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Methotrexate resistance in an *in vivo* mouse tumor due to a non-active-site dihydrofolate reductase mutation

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ABSTRACT **A series of methotrexate (MTX)-resistant L1210 leukemia murine ascites tumors were developed *in vivo* and analyzed for drug resistance. Three of 20 tumors studied expressed an altered dihydrofolate reductase (DHFR) and each was identical, having a C to T base transition at nucleotide 46 in the DHFR gene as demonstrated by PCR and direct sequencing. This transition results in a Gly to Trp substitution at amino acid 15 of the enzyme. Purified altered enzyme displays significantly lower binding affinity for the antifolates MTX, trimetrexate, edatrexate, and trimethoprim with respective K_i values 165-, 76-, 30-, and 28-fold higher than values obtained for enzyme isolated from parental tumor (wild-type enzyme). Substrate (dihydrofolate) and cofactor (NADPH) binding is also diminished for the mutant enzyme, although to a lesser extent (17.3- and 3.6-fold higher K_m, respectively). Gly-15 is highly conserved for all vertebrate species of DHFR but has no known interaction(s), either directly or indirectly, with bound cofactor, substrate, or inhibitor. Protein molecular modeling reveals that the affected residue is 9–12 Å away from the enzyme active site and located in a region analogous to the mobile Met-20 loop domain characterized for *Escherichia coli* DHFR.**

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate, an essential carrier of one-carbon units in the biosynthesis of thymidylate, purine nucleotides, and methyl compounds. DHFR has been the subject of intense study for >40 years as it is the target enzyme for several important drugs, including methotrexate (MTX; 4-amino-4-deoxy-10-methylfolic acid), trimethoprim [TMP; 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine], and pyrimethamine, for the treatment of cancer, bacterial infections, and malaria, respectively (1, 2). Efforts to develop improved and/or more selective inhibitors of DHFR are continuing and two, trimetrexate {TMTX; 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxylamino)methyl]quinazoline} and edatrexate (EDX; 10-ethyl-10-deazaaminopterin), are now in clinical trial as antineoplastic agents (3–5).

During the past decade a wealth of knowledge has emerged regarding the mechanisms of acquired resistance to MTX (1, 6). Four mechanisms have been identified for this antifolate: (*i*) gene amplification of DHFR, (*ii*) decreased drug transport of antifolates, (*iii*) reduced polyglutamylation of antifolates with decreased retention of drug, and (*iv*) altered DHFR with a decreased affinity for MTX. This information, however, has been derived largely by study of MTX-resistant cell lines obtained after *in vitro* selection of cells with stepwise increases in drug concentration. This contrasts to the dose schedules used in the treatment of cancer patients—namely, repeated fixed maximally tolerated doses of MTX. To this end, a series of L1210 leukemic cells resistant to MTX were developed *in vivo* through drug dosage schedules analogous to clinical schedules (7). Twenty independently derived sublines were selected for maximum resistance to MTX and all displayed an elevated DHFR activity. Ten of these sublines had only this phenotype, while seven sublines also demonstrated a reduced influx for MTX (7). The remaining three sublines (designated L1210/MTX-1, -2, and -3) were found to have a DHFR with reduced affinity for MTX on the basis of preliminary DHFR activity titration experiments with MTX using crude tumor lysates (8). Here we describe the basis for this decreased antifolate binding.

MATERIALS AND METHODS

Materials. General molecular biology reagents (including items for mRNA isolation and cDNA synthesis) and enzyme purification materials were from sources as indicated (9, 10). A slot-blot apparatus from Bio-Rad was used with nitrocellulose membranes supplied by Stratagene for gene copy number determinations. Radioactivity in hybridized blots was quantitated with a Betascope model 603 blot analyzer (Betagen, Waltham, MA). G. Attardi (California Institute of Technology) kindly provided human DHFR cDNA (from pHD80; see ref. 11) for use as a hybridization probe. All other materials were high-quality reagents from commercial sources or as noted in refs. 7, 9, and 10.

