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Distribution of Mutations in Human Thymidylate Synthase Yielding Resistance to 5-Fluorodeoxyuridine*

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Thymidylate synthase (TS) catalyzes methylation of dUMP to dTMP and is the target of cancer chemotherapeutic agents (*e.g.* 5-fluorouracil). Here, we used error-prone PCR to mutagenize the full-length human TS cDNA and then selected mutants resistant to 5-fluorodeoxyuridine in a bacterial complementation system. We found that resistant mutants contained 1–5 amino acid substitutions and that these substitutions were located along the entire length of the polypeptide. Mutations were frequent near the active site Cys^{195} and in the catalytically important Arg^{50} loop; however, many mutations were also distributed throughout the remainder of the cDNA. Mutants containing a single amino acid replacement identified the following 14 residues as unreported sites of resistance: Glu^{23}, Thr^{51}, Thr^{53}, Val^{84}, Lys^{93}, Asp^{110}, Asp^{116}, Pro^{194}, Ser^{206}, Met^{219}, His^{250}, Asp^{254}, Tyr^{258}, and Lys^{284}. Many of these residues are distant from the active site and/or have no documented function in catalysis or resistance. We conclude that mutations distributed throughout the linear sequence and three-dimensional structure of human TS can confer resistance to 5-fluorodeoxyuridine. Our findings imply that long range interactions within proteins affect catalysis at the active site and that mutations at a distance can yield variant proteins with desired properties.

Thymidylate synthase (TS[1]; EC 2.1.1.45) plays a central role in *de novo* biosynthesis of dTMP, an essential precursor for DNA synthesis. The enzyme catalyzes reductive methylation of dUMP by (*6R*)-N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate ((*6R*)-CH_2H_4-folate)) to produce dTMP and dihydrofolate. Inactivation of TS results in depletion of thymidine pools, inhibition of DNA synthesis, and subsequent cell death (1, 2). In accord with its mechanism of action, toxicity associated with TS inhibition is greatest in actively dividing cells, including many types of cancer cells. For this reason, TS has been a major target for the development of cancer chemotherapeutic agents.

Fluoropyrimidine-based analogs, such as 5-fluorouracil and 5-fluorodeoxyuridine (5-FdUR), are widely utilized for the treatment of advanced cancers. These analogs are metabolized to 5-fluorodeoxyuridylate (5-FdUMP), which forms a stable inhibitory complex with the 72-kDa TS homodimer and CH_2H_4-folate. Currently, new folate analogs are also being tested as specific inhibitors of TS (3, 4). These anti-folates interact with the folate-binding site to interfere with the utilization of the normal substrate, CH_2H_4-folate.

Use of TS inhibitors as chemotherapeutic agents is frequently limited by toxicity to bone marrow cells. Introduction of a drug-resistant TS into bone marrow stem cells could protect patients from the severe side effects of TS inhibitors. Based on this premise, mutations have been identified that render human TS resistant to 5-fluoropyrimidines and anti-folate analogs (5–10). Nearly all of these mutations map within the catalytic site. We have previously created 5-FdUR-resistant mutants by substituting random nucleotides for codons specifying amino acids adjacent to the catalytic cysteine (Cys^{195}) that is responsible for methyl transfer; these mutants include A197F, C199L, several replacements at Val^{204}, and Q211L (5). We similarly created 5-FdUR-resistant mutants in the highly conserved Arg^{50} loop that undergoes reorientation on dUMP binding to accept the incoming folate molecule; these mutants include replacements at Lys^{47}, Asp^{48}, and Gly^{52} (6). Earlier work involving mutagenesis of human HT1080 cells with ethylmethane sulfonate, followed by selection with the anti-folate Thymitaq (AG337), yielded three Arg^{50} loop mutants (D49G, G52S, and K47E) that were resistant to both the anti-folate and 5-FdUR (7). Two other mutants that are resistant to both anti-folates and 5-FdUR, I108A and F225W, were obtained by site-directed mutagenesis of highly conserved residues important in folate binding (8).

Two TS mutations have been reported (9, 10) that are not within the active site yet render human TS resistant to 5-FdUR. One of the first TS mutants discovered to confer 5-FdUR resistance was heterozygously expressed in the colon cancer cell line HCT116 (9). This mutant contains a single substitution, Y33H, within a loop near the amino terminus of the protein, at a distance from the catalytic site. The other mutation found in a 5-FdUR-resistant cell line was determined to encode a P303L substitution. Although metabolically unstable, the P303L protein was nonetheless able to confer resistance against both 5-FdUR- and anti-folate-based TS inhibitors in transfected cells (10). Like Y33H, the P303L substitution is not in close proximity to the catalytic site as determined from crystal structures (11–16).

The hypothesis guiding this study is that amino acid substitutions in human TS that yield 5-FdUR resistance can be located throughout the protein. This hypothesis is based on two considerations. The first is the existence of at least two single mutations in human TS that lie distant from the active site and also confer 5-FdUR resistance (9, 10). The second is that mi-

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[1] The abbreviations used are: TS, thymidylate synthase; CH_2H_4-folate, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; 5-FdUR, 5-fluoro-2′-deoxyuridine; 5-FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate.

crofluctuations throughout the protein, arising from the inherent flexibility of the backbone structure, may be required for maintaining substrate specificity during catalysis and may be affected by mutations distant from the catalytic site. To assess our hypothesis, we examined the distribution of amino acid substitutions that confer 5-FdUR resistance by using error-prone PCR to introduce mutations throughout the full length of the human TS cDNA. Mutations associated with resistance were then isolated by transfecting plasmids encoding the mutant enzymes into TS-deficient *Escherichia coli* and selecting for growth in the presence of 5-FdUR. Many of the mutant genes we thus created encode catalytically active TS, render *E. coli* 5-FdUR-resistant, and contain multiple substitutions. Among the TS variants containing a single amino acid substitution, we identified replacements at residues that have not been previously reported to be sites of resistance in human TS. In fact, this study more than doubles the number of single amino acid substitutions in human TS that have been demonstrated to yield 5-FdUR resistance. Moreover, in accord with our hypothesis, single mutations that render TS resistant to 5-FdUR were distributed throughout the protein. Whereas some of the amino acids we identified are located within the active site, others reside at a distance and have no previously recognized involvement in catalysis or 5-FdUR resistance.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—E. coli NM522 cells (Stratagene, La Jolla, CA) were used for cloning and library construction. *E. coli* χ2913recA cells lacking TS (Δ*thyA*572, *recA*56), kindly provided by Dr. Daniel Santi (University of California, San Francisco), were used in all complementation studies. Plasmid DNA was isolated by using Perfectprep Plasmid Mini (Eppendorf Scientific Inc., Westbury, NY) and Maxiprep kits (Qiagen, Chatsworth, CA). *Taq* DNA polymerase and dNTPs were from Promega (Madison, WI). DNA oligomers and T4 DNA ligase were from Invitrogen. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). DNA fragments were isolated by using the Quiaquick gel extraction kit from Qiagen. 5-FdUR, 5-FdUMP, and thymidine were purchased from Sigma, and (6*R*,*S*)-CH_2H_4-folate was obtained as the racemic mixture from Schircks Laboratories (Jona, Switzerland). ABI Prism Dye Terminator Cycle Sequencing kits for fluorescent sequencing were the products of PerkinElmer Life Sciences.

