

Enzymatic and Structural Characterization of rTS γ Provides Insights into the Function of rTS β

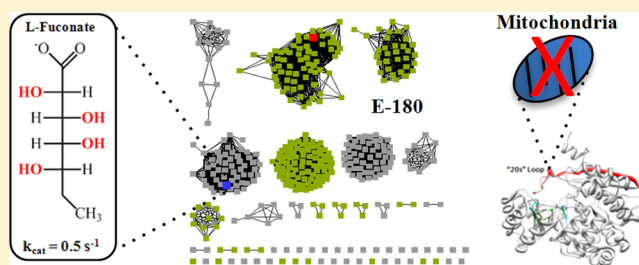
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S Supporting Information

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*. rTS β 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 rTS γ 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 rTS β and rTS γ , enabling more informed proposals for the possible function of rTS β 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: rTS α (Uniprot ID G2MQH2), rTS β (G2MQH3), and rTS γ (Q7LSY1).^{2,3} All three proteins share the same 341 C-terminal amino acid sequence but differ in their N-terminal sequences and lengths. rTS α appends an additional 20 residues to the N-terminal of the shared sequence making it a 361 residue polypeptide. rTS β has a different N-terminal sequence of 75 residues making it a 416 residue polypeptide, while rTS γ appends an additional 27 amino acids to the N-terminus of rTS β 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 rTS β 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, rTS β 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 rTS β expression and a decrease in the five year survival rate of colon cancer patients.⁵ The 27 residue longer N-terminus of rTS γ , compared to rTS β , is proposed to constitute a mitochondrial signaling sequence,⁹ suggesting that rTS β and rTS γ serve similar enzymatic functions. The function of rTS α , 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 Mg²⁺ 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 (β/α)- β -barrel domain for acid/base chemistry.^{12,13}

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

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MATERIALS AND METHODS

Cloning, Expression, and Purification of Human rTS γ (HsrTS γ). A DNA fragment containing residues 1–440 of HsrTS γ (IMAGE clone: 3454185) was subcloned into the pNIC28-Bsa4 vector (GenBank accession EF198106) incorporating an N-terminal TEV-cleavable His₆-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 HsrTS γ 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 HsrTS γ 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 HsrTS γ 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 HsrTS γ crystal were collected at the Diamond Light Source beamline I03 and processed with the XDS and SCALA. HsrTS γ crystallized in the hexagonal space group *P*₆₃22 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 XDS¹⁴ and Scala,¹⁵ respectively. SHELXD¹⁶ identified two heavy atom sites, and after phase refinement in SHARP¹⁷ 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 rTS β (HsrTS β). The gene encoding HsrTS β was PCR amplified from the HsrTS γ :pNIC28-Bsa4 plasmid using platinum Pfx polymerase (Invitrogen). The PCR reaction (30 μ L) contained 50 ng template, 1 mM MgCl₂, 1X Pfx Amp Buffer, 0.33 mM dNTP, 0.33 μ M of each primer (forward primer 5'-TGGGTACCG-AGAACCTGTACTTCCAACATATGCACACGGACCC-3'

and reverse primer 5'-CAGTGGTGGTGGTCTGGTGC-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 EcoRI (New England Biolabs) and ligated into NdeI/EcoRI digested pET17b (Novagen). The rTS β 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 μ g/mL ampicillin and used to inoculate 1 L LB containing 100 μ g/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 MgCl₂, 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 MgCl₂. Fractions were analyzed using SDS-PAGE. Fractions that contained rTS β were combined and dialyzed for 2 h at 4 °C against 4 L of 10 mM Tris-HCl, pH 7.9, containing 5 mM MgCl₂. 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 MgCl₂. Fractions were analyzed using SDS-PAGE. Fractions that contained HsrTS β 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 MgCl₂. 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 MgCl₂. Fractions were analyzed using SDS-PAGE. Fractions with pure HsrTS β were combined and dialyzed for 2 h at 4 °C against 4 L of 10 mM Tris-HCl pH 7.9, containing 5 mM MgCl₂, 100 mM NaCl, and 10% glycerol. Finally, the protein was concentrated to 7.6 mg/mL, flash frozen using liquid N₂, and stored at –80 °C prior to use.