Development of *in Vivo* Resistant Cells. A complete description is detailed in Rumberger *et al.* (7). Briefly, L1210 leukemia cells were transplanted by implantation i.p. of 10^6 cells in BD2 F_1 [(C57BL × DBA/2)F_1] mice (Harlan–Sprague–Dawley). Resistant L1210 cell lines were derived by transplantation of 10^6 cells into each of five mice that were treated 1 day later with the maximum tolerated dose of MTX (70 mg per kg of body weight) on a schedule of once per day every 3 days for a total of three injections. After the development of frank ascites and just prior to the death of these animals, tumor cells were harvested and 10^6 were cells transplanted i.p. into five additional mice; therapy was reinitiated. The process was continued until the median survival time obtained for each group after therapy was stabilized after a gradual reduction in survival following each transplantation generation. Cloning of each resistant L1210 line was carried out by limiting dilution in culture (12). Uncloned

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Abbreviations: DHFR, dihydrofolate reductase; H_2folate, dihydrofolate; MTX, methotrexate; TMP, trimethoprim; TMTX, trimetrexate; EDX, edatrexate; BSA, bovine serum albumin.

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and cloned resistant sublines were maintained in the presence of drug *in vivo* and transplanted in mice in the absence of drug for one passage generation prior to use of the cells in biochemical studies. Similar procedures and dosage quantities were used with BD2 F_1 mice to later reassess the degree of *in vivo* resistance for one of the cloned sublines (L1210/MTX-2).

PCR Primers. Oligonucleotide M1 (CTGCAGAAGCTTAGGAGGGGAGCAGAGAACTTGAAA) was used for first-strand cDNA synthesis in conjunction with oligonucleotide M2 (TGGTAGGATTTTATCCCCGCTGCCATC) for amplification of the mouse DHFR. The following oligonucleotide primers were used for direct sequencing of both DNA strands of the amplification product (numbering as in ref. 13): M300, ATTGAACAACCGGAATTGGCA; M301, TGCCAATTCCGGTTGTTCAA; M150, GTGATTATGGGTAGGAAA; M210, TCTGTCCTTTAAAGGTCG; M390, CACCTCAGACTCTTTGTGACA. Amplification of the first exon was accomplished with oligonucleotide primers MEX1 GATCACCGTCGTGGAACCGGTTAGC (anneals to the coding strand, bases 646–670) and MEX2 ACAGCTCAGGGCTGCGATTTCGCGCCAAA (anneals to the noncoding strand, bases 361–389). Primer MEX3 was used as a sequencing primer CGCTGCGATTTCGCGC (anneals to the coding strand).

Enzyme Purification. DHFR from parental cells was purified by the procedures of MTX affinity chromatography (with H_2folate elution), gel filtration, and ion-exchange chromatography (Bio-Rex 9) as described (10). For enzyme from the L1210/MTX-2 cell line, it was immediately apparent that it bound to the MTX affinity matrix less avidly and that a modified purification strategy would be necessary. While MTX affinity absorption was still used, the column dimensions, washing, and elution conditions were altered to maximize retardation of the mutant enzyme during chromatography. Further details of this procedure will be published elsewhere. Final DHFR preparations (parental and altered enzyme) were homogeneous by the criteria of SDS/PAGE and presumed ligand free [after Bio-Rex 9 treatment (10)]. A standard spectrophotometric assay was used to measure DHFR activity (10) during purification and for other experiments detailed below.

Characterization of Enzyme Properties. Stability of purified preparations of parental and altered enzyme was assessed by maintaining either dilute (20 milliunits/ml) or concentrated (500 milliunits/ml) enzyme on ice (in 50 mM Tris·HCl, pH 7.0/100 mM KCl) and periodically sampling the incubations for enzyme activity determinations (10). For diluted enzyme, four different conditions were examined: (*i*) no additives, (*ii*) supplementation with 50 μM NADPH, (*iii*) supplementation with bovine serum albumin (BSA) (50 μg/ml), and (*iv*) supplementation with both NADPH and BSA at the above concentrations. The more concentrated enzyme was maintained with no additives.

Substrate and cofactor Michaelis constants for purified enzyme preparations were obtained by nonlinear, least-squares regression analysis of steady-state kinetic data (14). Activity measurements [MATS assay buffer system (15), pH 7.4] were determined using 10-cm-pathlength cuvettes (25°C) to enable accurate monitoring at low substrate concentrations. Enzymatic activity in the presence of saturating concentrations of both H_2folate and NADPH was divided by molar enzyme concentration (16) to give k_{cat} with units of reciprocal time. Inhibition constants for MTX, TMTX, and EDX were evaluated by fitting to inhibition data the equation for tight-binding competitive inhibition, which takes into account the depletion of free inhibitor concentration due to formation of enzyme–cofactor–inhibitor complex (17). Inhibition experiments were conducted at high H_2folate concentrations (180–200 μM; 1-cm-pathlength cuvettes), yet K_i values presented take into account the affinity of competing substrate. K_i values for TMP were obtained by fitting to data the general equation for competitive inhibition (18).