Standard and Error-prone PCR—Standard PCRs contained 100 ng of plasmid DNA carrying the wild type TS cDNA; 1 μM concentrations of the primers HK-MUT5′ (5′-ATAACAATTTCACACAGGAAACAGC-TATGACC-3′) and HK-MUT3′ (5′-CAGGGTTTTCCCAGTCACGACGT-TGTAAAACG-3′); 250 μM each dCTP, dTTP, dATP, and dGTP; and 2 units of *Taq* DNA polymerase in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.01% Triton X-100 in a total volume of 100 μl. Error-prone PCR was performed by the method of Cadwell and Joyce (17) with modification. Conditions were those described for standard PCR, except for elevation of the $MgCl_2$ concentration to 7 mM, the addition of 0.5 mM $MnCl_2$, and unequal concentrations of the four dNTPs (1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.1 mM dGTP). PCR was performed in a PCT-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA) by using the following protocol: 3 min of initial denaturation at 94 °C followed by 45 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. To determine the mutation frequency under standard and mutagenic conditions, PCR products were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced by using the primer DLPCR-TS3R (5′-AAAAAAAAACCATGTCTCC-GGATCTCTGGTAC-3′).

Construction of the TS Mutant Library—DNA amplified by error-prone PCR was digested with *Sac*I and *Nde*I and subjected to electrophoreses in an agarose gel. The 1-kb TS fragment was extracted from the gel by using the Qiaquick Gel Extraction kit (Qiagen) and ligated into the *Nde*I- and *Sac*I-digested pGCHTS-TAA vector backbone (kindly provided from Dr. Daniel Santi) containing 115 bp of 5′-untranslated region from the *Lactobacillus casei* TS gene as a leader sequence. The ligated plasmid DNA was transformed into NM522 *Escherichia coli* cells (Stratagene) by electroporation (1.8 kV, 25 microfarads, 400 ohms; Gene Pulser; Bio-Rad). Twenty-five transformations were pooled, and the size of the library was determined to be 2.8×10^6 independent clones by plating an aliquot of the transformation mixture on 2× YT medium containing 50 μg/ml of carbenicillin (Island Scientific, Bainbridge Island, WA). To determine the frequency and types of mutations introduced by PCR, plasmid DNA was isolated from the *E. coli* mutant library and sequenced by using the ABI Prism Dye Terminator Cycle Sequencing Kit and the primer DLPCR-TS3R. For amplification of the library, 25 ml of the pooled transformation mixture was added into 500 ml of 1× YT medium containing 50 μg/ml carbenicillin and incubated overnight at 37 °C, and plasmid DNA was prepared. The plasmid DNA library (500 ng) was transformed into 100 μl of electrocompetent χ2913 cells. Transformation mixtures were pooled, grown overnight in appropriate antibiotics, and stored at −80 °C in 15% glycerol.

Genetic Selection in E. coli—E. coli χ2913 cells harboring plasmids encoding the TS library were grown overnight at 37 °C in 2× YT medium containing 50 μg/ml of carbenicillin and 50 μg/ml thymidine. Cultures were diluted 1:100 in the same medium and grown at 37 °C until the A_{600} reached 0.6–0.8. Aliquots (1 ml) of the exponentially growing cells were pelleted and resuspended in M9 salts and plated on three different media. On 1× YT containing 50 μg/ml of carbenicillin and supplemented with 50 μg/ml of thymidine, essentially all cells harboring the plasmid DNA should be recovered. On M9 plates containing carbenicillin, only cells expressing active TS proteins should grow, because the medium lacks thymidine. Drug-resistant clones were selected on M9 plates containing carbenicillin and increasing concentrations of 5-FdUR essentially as previously described (5, 6).

Site-directed Mutagenesis—Single amino acid substitutions were introduced by using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid DNA encoding the wild type TS cDNA was amplified by using PfuTurbo DNA polymerase and a set of primers containing the appropriate base substitution. The PCR product was treated with *Dpn*I to cleave the parental DNA template and the noncleaved DNA was transformed into XL-1 Blue competent cells. Cells containing plasmid DNA with the desired substitution were selected on the LB-ampicillin agar plates, and plasmid DNA was harvested and sequenced.

Purification of TS Proteins—To subclone mutant TS fragments into the pHis vector (a modified pUC12 vector provided by A. Hizi), the TS cDNA was PCR-amplified by using HK-MUT5′ and TS-Xho3′ (5′-CAGCTCGAGCTCCTTTGAAAGCACCCTAAAC-3′). After digestion with *Nde*I and *Xho*I, the amplified fragments were ligated into the *Nde*I-*Sal*I-digested pHis vector. The reconstructed plasmids were verified by both restriction analysis and DNA sequencing. Wild type and mutant TS proteins, as N-terminal hexahistidine fusions, were then purified by metal chelation chromatography on Ni^{2+} affinity resin (His-Bind resin and buffer kit; Novagen), as previously described, with minor modifications (5). An overnight culture of χ2913 cells expressing the His-TS fusion protein was diluted 1:100 into 250 ml of 2× YT medium containing carbenicillin and thymidine. After attaining an A_{600} of 0.2, cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, grown to an A_{600} of 0.8, harvested by centrifugation, resuspended in 12 ml of 1× binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9) and lysozyme (200 μg/ml; Sigma), and frozen at −80 °C. Frozen cells were thawed and lysed on ice for 3 h. The disrupted cells were centrifuged ($27{,}000 \times g$), and the supernatant was collected and applied to a charged 2.5-ml His-Bind column (1 × 2.5 cm; Novagen, Madison, WI). The resin was prepared by successive washes with 15 ml of deionized water, 10 ml of 1× charge buffer (50 mM $NiSO_4$), and 15 ml of 1× binding buffer. Following application of the crude extract, the column was washed with 30 ml of 1× binding buffer and 25 ml of a mixture containing 60% binding buffer and 40% wash buffer (60 mM imidazole, 500 μM NaCl, 20 mM Tris-HCl, pH 7.9). His-TS protein was eluted with five 1-ml aliquots of 1× elution buffer (1 M imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9). SDS-PAGE showed that TS protein was eluted in the second and third fractions. Fractions containing TS protein were combined and dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, and 10% glycerol for 16 h and then against the same buffer containing 1 mM dithiothreitol for an additional 10 h. The concentration of purified TS was determined by using the Bradford assay.