Screen for Dehydration. Reactions to test for dehydration activity for HsrTS β and HsrTS γ were performed in acrylic, UV transparent 96-well plates (Corning Incorporated) using a library of 72 acid sugars (Supporting Information Figure S2). Reactions (60 μ L total volume) contained 50 mM HEPES, pH 7.9, 10 mM MgCl₂, 1 μ M enzyme, and 1 mM acid sugar substrate (blanks with no enzyme). The plates were incubated at 30 °C for 16 h. After incubation, 240 μ L 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 $\epsilon = 10,200 \text{ M}^{-1} \text{ cm}^{-1}$) using an Infinite M200 PRO microplate reader (Tecan Group Ltd.).

Kinetic Assays of HsrTS γ . Kinetic constants for the dehydration of L-galactonate, D-arabinonate, L-arabate, 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. HsrTS β and HsrTS γ were assayed for shifts in melting temperature as previously described.²⁴ Each protein (5 μ g) was assayed as purified and,

also, in the presence of 5 mM MgCl₂ or 5 mM MgCl₂ and 5 mM D-erythrono hydroxamate.

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 MgCl₂ and 1 μM purified protein (rTSβ or rTSγ) in D₂O for 48 h at 37 °C. Immediately following incubation, samples were analyzed for deuterium incorporation into methotrexate or 5-fluorouracil via ¹H 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 MgCl₂, and 1 μM rTSβ in H₂O. 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. *HsrTSγ*, together with the canonical FucD from *Xanthomonas campestris* (*XcFucD*), is found within the fuconate dehydratase (FucD) cluster at an e-value threshold of 10⁻⁸⁰ (~40% identity) (Supporting Information Figure S3). *HsrTSγ* and *XcFucD* separate as the e-value threshold is decreased to 10⁻¹⁸⁰ (~70% identity) (Figure 1).²³ The sequences of *HsrTSγ* and *XcFucD* are 52% identical and 71% similar. In the mannonate dehydratase subgroup of the ENS, proteins that are 67%

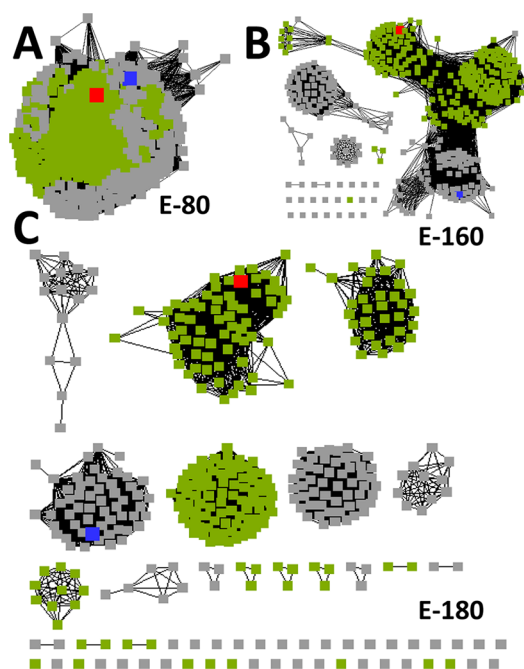


Figure 1. Panel A, sequence similarity network for the L-fuconate dehydratase subgroup at an e-value threshold of 10⁻⁸⁰ (~40% identity). Panel B, network at an e-value threshold of 10⁻¹⁶⁰ (~65% identity). Panel C, network at an e-value threshold of 10⁻¹⁸⁰ (~70% identity). The nodes for *XcFucD* (PDB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

identical and 79% can catalyze different enzymatic reactions.²⁵ Therefore, experimental characterization is necessary to assign an enzymatic function to *HsrTSγ*.

