that form the catalytic machinery, including a capping domain for substrate specificity and a $(\beta/\alpha)_7\beta$-barrel domain for acid/base chemistry.[12,13]

In order to provide functional insights into the medically relevant rTSβ and facilitate future studies, we isolated recombinant human rTSβ and rTSγ isoforms, identified rTSγ as an L-fuconate dehydratase using a medium throughput enzymatic screen, and determined the crystal structure of rTSγ at 1.74 Å resolution.

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■ MATERIALS AND METHODS

Cloning, Expression, and Purification of Human rTSγ (HsrTSγ). A DNA fragment containing residues 1−440 of *Hs*rTSγ (IMAGE clone: 3454185) was subcloned into the pNIC28-Bsa4 vector (GenBank accession EF198106) incorporating an N-terminal TEV-cleavable His_6-tag. The plasmid was transformed into *E. coli* BL21(DE3)-R3-pRARE2 cells, grown overnight at 18 °C in 12 L of TB medium after induction by 0.1 mM IPTG. Cells were harvested, lysed in buffer A (50 mM HEPES, pH 7.4, containing 500 mM NaCl, 5% glycerol, 10 mM imidazole, 0.5 mM TCEP, and an EDTA-free protease inhibitor), and centrifuged to remove insoluble debris.

The initial stage of purification involved passing the clarified cell extract through a 2.5 mL column of Ni-NTA resin pre-equilibrated with buffer A. Bound protein was eluted with buffer B (buffer A + 250 mM imidazole). The fractions containing *Hs*rTSγ were applied onto a HiLoad 16/60 Superdex 200 column pre-equilibrated with GF buffer (10 mM HEPES, pH 7.4, containing 500 mM NaCl, 5% glycerol, and 0.5 mM TCEP). Fractions containing *Hs*rTSγ were treated with TEV protease overnight at 4 °C, and passed over Ni-Sepharose resin pre-equilibrated with GF buffer. The tagless protein was then diluted to 50 mM NaCl, and applied to a 1 mL Resource-S column pre-equilibrated with IEX buffer (50 mM Tris−HCl pH 8.5, containing 50 mM NaCl). Protein was eluted with a linear gradient of 0−500 mM NaCl and concentrated to 35 mg/mL for storage at −80 °C. Further purification details can be found at http://www.thesgc.org/structures/4a35.

Crystallization, Data Collection, and Structure Determination. Crystals of *Hs*rTSγ were grown by vapor diffusion at 20 °C. A sitting drop containing 100 nL protein (35 mg/mL) and 50 nL well solution was equilibrated against well solution containing 25% (v/v) PEG3350 and 0.1 M Bis-Tris, pH 5.5. Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen. Diffraction data for the native *Hs*rTSγ crystal were collected at the Diamond Light Source beamline I03 and processed with the XDS and SCALA. *Hs*rTSγ crystallized in the hexagonal space group $P6_522$ with one polypeptide in the asymmetric unit. The structure was solved by single-wavelength anomalous dispersion (SAD) phasing.

Crystals were derivatized by incubation with reservoir solution supplemented with 1 mM thiomersal for 60 min. Diffraction data were collected at the Diamond Light Source beamline I03, and processed and scaled with XDS[14] and Scala,[15] respectively. SHELXD[16] identified two heavy atom sites, and after phase refinement in SHARP[17] and subsequent density modification with SOLOMON, [18] an electron density map of excellent quality was obtained and substantial parts of the model were automatically built with ARP/wARP.[19] The resulting model was refined against the native data, where manual model rebuilding was carried out with Coot,[20] and structure refinement with BUSTER (Global Phasing Ltd., Cambridge, UK).