Kinetic Analysis—TS activity was monitored spectrophotometrically by the increase in absorbance at 340 nm that occurs concomitant with the production of H_2-folate ($\Delta\epsilon = 6400\ M^{-1}\ cm^{-1}$) (18, 19). The reaction buffer contained 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.4), 25 mM NaCl, 6.5 mM formaldehyde, 1 mM EDTA, and 150 μM 2-mercaptoethanol. When the concentration of dUMP was varied, a high concentration of (6*R*,6*S*)-CH_2H_4-folate was used (600–2400 μM concentration of the racemic mixture); when the concentration of (6*R*,*S*)-CH_2H_4-folate was varied, the concentration of dUMP was 400 μM. Purified wild type or mutant TS protein was added to initiate the reaction at 25 °C. Initial velocity measurements were obtained with a PerkinElmer Life Sciences Lambda Bio 20 UV-visible spectrophotome-

TABLE I
Mutation spectra produced by amplification of human TS cDNA

The cDNA encoding human TS was PCR-amplified under standard and mutagenic conditions and cloned into pCRII-TOPO, and the 5′-terminal two-thirds of the DNA (nucleotides 1–630) was sequenced.

PCR conditions	No. of clones	Average no. of mutations/ clone	Mutation frequency	No. of substitutions						Deletions
				Transitions		Transversions				
				AT→GC	GC→AT	AT→TA	AT→CG	GC→TA	GC→CG	
			%							
Standard	36	0.2	0.031	6	0	0	0	0	1	0
Mutagenic	18	9.8	1.58	41	35	66	3	3	0	7

ter. Steady-state kinetic parameters were derived by a nonlinear least squares fit of the data to the Michaelis-Menten equation with KaleidaGraph 3.0 software (Abelbeck Software, Reading, PA).

Inhibition constants (K_i) for 5-FdUMP were obtained from a Dixon plot (20) by measuring initial velocities in the presence of dUMP, CH_2H_4-folate, and inhibitor at 25 °C. Two dUMP concentrations (30 and 300 μM) were used, and 5-FdUMP concentrations were varied. To initiate reactions, (6*R*,*S*)-CH_2H_4-folate was added to yield a final concentration of 600 μM.

RESULTS

Mutagenesis of Human TS by Error-prone PCR—To introduce amino acid substitutions throughout the TS protein, error-prone PCR was carried out by increasing the concentration of Mg^{2+}, adding Mn^{2+}, and using unequal concentrations of the four dNTPs. Manganese ions have been shown to increase misincorporation by a variety of DNA polymerases, and the error rates of these enzymes are proportional, over a limited range, to the relative concentrations of the dNTP substrates (21, 22). The mutations introduced by PCR under standard and mutagenic conditions were compared by sequencing the initial two-thirds of the TS cDNA (nucleotides 1–630 encoding amino acids 1–210). Under standard conditions with 1.5 mM Mg^{2+} and equimolar dNTPs, *Taq* DNA polymerase introduced a relatively small number of mutations; the average for the 36 clones sequenced was 0.2 mutations per clone. Under mutagenic conditions, however, the average was much higher (*i.e.* 9.8 mutations per clone), and the great majority of cDNAs carried more than seven mutations (Table I). Six of seven mutations found under standard conditions were A:T to G:C transitions, frequently introduced by *Taq* DNA polymerase (23, 24). In contrast, under mutagenic conditions, A:T to T:A transversions were most frequent, and a variety of other types of substitutions were detected, predicting a greater diversity of amino acid substitutions.

Construction of TS Mutant Library and Analysis of Unselected Clones—Following amplification by error-prone PCR, the TS cDNAs were cloned into a plasmid vector downstream of the *L. casei* TS untranslated sequence. The ligated products of the backbone and these variant PCR products constituted the TS mutant library, containing $\sim 2.8 \times 10^6$ independent clones. Plasmid DNA was isolated from 16 colonies obtained from growth on nonselective rich medium containing thymidine, and the number and types of nucleotide substitutions were determined by sequencing the 5′-proximal 630 nucleotides of the cDNA. The number of mutations per cDNA in the nonselected mutants is shown in Fig. 1*A*. Unselected clones had an average of 4.3 nucleotide changes; all of those detected were base substitutions with the exception of two deletions. At the amino acid level, this corresponds to 3.2 substitutions within the segment sequenced, suggesting that, on average, the full-length proteins contained about 4.8 amino acid replacements. Amino acid substitutions in the unselected clones were distributed fairly uniformly throughout the sequenced region (data not shown).

Selection and Analysis of Active TS Mutants—Expression of catalytically active TS protein enables TS-deficient *E. coli* χ2913 host cells to form colonies on M9 minimal plates in the absence of added thymidine. On plating the TS mutant library, approximately half the number of colonies was formed on M9 minimal medium lacking thymidine relative to the thymidine-

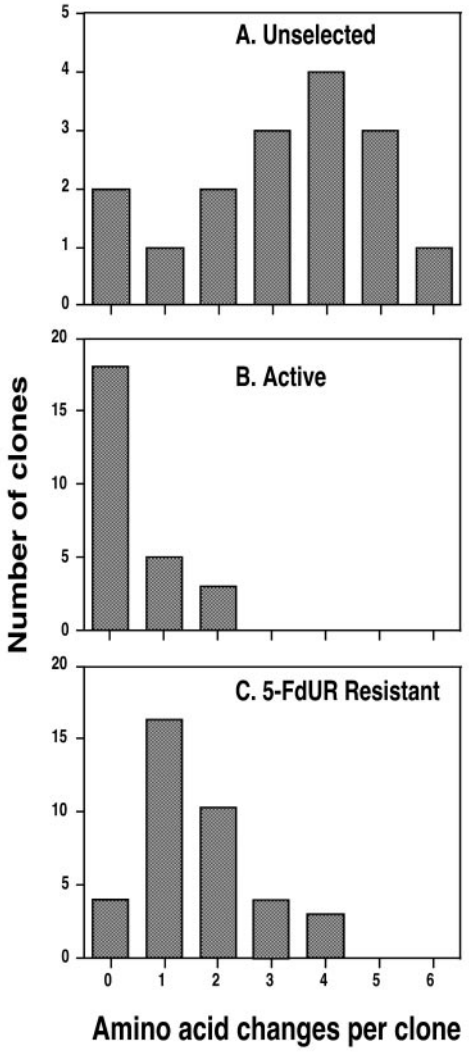

FIG. 1. **Number of amino acid substitutions in human TS mutants.** A mutant library was obtained by error-prone PCR amplification of the human TS cDNA and cloned into an expression vector. *A*, unselected mutants were obtained from transformed *thy*$^-$ *E. coli* χ2913 cells grown on rich medium. *B*, active mutants were obtained from bacteria grown on M9 minimal medium. *C*, 5-FdUR-resistant mutants were collected from cells grown on M9 medium containing 200 or 300 nM 5-FdUR. To confirm that the plasmid DNA recovered from *E. coli* was responsible for resistance, the DNA was retransformed into fresh host cells, and survival was determined on varying concentrations of 5-FdUR. Thirty-seven clones showing resistance to ≥150 nM upon retransformation were chosen for further analysis. The 5′-proximal 630 bases of the plasmids recovered from unselected, active, and resistant mutants were sequenced. Since the full length of TS cDNA is 939 bases, 1.5 times as many substitutions are expected to be present throughout the protein, and this is indicated in the number of substitutions in this figure.