HsrTSγ was screened for dehydration activity with a library of 72 acid sugars. Positive screening hits were verified via ¹H 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

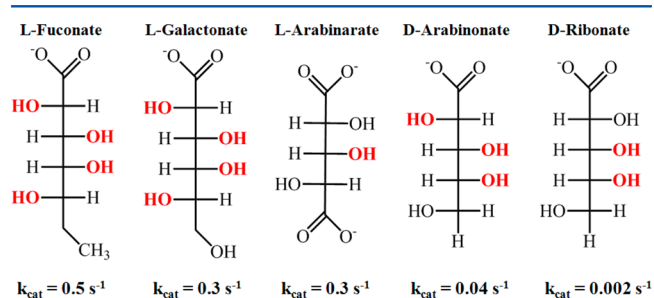


Figure 2. Structures of the top dehydration screening hits for rTSγ. The first order rate constants for dehydration are shown below the corresponding acid sugar. Carbons with conserved stereochemistry to L-fuconate have their hydroxyl groups highlighted in red.

exception of L-arabarate were similarly identified as substrates for *XcFucD* substrates.²³ L-fuconate shows the greatest catalytic efficiency ($k_{\text{cat}}/K_M = 2.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), which is an order of magnitude greater than that for the second best substrate, L-galactonate ($k_{\text{cat}}/K_M = 1.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) (Table 1). Together

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 *HsrTSγ* is 10-fold lower than for *XcFucD* ($4.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$).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 *HsrTSγ* that may not be present in the recombinant protein expressed in bacteria.

Crystal Structure of *HsrTSγ*. The crystal structure of *HsrTSγ* with Mg²⁺ 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 *XcFucD* being the closest structural relative, as identified by the DALI server²³ (root-mean-square deviation of 1.03 Å for 430 C^α atoms, Z-score 62.0, PDB 2HXT) (Figure 3a). As a member of the MR subgroup, *HsrTSγ* contains the following canonical signature sequences in the core (β/α)₇β-barrel domain that are essential for catalysis (Supporting Information

Table 2. Data Collection and Refinement Statistics for rTS γ^a

| | |
|---------------------------|------------------------|
| Data collection | |
| space group | $P6_322$ |
| no. of molecules | 1 |
| in asym. unit | |
| cell dimensions | |
| a (Å) | 84.77 |
| b (Å) | 84.77 |
| c (Å) | 316.30 |
| β° | $\gamma=120$ |
| 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 ($^\circ$) | 1.11 |
| PDB accession code | 4A35 |

^aBracketed values represent highest resolution shell.

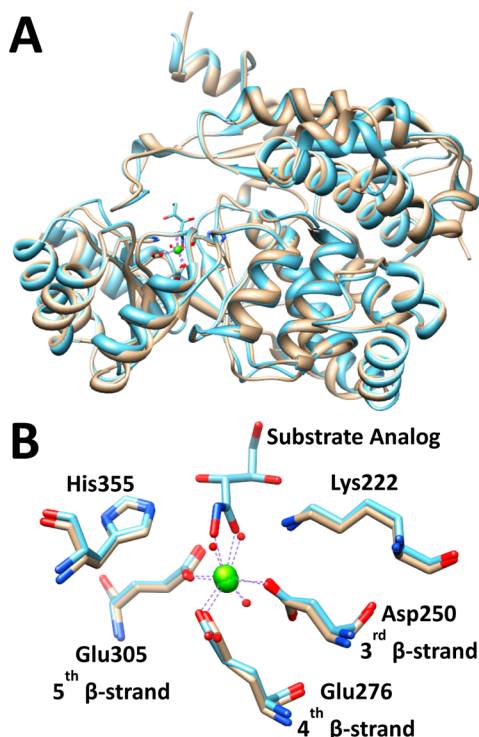


Figure 3. Panel A, an overlay of the structure of rTS γ (PDB 4A35, tan) and XcFucD bound to the substrate analog L-erythronohydroxamate (PDB 2HXT, blue). The $C\alpha$ RMSD is 1.03 Å. Panel B shows an overlay of the active site residues of the structures. The $C\alpha$ RMSD for these metal binding and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

Figure S1): (1) a KxK motif (Lys 220-Val 221-Lys 222; HsrTS γ 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 HsrTS γ and XcFucD structures is 0.31 Å, supporting their catalytic roles in HsrTS γ . Based on the characterization of XcFucD, HsrTS γ 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 HsrTS γ was simply an isoform of HsrTS β appended with a mitochondrial signaling sequence at its N-terminus.⁹ Structural analysis of HsrTS γ 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