Cloning, Expression, and Purification of Human rTSβ (HsrTSβ). The gene encoding *Hs*rTSβ was PCR amplified from the *Hs*rTSγ:pNIC28-Bsa4 plasmid using platinum *Pfx* polymerase (Invitrogen). The PCR reaction (30 μL) contained 50 ng template, 1 mM $MgCl_2$, 1X Pfx Amp Buffer, 0.33 mM dNTP, 0.33 μM of each primer (forward primer 5′-TGGGTACCG-AGAACCTGTACTTCCAACATATGCACACGGACCC-3′ and reverse primer 5′-CAGTGGTGGTGGTCGTGGTGC-TCGAGT3′), and 1.25 units Pfx polymerase (Invitrogen Platinum Pfx DNA Polymerase kit). Amplifications were performed according to the manufacturer's guidelines. The amplification product was digested by NdeI and *Eco*RI (New England Biolabs) and ligated into NdeI/*Eco*RI digested pET17b (Novagen). The rTSβ gene in pET17b was expressed in *Escherichia coli* BL21 (DE3). Small-scale cultures were grown at 37 °C for 18 h in 5 mL of LB containing 100 μg/mL ampicillin and used to inoculate 1 L LB containing 100 μg/mL ampicillin. The 1 L cultures were grown for an additional 18 h at 37 °C without induction. The cells were harvested by centrifugation at 5000 rpm for 10 min and resuspended in 70 mL of binding buffer (6 mM imidazole, 20 mM Tris−HCl, 5 mM $MgCl_2$, and 500 mM NaCl, pH 7.9). The resuspended cells were lysed by sonication and centrifuged at 17,000 rpm for 30 min. The supernatant was loaded onto a 300 mL DEAE-Sepharose column (Amersham Biosciences) and eluted with a NaCl gradient (0 - 1 M over 1.6 L) in 10 mM Tris−HCl, pH 7.9, containing 5 mM $MgCl_2$. Fractions were analyzed using SDS-PAGE. Fractions that contained rTSβ were combined and dialyzed for 2 h at 4 °C against 4 L of 10 mM Tris−HCl, pH 7.9, containing 5 mM $MgCl_2$. The dialyzed protein was then loaded onto a 30 mL Q-Sepharose column (Amersham Biosciences) and eluted with a NaCl gradient (0−1 M over 500 mL) in 10 mM Tris−HCl, pH 7.9, containing 5 mM $MgCl_2$. Fractions were analyzed using SDS-PAGE. Fractions that contained *Hs*rTSβ at high purity were combined and dialyzed for 2 h at 4 °C against 4 L of 10 mM Tris−HCl, pH 7.9, containing 5 mM $MgCl_2$. Ammonium sulfate was added to a final concentration of 1 M, and the protein was loaded onto a 30 mL phenylsepharose column (Amersham Biosciences). The protein was eluted with a gradient of ammonium sulfate (1−0 M over 500 mL) in 10 mM Tris−HCl, pH 7.9, containing 5 mM $MgCl_2$. Fractions were analyzed using SDS-PAGE. Fractions with pure *Hs*rTSβ were combined and dialyzed for 2 h at 4 °C against 4 L of 10 mM Tris−HCl pH 7.9, containing 5 mM $MgCl_2$, 100 mM NaCl, and 10% glycerol. Finally, the protein was concentrated to 7.6 mg/mL, flash frozen using liquid N_2, and stored at −80 °C prior to use.

Screen for Dehydration. Reactions to test for dehydration activity for *Hs*rTSβ and *Hs*rTSγ were performed in acrylic, UV transparent 96-well plates (Corning Incorporated) using a library of 72 acid sugars (Supporting Information Figure S2). Reactions (60 μL total volume) contained 50 mM HEPES, pH 7.9, 10 mM $MgCl_2$, 1 μM enzyme, and 1 mM acid sugar substrate (blanks with no enzyme). The plates were incubated at 30 °C for 16 h. After incubation, 240 μL of a semicarbazide solution (1% semicarbazide w/v, 1% sodium acetate w/v) was added to each well and the plate was incubated for 1 h at room temperature. The absorbance at 250 nm was measured (semicarbazone ε = 10,200 M^{-1} cm^{-1}) using an Infinite M200 PRO microplate reader (Tecan Group Ltd.).