Determination of DHFR Gene Copy Number. Genomic DNA was isolated according to a Maniatis protocol (19). Prehybridization and hybridization conditions for slot-blot analysis were essentially as described by Wahl *et al.* (20). Human cDNA probe [^{32}P random-primed (21); specific activity, 2–3 $\times$ 10^8 cpm per μg of DNA] was incubated with the filters for 4 hr. Filters were washed twice (to remove unbound label) with 2$\times$ standard saline citrate (SSC)/0.5% SDS for 30 min (25°C), followed by two washes at 68°C (30 min each). Finally, filters were washed in 0.2$\times$ SSC/0.1% SDS at 68°C for 1 hr and then radioactivity was quantitated for each slot.

Molecular Modeling. A three-dimensional model of the human DHFR active site was used as described (10). A comparison of this model with the published structure of murine DHFR (22) revealed a high degree of structural homology between these two protein species. Models were displayed on a silicon graphics personal Iris using the Polygen Quanta software.

RESULTS

Magnitude of Resistance (*in Vivo*). As *in vivo* derived resistant cells had been cloned and manipulated in cell culture, the animal model was used to reevaluate the level of *in vivo* resistance to (*i*) compare with the sensitivity of the original (parental) cells and (*ii*) compare with the magnitude of resistance displayed on first deriving the resistant tumor lines. This experiment was conducted by reimplanting cloned cells into untreated mice and following procedures similar to that described for development of *in vivo* resistant cells (see *Materials and Methods*). A complete examination of *in vivo* resistance was undertaken for one of the cloned sublines (L1210/MTX-2) and found to be quite marked relative to parental cells (Fig. 1). Mice from all groups were treated with the maximum tolerated dose of MTX. The group of mice inoculated with the L1210/parental tumor, if left untreated, died within 1 week. If treated (12 mg per kg of body weight; every other day, starting 24 hr after inoculation, for a total of three doses) the survival of mice is prolonged to almost 3 weeks. In contrast, the mice inoculated with L1210/MTX-2 showed no response to MTX. Death occurred within 1 week whether or not they were treated with MTX, indicative of complete resistance to the highest tolerated dose.

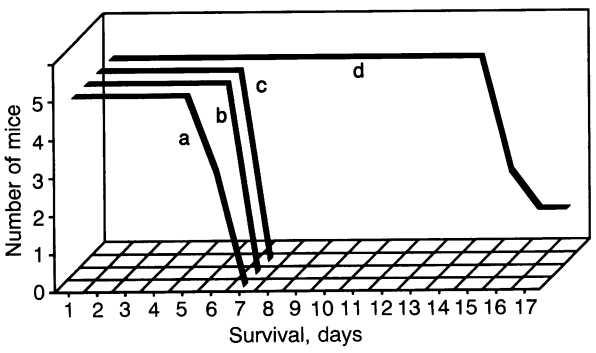

FIG. 1. Survival of mice implanted with L1210/parental (wild type) or L1210/MTX-2 tumor and their response to treatment with or without MTX. Mice inoculated with L1210/parental tumor, if left untreated, died within 1 week (curve a); however, if treated (see *Results*) survival time is prolonged almost 3 weeks (curve d). In contrast, the mice inoculated with the L1210/MTX-2 tumor show no response to MTX and death occurred within 1 week whether or not they were treated with MTX (L1210/MTX-2 untreated, curve b; treated, curve c).

DNA Sequence Analysis of MTX-Resistant L1210 Tumors. *In vitro* amplification of the entire translated region of the mouse DHFR cDNA was performed using PCR for both parental and L1210/MTX-1, -2, and -3 cell lines. Primers used for amplification annealed to the 5′ and 3′ untranslated regions and did not overlap with any coding sequence. After this conventional amplification reaction, the asymmetric PCR technique (23) was used to yield single-stranded DNA for sequencing. While various silent mutations were detected, a common mutation was found in all three (L1210/MTX-1, -2, and -3) cell lines: a G → T transversion at nucleotide 46, resulting in a substitution of Trp for Gly at amino acid 15 (Fig. 2).