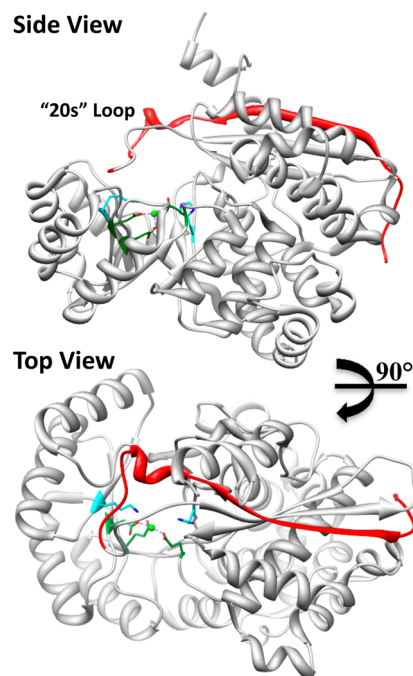


Figure 4. The structure of rTS γ (PDB 4A35) from side and top views. Each structure has the first 27 amino acids, which would be missing in rTS β , highlighted in red. The metal binding residues are highlighted in green and catalytic residues in cyan.

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 HsrTS γ . This is supported by the lack of definitive mitochondrial targeting signals identified from various bioinformatics servers (data not shown).

Pursuit of rTS β Function. The importance of the N-terminal 27 residues for HsrTS γ reactivity and integrity implies that HsrTS β , 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 HsrTS β . Our initial attempt to subclone the gene encoding HsrTS β , as described for HsrTS γ (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 HsrTS γ 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 HsrTS β 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 HsrTS β . We also characterized HsrTS β and HsrTS γ by differential scanning fluorimetry (Supporting Information Table S1) and showed that HsrTS β is more thermolabile than HsrTS γ ($\Delta T_m = 5^\circ\text{C}$) and, unlike HsrTS γ , is not thermally stabilized by the addition of D-erythronohydroxamate, an enediolate intermediate analog, thereby suggesting that HsrTS β does not bind the ligand. Taken together, our data substantiate the hypothesis that the N-terminal 27 amino acid residues in rTS γ are necessary for catalytic activity and confer stability to the protein.

Several studies have observed uncharacteristically high levels of HsrTS β expression in tumor cell lines that also exhibit resistance to either methotrexate or 5-fluorouracil.^{2–7} This correlation suggests the possibility that HsrTS β , which does not exhibit the L-fuconate dehydratase activity of HsrTS γ , could instead harbor an alternative metabolic activity on these small molecules in a manner that inactivates them. The observation that HsrTS β 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 HsrTS γ . 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 D₂O 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 ¹H NMR in the presence of either HsrTS γ or HsrTS β . Furthermore, to test a racemization mechanism in which deuterium exchange does not occur, methotrexate was incubated with HsrTS β or HsrTS γ 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 rTS β is not involved in chemical modification of methotrexate or 5-fluorouracil.

Dolnick proposed that HsrTS β 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 HsrTS γ and sequence homology to members of the MR subgroup, HsrTS β likely lacks an enzymatic activity. This, however, does not prohibit HsrTS β 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 rTS α and rTS β , and conversely, rTS α and rTS β could form a dimer (as observed in the X-ray structure for rTS γ) and coimmunoprecipitate with TS.⁴ Furthermore, preliminary data suggesting that rTS proteins interact with dihydrofolate reductase (DHFR) could explain the correlation between rTS β overexpression and methotrexate resistance.³⁵ The characterization of HsrTS γ as an L-fuconate dehydratase, coupled with the inability of HsrTS β 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 rTS γ 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 rTS γ compared to rTS β are important for catalysis and stability, refuting previous proposals that rTS γ is merely a variant of rTS β with an N-terminal mitochondrial signaling domain. rTS β has been implicated in the resistance of tumor cell lines to methotrexate and 5-fluorouracil. Unfortunately, rTS β does not catalyze any chemical modification of these chemotherapeutics. Therefore, we believe attempts to sensitize resistant cells lines via inhibitors to rTS β will not be successful. Based on the crystal structure of rTS γ and the enzymatic screening performed in this study, we do not believe rTS β 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, HsrTS γ constructs, and surface representations of HsrTS γ 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 HsrTS γ 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 Q7LSY1.

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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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