Kinetic Assays of HsrTSγ. Kinetic constants for the dehydration of L-galactonate, D-arabinonate, L-arabarate, and D-ribonate were measured using a discontinuous assay containing the semicarbazide assay.[21,22] Kinetic constants for the dehydration of L-fuconate were determined using a coupled enzyme assay as described by Yew and co-workers.[23]

Differential Scanning Fluorimetry. *Hs*rTSβ and *Hs*rTSγ were assayed for shifts in melting temperature as previously described.[24] Each protein (5 μg) was assayed as purified and, also, in the presence of 5 mM $MgCl_2$ or 5 mM $MgCl_2$ and 5 mM D-erythronohydroxamate.

Methotrexate and 5-Fluorouracil Assays. Methotrexate (Sigma-Aldrich) or 5-fluorouracil (Sigma-Aldrich) (10 mM) was incubated (800 μL) with 50 mM deuterated Tris−DCl, pD 7.9, 5 mM $MgCl_2$ and 1 μM purified protein (rTSβ or rTSγ) in D_2O for 48 h at 37 °C. Immediately following incubation, samples were analyzed for deuterium incorporation into methotrexate or 5-fluorouracil via ^{1}H NMR. In the case of a reaction that occurs without deuterium incorporation (with methotrexate), the optical activity at 589 nm was measured for an 800 μL reaction containing 10 mM methotrexate, 50 mM HEPES, pH 7.9, 10 mM $MgCl_2$, and 1 μM rTSβ in H_2O. Measurements were made at room temperature using a Jasco P-1010 polarimeter (Jasco Inc.) configured with a halogen lamp and 589 nm sodium d-line filter.

RESULTS AND DISCUSSION

Characterization of rTSγ. A sequence similarity network of all proposed acid sugar dehydratases within the ENS is shown in Supporting Information Figure S3. Clusters in the network were assigned specific acid sugar dehydratase functions based on homology to known acid sugar dehydratases, including conservation of metal binding and catalytic residues. *Hs*rTSγ, together with the canonical FucD from *Xanthomonas campestris* (*Xc*FucD), is found within the fuconate dehydratase (FucD) cluster at an e-value threshold of 10^{-80} (∼40% identity) (Supporting Information Figure S3). *Hs*rTSγ and *Xc*FucD separate as the e-value threshold is decreased to 10^{-180} (∼70% identity) (Figure 1).[23] The sequences of *Hs*rTSγ and *Xc*FucD are 52% identical and 71% similar. In the mannonate dehydratase subgroup of the ENS, proteins that are 67% identical and 79% can catalyze different enzymatic reactions.[25] Therefore, experimental characterization is necessary to assign an enzymatic function to *Hs*rTSγ.

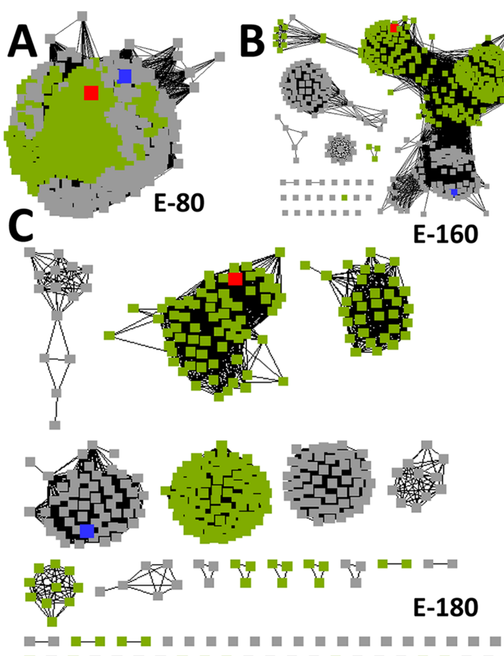

Figure 1. Panel A, sequence similarity network for the L-fuconate dehydratase subgroup at an e-value threshold of 10^{-80} (∼40% identity). Panel B, network at an e-value threshold of 10^{-160} (∼65% identity). Panel C, network at an e-value threshold of 10^{-180} (∼70% identity). The nodes for *Xc*FucD (PDB 2HXT) and rTSγ are colored red and blue, respectively.