The G → T transversion at nucleotide 46 was confirmed by amplifying and directly sequencing exon 1 for all resistant cell lines (data not shown). Furthermore, sequence analysis indicated that this mutation would give rise to the gain of a *Pfl*MI site, which was confirmed by restriction digests of PCR amplified genomic DNA for L1210/parental and L1210/MTX-2 DNA (data not shown). All further studies of the altered mouse DHFR were conducted with one of the cloned sublines (L1210/MTX-2).

DHFR Gene Copy Number. Previous preliminary studies with the three cloned lines had identified decreased binding of MTX for DHFR contained in cell extracts as well as an increased specific activity of DHFR (enzyme activity/total cytosolic protein) relative to parental cells (7). To determine whether gene amplification (24) was a factor contributing to increased specific activity, slot-blot analysis of genomic DNA was conducted to determine gene copy number. Interpolation of a standard plot constructed from counts obtained with variable amounts of L1210/parental DNA indicated that 5 μg of L1210/MTX-2 DNA hybridized approximately the same amount of radioactive cDNA probe as 35–38 μg of L1210/parental DNA (data not shown). This represents a 7- to 8-fold increase in gene copy number.

Stability and Kinetic Properties of Enzyme. Purified altered enzyme, relative to parental enzyme, was unstable at concentrations <20 milliunits/ml (≈2.3 μg of DHFR protein per ml) and lost almost 60% of its activity on ice over a 4-hr period (Fig. 3*B*). Neither 2-mercaptoethanol nor dithiothreitol at 100 mM enhanced stability of enzyme (data not shown), although BSA (50 μg/ml) with or without NADPH (50 μM) almost halved the rate of activity loss. NADPH alone did not stabilize altered enzyme. Purified parental L1210 enzyme (also at 20 milliunits/ml) was generally more stable under all conditions examined (Fig. 3*A*). NADPH alone did enhance stability, and the best combination of agents examined proved to be BSA and NADPH. Concentrated preparations of both enzyme species (500 milliunits/ml; Fig. 3) were stable for hours on ice and for at least 6 months at −70°C.

The steady-state kinetic properties of purified mouse DHFRs are presented in Table 1. The Michaelis constants (K_m) for substrate and cofactor are increased 17.3-fold and 3.6-fold, respectively, for altered enzyme. Interestingly, the overall rate for substrate to product conversion (k_{cat}) is almost 50% greater for mutant enzyme at pH 7.4. This suggests that the increased activity of DHFR measured in crude extracts of the resistant tumor may also be apportioned to an increased intrinsic catalytic ability of the mutant species. The catalytic efficiency, however (k_{cat}/K_m, with respect to H_2folate), is slightly more than 12-fold lower for purified mutant enzyme.

There is a dramatic difference in antifolate binding affinities for the enzymes (Table 1). K_i values for MTX, the bacterial inhibitor TMP, TMTX, and EDX are elevated 165-, 28-, 75- and 30-fold, respectively, for mutant enzyme. As the magnitude and rank order of inhibition differ for these