supplemented medium. Thus, ~50% of the TS mutants exhibited sufficient catalytic activity to complement the TS-deficient phenotype. Of the active mutants analyzed, 8 of 26 clones carried amino acid substitutions in the 630-bp sequence (Fig. 1*B*). No amino acid substitutions were detected in the other 18 clones, although two clones contained silent mutations. This limited survey indicates that most of the active mutant clones contained less than one amino acid substitution and that these substitutions do not prevent the catalytic activity required for complementation.

Isolation and Analysis of 5-FdUR-resistant TS Mutants—To isolate mutants that render *E. coli* χ2913 resistant to 5-FdUR, the TS library was plated on M9 minimal medium containing 200 or 300 nM 5-FdUR. As previously reported (5, 6), cells expressing wild-type TS exhibited a diminished ability to form colonies on M9 plates containing more than 150 nM 5-FdUR. Resistant colonies were selected, and plasmid DNA was isolated and retransformed into fresh χ2913 cells to confirm that the plasmid was the source of the drug-resistant phenotype. Thirty-seven independent clones were picked that showed increased resistance upon re-exposure to 5-FdUR, and the 5′-terminal 630 bp of the cDNA was sequenced. 0–4 amino acid substitutions were observed in the sequenced segment of resistant clones, the average being 1.6 (Fig. 1*C*).

To obtain a profile of amino acid replacements conferring resistance, the remaining, 3′-proximal third of the cDNA from each of the 37 mutants was sequenced. In two of the clones, we failed to detect any substitutions; the mechanisms of resistance in these two clones remain to be determined. A summary of all predicted amino acid substitutions found in 35 of the mutants that exhibited resistance to ≥150 nM 5-FdUR is shown in Table II. Resistance is recorded as percentage survival relative to percentage survival of host cells harboring wild-type TS at 150 nM 5-FdUR or greater. Expression of TS in 10 of the resistant mutants was analyzed by Western blotting with antiserum directed against human TS. The level of expression of wild-type TS was more than 2-fold greater than that of any of the mutants, estimated by visual inspection of the gel (data not shown). The mutant proteins may be more labile than the wild type or more susceptible to proteolytic degradation.

Distribution of PCR-generated Mutations Yielding 5-FdUR Resistance—The distribution of amino acid substitutions in the 5-FdUR-resistant clones generated by error-prone PCR is indicated in Fig. 2. In the case of mutants with multiple substitutions, not all of the replacements are expected to be directly involved in resistance, some presumably having been co-selected. Importantly, substitutions were dispersed throughout the protein. The distribution included "hot spots," where clustered mutations appeared to be more frequent than would be expected on a random basis, as well as "cold spots." Several regions exhibited a high density of mutations. The residues between and including Val^{45} and Thr^{55} constituting the Arg^{50} loop harbored eight different substitutions. The replacements included T51S, commonly observed and present in most resistant mutants in previous experiments (6). These results are in accord with previous reports showing that the evolutionarily conserved Arg^{50} loop is a major site of mutations that confer 5-FdUR resistance (6, 7). The residues from Arg^{78} to Leu^{88} accumulated nine replacements. This region has not been reported to be involved in 5-FdUR resistance; structural analysis indicates that it is located on the active site pocket and interacts with folate-based inhibitors, providing a plausible rationale for resistance (1, 11–16). The N-terminal residues from Gly^{5} through Gln^{18} accumulated 10 substitutions; this segment is disordered in existing crystal structures and has no known function (11–16). There is no obvious correlation between the frequency of either single or multiple substitutions observed in resistant mutants and the presence of β-sheets or α-helices. However, amino acid residues that are completely conserved during evolution are less likely to incur substitutions resulting in 5-FdUR resistance.

TABLE II

5-FdUR-resistant human TS mutants created by error-prone PCR

Thirty-seven 5-FdUR-resistant TS mutants were obtained by complementation of the growth defect of *thy*$^-$ *E. coli* plated on medium containing 5-FdUR; the 35 mutants harboring amino acid substitutions within the TS cDNA are listed. The resistance conferred by each mutant was determined in direct, side-by-side comparison of mutant and wild-type survival. All comparisons were carried out in duplicate at varying concentrations of 5-FdUR, and most comparisons were repeated in two or more separate experiments. As previously reported (5, 6), cells expressing wild-type TS exhibited diminished ability to form colonies on M9 plates containing 150 nM 5-FdUR. Only those experiments in which the survival of the wild-type was lower than 1.5% were included in this tabulation. Ligand interactions with wild-type TS are described in Protein Data Bank (25) entries 1JU6 and 1HVY: for dUMP, residues at positions 50, 87, 109, 135, 192, 195, 196, 214, 215, 216, 218, 222, 223, 226, 256, 258, 175′, and 176′; for cofactor, positions 50, 51, 77, 79, 80, 87, 107, 108, 109, 112, 192, 218, 221, 222, 225, 226, 258, 306, 307, 308, 309, 311, and 312.