*Hs*rTSγ was screened for dehydration activity with a library of 72 acid sugars. Positive screening hits were verified via ^{1}H NMR, and steady-state kinetic constants were obtained for the confirmed substrates. The following sugars were positive hits in the screen: L-fuconate, L-galactonate, D-arabinonate, L-arabarate, and D-ribonate (Figure 2). All sugars with the exception of L-arabarate were similarly identified as substrates for *Xc*FucD substrates.[23] L-fuconate shows the greatest catalytic efficiency (k_{cat}/K_M = 2.5 × 10^3 M^{-1} s^{-1}), which is an order of magnitude greater than that for the second best substrate, L-galactonate (k_{cat}/K_M = 1.0 × 10^2 M^{-1} s^{-1}) (Table 1). Together

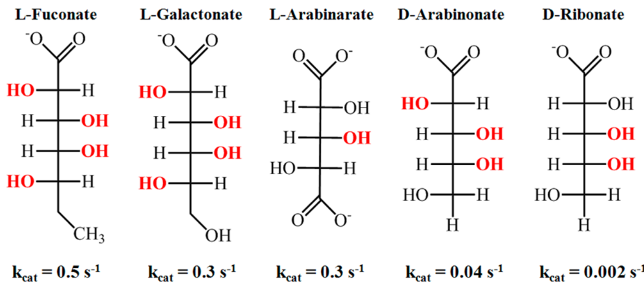

Figure 2. Structures of the top dehydration screening hits for rTSγ. The first order rate constants for dehydration are shown below the corresponding acid sugar. Carbons with conserved stereochemistry to L-fuconate have their hydroxyl groups highlighted in red.

Table 1. Steady-State Kinetic Parameters for rTSγ

substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
L-fuconate	0.5 ± 0.006	0.2 ± 0.01	2.5 × 10^3
L-galactonate	0.3 ± 0.01	3.0 ± 0.2	1.0 × 10^2
L-arabinarate	0.3 ± 0.004	4.0 ± 0.5	7.5 × 10^1
D-arabinonate	0.04 ± 0.002	2.0 ± 0.1	2.0 × 10^1
D-ribonate	0.002 ± 0.001	0.4 ± 0.2	5.0

with conservation of active site catalytic and metal binding residues, the kinetic data establish rTSγ as an L-fuconate dehydratase. In humans, as well as bacteria, L-fuconate dehydratase is important for the metabolism of L-fucose, which is found on the cellular surface of mammalian, insect, and plant cells.[23,26,27]

The k_{cat}/K_M value for L-fuconate in *Hs*rTSγ is 10-fold lower than for *Xc*FucD (4.5 × 10^4 M^{-1} s^{-1}).[23] This difference in catalytic efficiency may not be surprising given the moderate percent identity shared between the two enzymes. Additionally, the 10-fold reduction could also be explained by post-translational modifications in native *Hs*rTSγ that may not be present in the recombinant protein expressed in bacteria.

Crystal Structure of HsrTSγ. The crystal structure of *Hs*rTSγ with Mg^{2+} was solved by Hg-SAD phasing to a resolution of 1.74 Å (Table 2, PDB 4A35). The structure confirms its membership within the MR subgroup of the ENS, with *Xc*FucD being the closest structural relative, as identified by the DALI server[23] (root-mean-square deviation of 1.03 Å for 430 C$^\alpha$ atoms, Z-score 62.0, PDB 2HXT) (Figure 3a). As a member of the MR subgroup, *Hs*rTSγ contains the following canonical signature sequences in the core $(\beta/\alpha)_7\beta$-barrel domain that are essential for catalysis (Supporting Information

Table 2. Data Collection and Refinement Statistics for rTSγ^{a}

Data collection	
space group	$P6_522$
no. of molecules in asym. unit	1
cell dimensions	
a (Å)	84.77
b (Å)	84.77
c (Å)	316.30
β°	γ=120
resolution (Å)	1.74
no. of unique reflections	69165
R_{merge}	0.04 (0.79)
completeness (%)	99.0 (93.9)
refinement	
resolution (Å)	20.00−1.74 (1.78−1.74)
R_{cryst}	0.152 (0.240)
R_{free}	0.176 (0.288)
no. atoms	
protein	3468
waters	594
bound ligands	MG, EDO
ligand atoms	30
r.m.s deviations	
bond lengths (Å)	0.015
bond angles (°)	1.11
PDB accession code	4A35

[a]Bracketed values represent highest resolution shell.