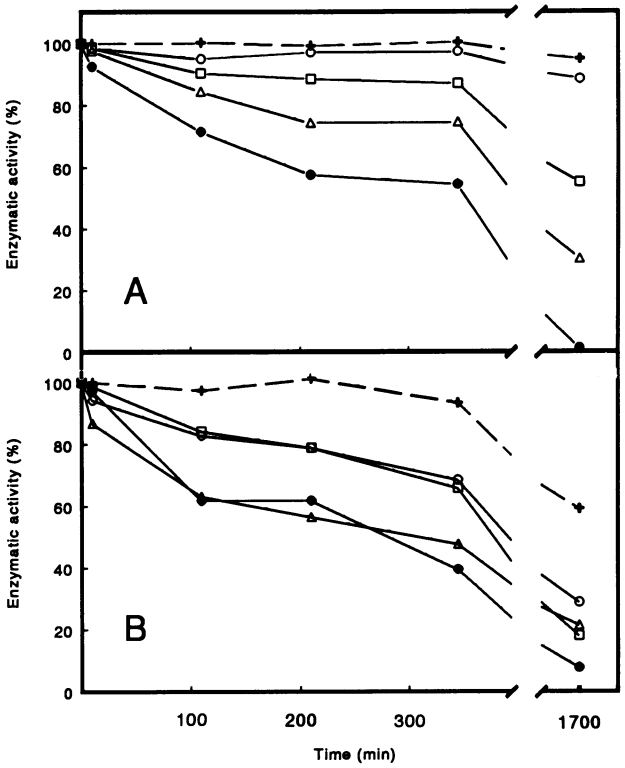

FIG. 3. Stability of purified preparations of parental (wild type) enzyme (*A*) and altered (L1210/MTX-2) enzyme (*B*) at 4°C. The profile for an undiluted portion of enzyme (500 milliunits/ml; both species) is shown (+). For diluted enzyme (20 milliunits/ml; both species, solid lines), four conditions were tested: ●, no additives; △, 50 μM NADPH; □, BSA (50 μg/ml); ○, 50 μM NADPH plus BSA (50 μg/ml). Wild-type enzyme was more stable under all conditions examined, and relatively smaller stabilizing effects were observed with additives for diluted altered enzyme by comparison with wild-type enzyme.

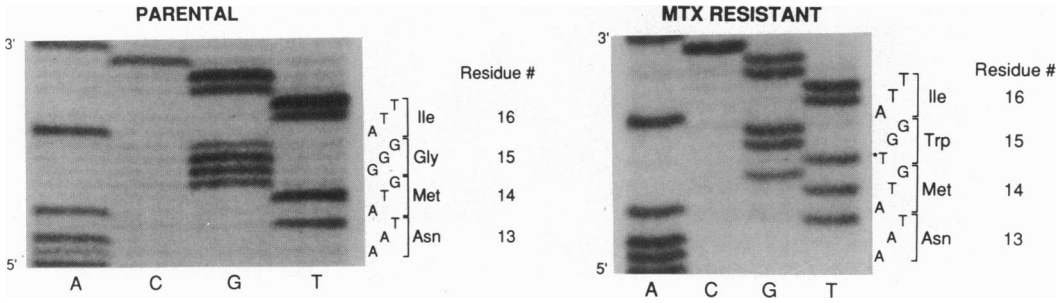

FIG. 2. Direct DNA sequence analysis of L1210/parental (wild type) and L1210/MTX-2 (MTX resistant) mouse DHFR cDNAs as described in *Results*. Depicted is the sequence region where the only functional mutation was detected. The complete amino acid sequence for mouse and a comparison with other DHFR species are given in ref. 2.

Table 1. Enzyme kinetic and inhibition properties for purified parental (wild type) and Gly-15 to Trp DHFR

Enzyme	K_m		K_{cat},	H_2folate K_{cat}/K_m,	K_i‡			
	H_2folate,* μM	NADPH,† μM	sec^{-1}	$sec^{-1}·μM^{-1}$	MTX, pM	TMTX, pM	EDX, pM	TMP, μM
Parental	0.36 ± 0.03	0.54 ± 0.08	2.2	6.1	12.9	23.0	25.1	1.3
Gly-15 → Trp	6.22 ± 0.25	1.94 ± 0.25	3.2	0.5	2130 (165)	1744 (76)	755 (30)	36.9 (28)

*Initial velocity data, ±1 SE.
†Initial velocity and progress curve experiments, ±1 SE.
‡Numbers in parentheses represent inhibition ratios, K_i(Gly-15 to Trp)/K_i(parental).

compounds, the relative binding energy contributions of the individual active site interactions of drug and parental DHFR and drug and Gly-15 to Trp mutant DHFR must be changed.

Molecular Modeling. Fig. 4 depicts a three-dimensional model of a portion of the human DHFR active site. Position 15 is clearly distant from sites of substrate, cofactor, and inhibitor binding. The approximate distances between the position 15 backbone carbon and the closest portion of cofactor and MTX are 7.2 and 12.3 Å, respectively. The region surrounding Gly-15 is quite crowded and there appears to be no energetically favorable location for the substituted Trp side chain. It therefore seems unlikely that the area depicted could adequately accommodate the introduction of this bulky hydrophobic group without an effect on local secondary and/or tertiary structure.