Substitution(s)	Mutant number	Survival[a] (mutant/WT)
Wild type		
none		
Single mutants		
E23G	357 (a)	>110
D48V	312 (a)	>98
V84A	319 (b)	54
K93E	209 (c)	340
D110E	330 (b)	530
P194Q	226 (d)	36
S206G	229 (e)	>24
H250L	339 (a)	>7
K284N	331 (a)	>14
Double mutants		
P12L, N302S	307 (f)	>37
D21G, M311L	217 (d)	56
V45A, D254N	255 (e)	>210
T53S, Y258F	302 (g)	220
G54C, M236I	329 (f)	>180
F80S, F91L	219 (h)	>90
F80S, L88S	353 (f)	>250
L85M, L88S	361 (i)	>62
T96S, A228T	370 (e)	>300
S103T, V204A	316 (f)	>140
F142S, F225I	317 (f)	>200
P172S, D254E	358 (e)	>180
P193S, A231G	308 (g)	270
Triple mutants		
G5D, L13R, A231T	315 (g)	280
G5S, R78C, M219I	228 (e)	>82
S6N, D69Q, Q211L	313 (f)	>140
A17T, D116A, D254E	318 (b)	770
Q18R, R78H, I262T	238 (j)	54
T55I, V106A, K284I	222 (h)	>48
S66G, K104R, E128G	369 (f)	>210
Quadruple and higher		
L8Q, D21G, T53A, R78L	203 (e)	>320
L8Q, W81G, L131V, Y230F	354 (f)	>170
L13Q, P27L, R42H, V84A, F248L	356 (f)	>190
V45I, T125I, A144S, K308R	326 (b)	16
T51S, K82Q, K99D, N171S	362 (g)	180
T170S, V204M, S206G, M219V	359 (f)	>160

[a] Survival is expressed as fold enhancement in survival of host cells harboring mutant TS, relative to survival conferred by wild-type TS (WT). The percentage survival of the wild type at the indicated concentrations of 5-FdUR, given in parentheses following the individual mutant numbers, was as follows: (a) <0.7% at 150 nM; (b) 0.1% at 150 nM; (c) 0.2% at 150 nM; (d) 0.9% at 300 nM; (e) <0.2% at 150 nM; (f) <0.5% at 200 nM; (g) 0.4% at 150 nM; (h) <0.4% at 150 nM; (i) <0.1% at 150 nM; (j) 1.5% at 200 nM. Survival listed for each mutant is the average of replicate determinations.

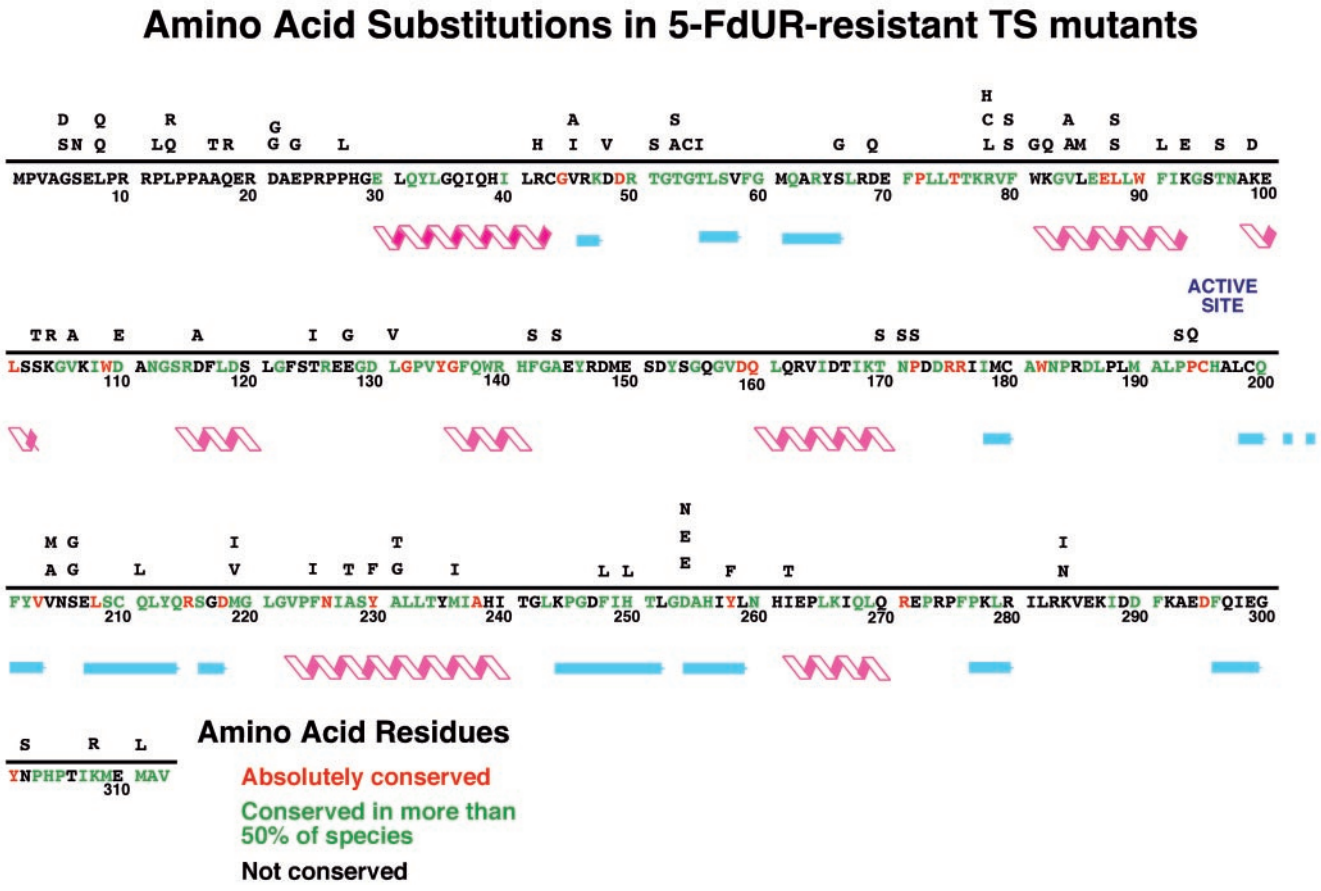

FIG. 2. **Distribution of amino acid substitutions in 5-FdUR-resistant TS mutants.** All amino acid substitutions detected in 5-FdUR-resistant mutants generated by error-prone PCR are presented. The wild-type sequence is shown *below* the *line*. *Red letters* indicate residues absolutely conserved among 29, evolutionarily diverse TS proteins (1); *green letters* indicate residues conserved in more than 50% of the TS sequences compiled, representing proteins from viruses to mammals (1); *black letters* indicate nonconserved residues. *Spiral shapes*, α-helices; *bars*, β-sheets (14).

Resistance of T53S,Y258F Reflects the Contribution of Both Single Mutations—Among the newly identified clones, the double mutant T53S,Y258F (clone 302) exhibited a high level of resistance at elevated concentrations of 5-FdUR. In multiple experiments, T53S,Y258F was as resistant or more resistant to elevated concentrations of 5-FdUR than any clone tested. As shown in Fig. 3*A*, *E. coli* cells carrying the T53S,Y258F mutant grew on minimal plates containing 250 nM 5-FdUR, whereas, in a side-by-side comparison, the double mutant T51S,G52S, the most resistant clone found in our previous studies (7), formed colonies at 200 nM 5-FdUR but not 250 nM 5-FdUR. The same was true for another clone isolated in the present work that exhibited high level resistance, the quadruple mutant T51S,K82Q,K99E,N171S (clone 362), as well as for another triple mutant G5D,L31R,A231T (clone 315; results not shown).