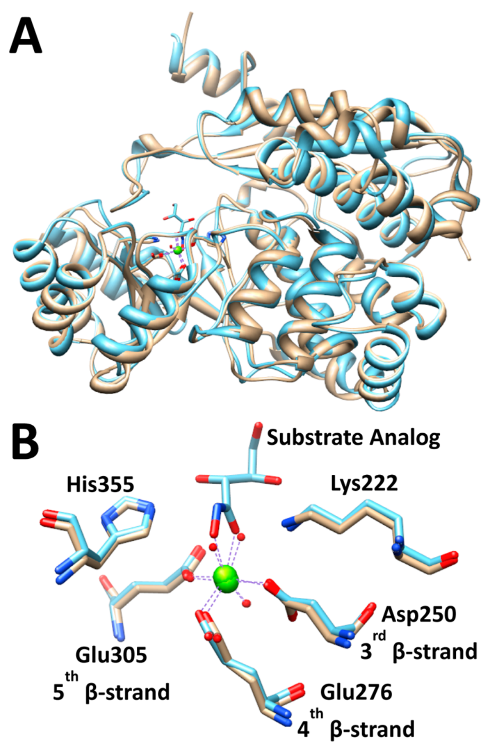

Figure 3. Panel A, an overlay of the structure of rTSγ (PDB 4A35, tan) and *Xc*FucD bound to the substrate analog L-erythronohydroxamate (PDB 2HXT, blue). The Cα RMSD is 1.03 Å. Panel B shows an overlay of the active site residues of the structures. The Cα RMSD for these metal binding and catalytic residues is 0.31 Å. Residue number is from the structure of rTSγ.

Figure S1): (1) a KxK motif (Lys 220-Val 221-Lys 222; *Hs*rTSγ residue numbering hereafter) at the end of the second β-strand of the barrel domain for base-catalyzed proton abstraction, (2) acidic residues Asp 250, Glu 276, and Glu 305 at the ends of the third, fourth, and fifth β-strands of the barrel domain, respectively, which provide the ligands for the essential Mg^{2+} (Figure 3b), and (3) a His-Asp dyad (His 355-Asp 328) at the ends of the seventh and sixth β-strands of the barrel domain, respectively, which is the general acid catalyst for dehydration of the enediolate intermediate.[28] The RMSD of these conserved residues between the *Hs*rTSγ and *Xc*FucD structures is 0.31 Å, supporting their catalytic roles in *Hs*rTSγ. Based on the characterization of *Xc*FucD, *Hs*rTSγ is assumed to utilize Lys 222 as the general basic catalyst for proton abstraction and His 355 as the general acid catalyst for dehydration (Figure 3b).

Liang and co-workers previously postulated that *Hs*rTSγ was simply an isoform of *Hs*rTSβ appended with a mitochondrial signaling sequence at its N-terminus.[9] Structural analysis of *Hs*rTSγ reveals that the extra 27 residues in the γ isoform comprise the first β-strand within the capping domain, a short α helix, and a large portion of the "20s" loop. This β-strand is part of a three-stranded β-sheet in the capping domain and packs closely against the C-terminus, while the "20s" loop, a conserved structural feature in MR subgroup members, is known to confer substrate specificity while occluding solvent from the active site[29−32](Figure 4). Considering the structural