To analyze the contribution of individual amino acid substitutions to the 5-FdUR resistance of the double T53S,Y258F mutant, we generated the T53S and Y258F single mutants by site-directed mutagenesis. As illustrated in Fig. 3*B*, both the T53S and Y258F single mutants were less resistant than the double mutant but more resistant than wild type TS. These results indicate that both substitutions contribute to the high level of resistance exhibited by T53S,Y258F.

Asp^{254} Is a Site of 5-FdUR Resistance—Replacements at Asp^{254} were detected in three multiply substituted, resistant clones generated by error-prone PCR (D254E in two clones and D254N in one). We generated several single mutants at this site and assessed their resistance. As shown in Fig. 3*C*, the three mutants D254A, D254E, and D254N all showed higher resistance than wild type TS and were more resistant than a parent clone (clone 318), which had two substitutions in addition to D254E (A17T,D116A,D254E).

Kinetic Analysis of Mutant TS Proteins—To establish that the resistance to 5-FdUR observed in *E. coli* reflects altered function of the mutant proteins, several TS variants were purified as N-terminal hexahistidine fusions. Purity was estimated to be greater than 80% by densitometric scanning of Coomassie Blue-stained gels. Steady-state kinetic parameters for the wild-type and mutant TS proteins are recorded in Table III. The k_{cat} values observed for the mutants and the K_m values for dUMP do not differ markedly from that of wild type. However, the K_m values of the mutants for CH_2H_4-folate were 5–13-fold higher than that for wild type TS. K_m values were ~10-fold higher for the double mutant T53S,Y258F (clone 302) and the quadruple mutant T51S,K82Q,K99D,N171S (clone 362), which were among the most resistant variants. In accord with the *E. coli* survival data, the K_i of all mutants for 5-FdUMP was greater than that of wild type TS. Mutant T53S,Y258F (clone 302), which exhibited high resistance to 5-FdUR in our complementation assay, showed a 6-fold increase. T51S demonstrated the largest increase over wild type (11-fold), whereas the less resistant single mutant, Y258F (see Fig. 3*B*), showed a lesser increase (3.1-fold).

Single Mutants That Yield 5-FdUR Resistance—The hypothesis we set out to assess is that mutations in TS that confer 5-FdUR resistance are distributed throughout the protein and are not confined to regions directly involved in catalysis. In this work, we created single mutants that newly identify a total of

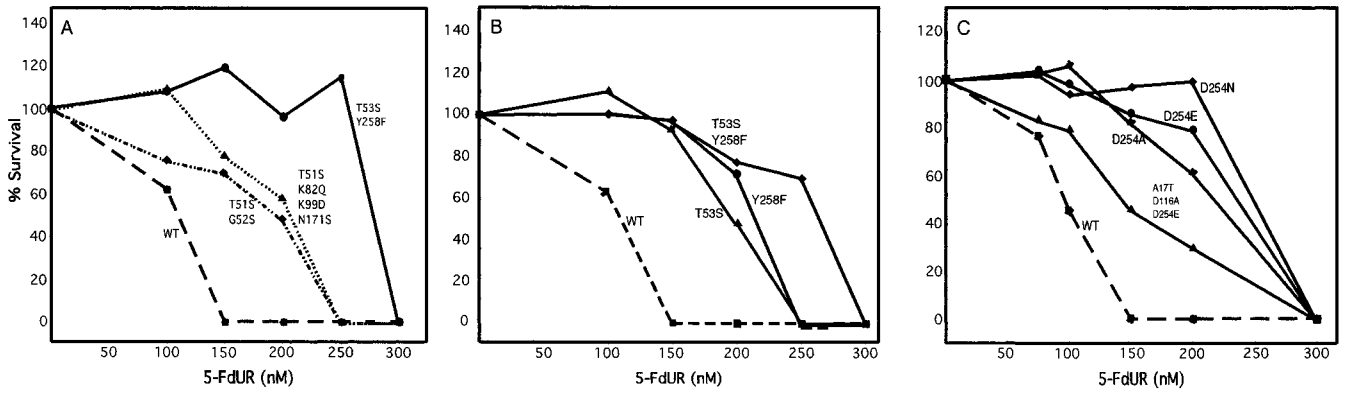

FIG. 3. **Survival of 5-FdUR-resistant TS mutants.** *E. coli* χ2913 cells expressing wild-type or mutant TS protein were grown on M9 minimal plates containing 5-FdUR for 48 h. Survival was determined by counting colonies at each dose of 5-FdUR and is expressed as a fraction of the survival of cells grown on M9 minimal medium without 5-FdUR. *A*, comparison of mutants isolated in the present and previous experiments (6). Survival of the double mutant T53S,Y258F (clone 302) is compared with that of the quadruple mutant T51S,K82Q,K99D,N171S (clone 362); the double mutant T51S,G52S, the most resistant in previous experiments (6); and the wild type. *B*, comparison of T53S,Y258F with corresponding single mutants. The single mutants T53S and Y258F were made by site-directed mutagenesis, and their survival was compared with that of the double mutant. *C*, resistant mutants carrying single substitutions at Asp^{254}. Three substitutions were observed at Asp^{254} among the PCR-generated 5-FdUR-resistant mutants, two of them Asp to Glu and the third Asp to Asn. We used site-directed mutagenesis to create the D254E, D254N, and D254A single mutants and compared their survival with that of the wild type and the triple mutant A17T,D116A,D254E.

TABLE III
Rate and inhibition constants for the wild-type and mutant forms of TS

	K_m dUMP	K_m CH_2H_4-folate	k_{cat}	K_i 5-FdUMP	Difference
	μM	μM	s^{-1}	μM	*-fold*
Wild type	2.0	12	0.46	0.16	1
302 (T53S, Y258F)	1.1	100	0.30	1.00	6.3
362 (T51S, K82Q, K99D, N171S)	0.7	150	0.28	0.88	5.5
T51S	2.6	54	0.34	1.69	11
Y258F	1.0	55	0.44	0.50	3.1

14 amino acids as residues where 5-FdUR resistance can arise. Eight residues were identified in mutants created by error-prone PCR; these are Glu^{23}, Val^{84}, Lys^{93}, Asp^{110}, Pro^{194}, Ser^{206}, His^{250}, and Lys^{284}. Six of these (Glu^{23}, Val^{84}, Lys^{93}, Asp^{110}, Ser^{206}, and Lys^{284}) are located at a distance from the active site and are not associated with catalysis. (Note that Asp^{48}, listed in Table II, was previously identified (6).) We also identified other single amino acid substitutions that may confer 5-FdUR resistance among the PCR variants containing multiple mutations. Four of these (Thr^{51}, Thr^{53}, Asp^{254}, and Tyr^{258}) were chosen and verified to be authentic sites of resistance by using site-directed mutagenesis to create single mutants; these amino acids reside within the active site and have been implicated in catalysis (1, 11–15). An additional two residues (Asp^{116} and Met^{219}) were also shown by site-directed mutagenesis to yield resistance mutations (D116A and M219V, respectively). The location in the three-dimensional structure of all of the single mutations analyzed here, together with the location of other previously identified resistance mutations, is shown in Fig. 4.

DISCUSSION

A major goal of work on human TS is to elucidate the structural and mechanistic bases of interactions with inhibitors, such as 5-FdUR, that are used as cancer chemotherapeutic agents. This goal is important for understanding both basic structure-function relationships in TS and drug resistance. Efforts to understand 5-FdUR resistance include the creation and analysis of resistant mutants, and this approach has centered on amino acid substitutions in catalytically important regions of the protein (*e.g.* Refs. 5–8). Here, we used a different strategy, based on the hypothesis that amino acid replacements yielding 5-FdUR resistance are located throughout the TS primary sequence. We used error-prone PCR to mutagenize the entire human TS cDNA and isolated 35 resistant mutants in an *E. coli*-based complementation assay. In fact, we did find that the resistant variants, listed in Table II, harbor substitutions throughout the linear sequence (Fig. 2) and three-dimensional structure (Fig. 4) of the protein. Some of the 74 different replacements (or combinations thereof) are responsible for 5-FdUR resistance, including the substitutions in single mutants, whereas other replacements are presumably co-selected and not relevant to resistance. Recent crystallographic analyses of TS, especially closed ternary complexes with dUMP and folate-based inhibitors (14, 16), allow us to evaluate these replacements with a view toward understanding possible structure-function relationships and establishing fresh targets for directed mutagenesis. We have used the atomic coordinates of the wild-type amino acids in tightly closed complexes with dUMP and a folate analog inhibitor (see Refs. 14 and 16; Protein Data Bank (25) entries 1HVY and 1JU6, respectively) to locate each amino acid replacement in the three-dimensional structure and to consider possible bases for resistance.

Single Mutants—The single mutants generated by PCR (see Table II) contain amino acid replacements that are distributed throughout the protein. Possible resistance mechanisms can be envisioned for some of the replacements, whereas no mechanisms are apparent for others. For example, substitution of valine for glutamate in the D48V mutant would disrupt the hydrogen bonding network within the Arg^{50} loop that mediates dUMP binding and also affect interactions with the second subunit of the obligate homodimer (14, 16); it has been proposed that mutations at residues 47–52 may confer resistance by destabilizing the closed ternary complex (16). At position 84 in the V84A mutant, wild-type valine interacts with two residues, Phe^{80} and Phe^{225}, that contact the cofactor. Altered interactions with folate are known to confer 5-FdUR resistance (8) and could account for the resistance in the mutant. At

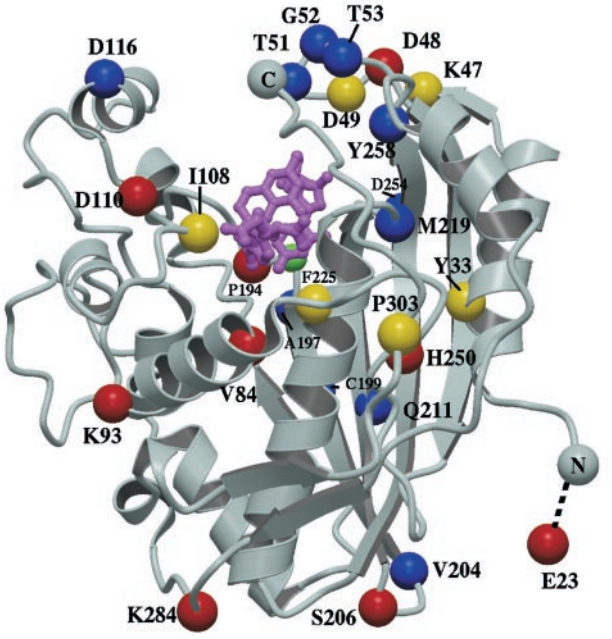

FIG. 4. **Location of amino acid substitutions in single 5-FdUR-resistant mutants in the three-dimensional structure of human TS.** A *ribbon drawing* of one subunit from the 1.9-Å structure of dimeric human TS complexed with dUMP and ralitrexed, an anti-folate drug (14), is shown, with the dimer interface furthest from the viewer. Residues 1–25 are disordered and are not in the crystallographic model; *N* and *C*, the amino- and carboxyl termini of residues 26–313, respectively. The essential, active site Cys^{195} is represented by a *green ball* at its α-carbon position. The ligands are shown in *purple*. All of the published amino acid substitutions in single, 5-FdUR-resistant mutants are mapped. The *red balls* denote PCR-generated mutations reported here; the *blue balls* denote mutations generated in this study by site-directed mutagenesis and mutations previously reported from this laboratory (5, 6); the *yellow balls* denote mutations described by others (7–10). The coordinate set for the ternary complex was obtained from the Protein Data Bank; chain 1HVY:A is modeled (25). The drawing, generously provided by Dr. Elinor Adman, was made by using the programs Molscript (29) and Raster 3D (30).

position 110, wild-type aspartate interacts with Trp^{109}, which in turn interacts with both dUMP and co-factor, perhaps accounting for resistance of the D110E mutant. At position 194 in the active site, wild-type proline interacts with the catalytic cysteine, presumably accounting for the resistance of the P194L mutant. At position 250, wild-type histidine interacts with several residues that contact dUMP, including Asp^{226}, presumably accounting for the resistance of the H250L mutant. In contrast, resistance mechanisms for the single mutants E23G, K93E, S206G, and K284N are not apparent. As shown in Fig. 4, E23G is in the disordered N-terminal region, whereas K93E, S206G, and K284N are located in loops at the periphery of the TS monomer and have no obvious relationship to catalysis or resistance.