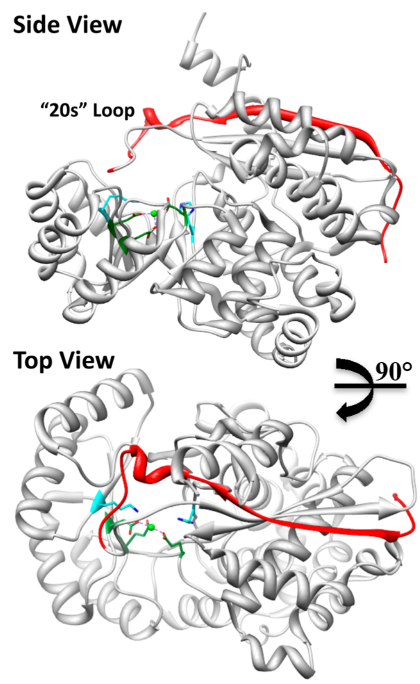

Figure 4. The structure of rTSγ (PDB 4A35) from side and top views. Each structure has the first 27 amino acids, which would be missing in rTSβ, highlighted in red. The metal binding residues are highlighted in green and catalytic residues in cyan.

integrity of this region and its close proximity to the active site, we propose that the extra 27 residues are not involved in mitochondrial targeting, but instead are necessary for the enzyme architecture and also possibly confer L-fuconate dehydratase activity to *Hs*rTSγ. This is supported by the lack of definitive mitochondrial targeting signals identified from various bioinformatics servers (data not shown).

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Pursuit of rTSβ Function. The importance of the N-terminal 27 residues for *Hs*rTSγ reactivity and integrity implies that *Hs*rTSβ, lacking this region, may have a deformed active site (e.g., highly solvent exposed, Supporting Information Figure S4) and be devoid of L-fuconate dehydratase activity. Accordingly, we pursued enzymatic characterization of recombinantly produced *Hs*rTSβ. Our initial attempt to subclone the gene encoding *Hs*rTSβ, as described for *Hs*rTSγ (i.e., N-terminal His-tagged fusion protein), resulted in insoluble protein. This suggests the importance of the N-terminal 27 residues for protein stability, in addition to its role in enzyme activity, and is also consistent with our observations that truncation constructs of *Hs*rTSγ lacking the N-terminal 5, 8, or 12 residues rendered the γ isoform to be highly insoluble in *E. coli* (Supporting Information Figure S5). We managed to subclone *Hs*rTSβ as a tag-less construct which resulted in a low level of expression in *E. coli*. The purified protein was screened for dehydration activity on the library of 72 acid sugars as described above. Based on the expectation that the complete 20s loop is necessary for catalysis, no dehydration activity was detected for *Hs*rTSβ. We also characterized *Hs*rTSβ and *Hs*rTSγ by differential scanning fluorimetry (Supporting Information Table S1) and showed that *Hs*rTSβ is more thermolabile than *Hs*rTSγ (ΔT_m = 5 °C) and, unlike *Hs*rTSγ, is not thermally stabilized by the addition of D-erythronohydroxamate, an enediolate intermediate analog, thereby suggesting that *Hs*rTSβ does not bind the ligand. Taken together, our data substantiate the hypothesis that the N-terminal 27 amino acid residues in rTSγ are necessary for catalytic activity and confer stability to the protein.

Several studies have observed uncharacteristically high levels of *Hs*rTSβ expression in tumor cell lines that also exhibit resistance to either methotrexate or 5-fluorouracil.[2−7] This correlation suggests the possibility that *Hs*rTSβ, which does not exhibit the L-fuconate dehydratase activity of *Hs*rTSγ, could instead harbor an alternative metabolic activity on these small molecules in a manner that inactivates them. The observation that *Hs*rTSβ expression was found to be induced by TS inhibitors indicates the protein may indeed be acting on these drug molecules.[2,4,33] To explore this possibility, methotrexate and 5-fluorouracil were analyzed for their ability to act as substrates for *Hs*rTSγ. Methotrexate has a proton alpha to the carboxylate group of its glutamate side chain, which could be racemized by an ENS member. Because methotrexate has more than twice the molecular weight of L-fuconate, a truncation of the "20s" loop, such as that seen in the β isoform, could expose a larger binding surface to accommodate methotrexate in the active site. Reactions were performed in D_2O to determine if exchange of the alpha proton (racemization) were occurring. Also, 5-fluorouracil was tested for defluorination. No incorporation of deuterium was observed via ^{1}H NMR in the presence of either *Hs*rTSγ or *Hs*rTSβ. Furthermore, to test a racemization mechanism in which deuterium exchange does not occur, methotrexate was incubated with *Hs*rTSβ or *Hs*rTSγ in a polarimeter, but no change in optical rotation was observed. These negative results, in conjunction with the lack of identification of an acid sugar substrate, allow us to conclude that rTSβ is not involved in chemical modification of methotrexate or 5-fluorouracil.