By using site-directed mutagenesis to create single mutants, we showed that several amino acids that were replaced in multiply mutated PCR variants are authentic sites of 5-FdUR. Among these site-directed replacements is T51S, the most common mutation we have previously observed and present in the highly resistant variant T51S, G52S (6). Thr^{51} participates in hydrogen bonding within the Arg^{50} loop and with Val^{313} after ligand-induced conformational changes of the C-terminal segment (1, 11–16); 5-FdUR resistance could arise at Thr^{51} from several mechanisms, including an effect on this conformational change. Two replacements at Asp^{254} (D254N and D254E) were observed in multiply mutated variants (255, 318, 358), and we found that both, as well as the D254A substitution, conferred resistance as single mutations (Fig. 3*C*). Asp^{254} is hydrogen-bonded to His^{256}, which is in turn hydrogen-bonded to dUMP-binding $Arg^{175'}$ in the second subunit; 5-FdUR resistance may arise from disruption of these interactions. We also observed that each replacement in the double mutant T53S,Y258S conferred resistance as a single mutation (Fig. 3*B*). Whereas the resistance of T53S may be attributable to disturbance of hydrogen bonding in the Arg^{50} loop, the resistance of Y258F is probably due to loss of hydrogen bonding between the tyrosine hydroxyl group and dUMP and/or $Arg^{175'}$. Interestingly, the Y261F substitution (analogous to the Y258F replacement that we obtained here) was not tested among the 14 *L. casei* TS mutants previously examined (26). The D116A substitution, like some of the PCR-generated single replacements, lies at the surface of the protein, and its contacts do not suggest why it confers resistance. Last, the M219V replacement affects Met^{219}, which has multiple contacts with Tyr^{33}, a known site of resistance, and with His^{256}, which interacts with dUMP.

Replacements in Multiply Mutated Variants—The resistance arising from multiple substitutions is difficult to analyze. Nonetheless, evaluation of the replacements in the double and higher mutants listed in Table II and Fig. 2 can afford potentially useful inferences and point to particular amino acids for further examination. It is a logical assumption that regions where mutations cluster may have special relevance for resistance. One such region includes residues Val^{45}–Thr^{55} constituting the Arg^{50} loop. Wild-type residues at the six mutated positions (positions 45, 48, 51, and 53–55) participate in a network of interactions, primarily hydrogen bonding, within the loop and also between the Arg^{50} loop and the second subunit that contains dUMP-binding residues 175′ and 176′. Replacements at these six positions might affect interactions with both dUMP and 5-FdUMP and also destabilize the ternary complex (16), thereby conferring resistance. A conspicuous cluster of mutations was noted at positions Arg^{78}–Leu^{88}, where seven residues harbor nine different replacements. Phe^{80} in this region interacts with the cofactor, whereas Glu^{87} may have destabilizing interactions with both the cofactor and dUMP. Wild-type amino acids at four mutated positions (positions 78, 81, 82, and 84) contact Phe^{80}, whereas those at three mutated positions (positions 84, 85, and 88) interact with Glu^{87}. Altered interactions with folate and/or dUMP may account for the resistance conferred by replacements in this region. Of particular interest with respect to folate binding is the occurrence of the M311L replacement in the double mutant 217. Met^{311}, together with three wild-type residues that yield 5-FdUR-resistant mutants (8), form a hydrophobic collar around the *p*-aminobenzoic acid ring in the folate analog in a new ternary complex (16). A third group of 14 replacements was observed in the N-terminal 27 amino acids, which are disordered in crystal structures (1, 11–16). This observation raises the possibility that at least some wild-type amino acids in the N-terminal region may have an unrecognized function(s) in 5-FdUR resistance, a possibility substantiated by identification of the single mutant E23G. A related possibility is that the N terminus may have a specific function(s) and an ordered structure(s) in conformational states that have not been captured in existing crystals.

Another possible source of 5-FdUR resistance in multiply mutated variants is altered interactions between the subunits of the obligate TS homodimer; these interactions may be important for positioning of dUMP-binding residues in one or both subunits and may thus affect 5-FdUR resistance. For example, Val^{45} in the Arg^{50} loop interacts with Val^{204} in the second subunit, and we have previously isolated a 5-FdUR-resistant V204D mutant (5). The Val^{204}–Val^{45} hydrophobic interaction was disrupted in four different multiply substituted mutants isolated here, two involving Val^{45} (mutants 255 and 326) and

two involving Val 204 (mutants 316 and 359). In another example of subunit interactions, Glu^{211} resides at the dimer interface where it contacts Phe^{59} from the second subunit. We have previously identified the 5-FdUR resistance mutation Q211L in a single mutant (5) and isolated it again here in the triple mutant 313.

All told, we can offer a plausible structure-based suggestion for the resistance of most of the 35 mutants in Table II, although only 5 of 29 contain a single substitution. These mutants contain a replacement at a position where the wild-type residue is either (*a*) in direct contact with dUMP or a residue that contacts dUMP, and/or (*b*) in direct contact with folate or a residue that contacts folate, and/or (*c*) interacts with the second subunit at the dimer interface. We and others have previously documented 5-FdUR resistance mutations in these three categories (5–8). We rationalize our observations concerning resistance mutations at ligand-interacting residues as follows. Wild-type amino acids that interact directly with substrates or co-factors are likely to be important for catalytic activity and to yield relatively few replacements that conserve catalytic efficiency, alter substrate/inhibitor preference, and afford resistance. This relative lack of mutability notwithstanding, we and others have identified drug resistance mutations at such residues by intensive, targeted mutagenesis. On the other hand, residues that contact ligand-interacting residues may yield a relatively large number of substitutions that simultaneously preserve activity, modulate substrate/inhibitor interactions, and confer drug resistance; mutations at such residues may be highly prevalent in the present work, where each residue in the protein has a limited probability of being substituted.

We can also suggest possible sources of 5-FdUR resistance for the four single amino acid replacements that are far removed from the active site in current crystal structures. We have discussed the E23G mutation in the disordered N-terminal segment and noted that three others (K93E, S206G, and K284N) are located in loops at the surface of the monomer (Fig. 4). If TS functions in a multiprotein complex *in vivo*, these loops may be involved in protein-protein interactions that alter the conformation of TS and affect resistance indirectly. It is also possible that the loops are involved in the conformational dynamics of the protein and affect motions that influence the probability of reactions at the active site (27). Studies of single enzyme molecules by confocal fluorescence spectroscopy indicate that enzymes undergo sets of transient oscillations during each catalytic step (28). Amino acid substitutions at a distance may affect these oscillations and thereby alter substrate and inhibitor discrimination at the active site. Perhaps yet-to-be determined mechanisms that govern conformational changes during catalysis can be affected by amino acid replacements throughout the protein.

Summary Statement—A major conclusion of this work is that amino acid replacements dispersed throughout the linear sequence and three-dimensional structure of TS can confer resistance to 5-FdUR and hence affect discrimination at the active site. It is possible that a comparable multiplicity of residues may affect discrimination and additional functions at the active site of other proteins as well. This possibility has important implications for the study of structure-function relationships and for the design of mutagenesis studies intended to create mutant proteins with desired properties. Our results suggest that efforts to understand protein function and to create new variants with desired attributes might profitably include approaches such as the one illustrated here that explore mutation of entire proteins without preconception. Thereafter, individual amino acid residues or regions of interest can be targeted by more directed mutagenesis. In the case of human TS, the increased insights into structure-function relationships achieved with this approach may ultimately allow us to create multiply mutated variants harboring synergistic alterations in several protein domains.

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