Dolnick proposed that *Hs*rTSβ could function by transferring a carboxylate group from S-adenosylmethionine to a lipophilic acceptor.[33−35] We find this unlikely, considering that MR subgroup members catalyze the abstraction of a proton alpha to a carboxylate group, which is not a partial reaction known to result in decarboxylation. Based on the in vitro function of *Hs*rTSγ and sequence homology to members of the MR subgroup, *Hs*rTSβ likely lacks an enzymatic activity. This, however, does not prohibit *Hs*rTSβ from participating in protein−protein interactions that may produce the observed resistance phenotype. In fact, TS can physically associate with proteins of the same molecular weight as rTSα and rTSβ, and conversely, rTSα and rTSβ could form a dimer (as observed in the X-ray structure for rTSγ) and coimmunoprecipitate with TS.[4] Furthermore, preliminary data suggesting that *rTS* proteins interact with dihydrofolate reductase (DHFR) could explain the correlation between rTSβ overexpression and methotrexate resistance.[35] The characterization of *Hs*rTSγ as an L-fuconate dehydratase, coupled with the inability of *Hs*rTSβ to catalyze this reaction, points to a mechanism of resistance that does not involve catalysis, but rather interactions with one of the above proposed partners. Future in vitro studies are needed to confirm the rTS/TS or rTS/DHFR protein−protein interactions and characterize their effect on TS or DHFR function.

■ CONCLUSION

Human rTSγ has been characterized in vitro as an L-fuconate dehydratase that belongs to the mandelate racemase subgroup of the enolase superfamily. The additional N-terminal amino acids in rTSγ compared to rTSβ are important for catalysis and stability, refuting previous proposals that rTSγ is merely a variant of rTSβ with an N-terminal mitochondrial signaling domain. rTSβ has been implicated in the resistance of tumor cell lines to methotrexate and 5-fluorouracil. Unfortunately, rTSβ does not catalyze any chemical modification of these chemotherapeutics. Therefore, we believe attempts to sensitize resistant cells lines via inhibitors to rTSβ will not be successful. Based on the crystal structure of rTSγ and the enzymatic screening performed in this study, we do not believe rTSβ is catalytically active. Therefore, hypotheses that involve protein−protein interactions are more likely the source of the observed phenotypes and should be investigated further.

■ ASSOCIATED CONTENT

Supporting Information

The sequence alignments, acid sugar library, acid sugar dehydratase SSN, *Hs*rTSγ constructs, and surface representations of *Hs*rTSγ can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org

Accession Codes

The X-ray coordinates and structure factors for *Hs*rTSγ have been deposited in the Protein Data Bank (PDB 4A35). This manuscript describes characterization of in vitro enzymatic activities of proteins with the following UniProt accession IDs: G2MQH2, G2MQH3, and Q7L5Y1.

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Author Contributions

J.A.G. and W.W.Y. conceived of the study. D.J.W., W.W.Y., and J.A.G. designed the experiments. D.J.W., D.S.F., J.K., and J.R.C.M. performed the experiments. D.J.W., W.W.Y., and J.A.G. analyzed the data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ENS, enolase superfamily; FucD, fuconate dehydratase; EDO, 1,2-ethanediol; MR, mandelate racemase; RMSD, root-mean-square deviation; rTS, reverse thymidylate synthase; TS, thymidylate synthase

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