DISCUSSION

Critical observations regarding the mechanism of antifolate resistance have been accumulating over the past 40 years. To date, five MTX-resistant cell lines, developed *in vitro*, have been identified and found to contain an altered DHFR with reduced affinity for antifolates (9, 25–28). In each of these cases, sequencing of the DHFR gene has revealed a single nucleotide base change that causes a single amino acid replacement for the protein products. In all cases, it has been inferred from three-dimensional structural data that the altered residue participates directly in inhibitor binding or that the mutated residue directly interferes with inhibitor binding through steric hindrance or electrostatic repulsion (29). The Gly-15 to Trp substitution reported here is unique to these previously identified mutants in that the altered residue is considerably distant from the catalytic center (and inhibition site).

The data presented here provide strong evidence that the marked *in vivo* resistance (Fig. 1) exhibited is due to mildly amplified expression of an altered DHFR. To what extent resistance can be attributed to the enzymatic properties of the altered enzyme or to the increased expression cannot be answered at this stage, although it is interesting to note that, when examined, all other DHFR mutants identified in antifolate-resistant cell lines have been associated with increased levels of mutant protein expression (presumably through gene amplification). In these latter cases, it is generally assumed that overproduction of the mutant enzyme is necessary to offset the poorer catalytic properties of altered enzyme. In contrast, the mutant identified in this study has relatively good catalytic activity with k_{cat}, even greater than

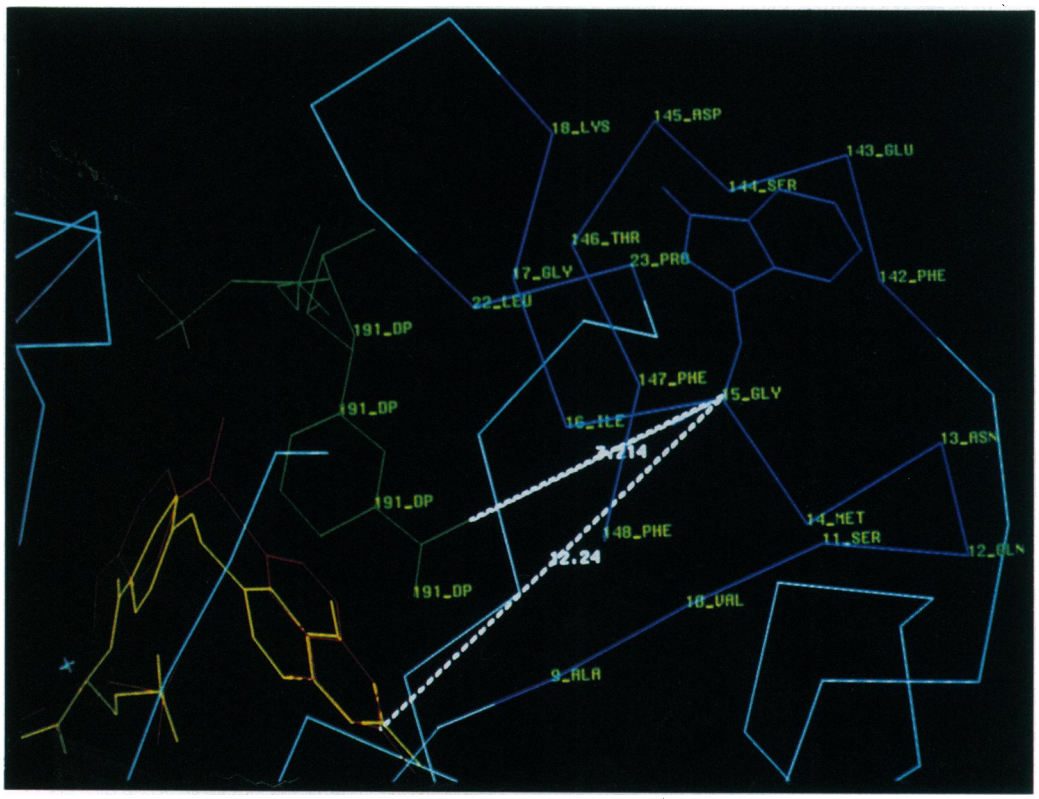

FIG. 4. Molecular modeling of a portion of human DHFR active site. A detailed view of the α-carbon backbone structure highlighting residues within 8 Å of Gly-15 is given (dark blue); residues are identified by their respective amino acid (2). A Trp residue is superimposed on Gly-15 for spatial comparison only. MTX is shown in red, folate is in yellow, and NADPH is in green. Dashed lines are represented with distances in angstroms. Examination of the ψ (144.2°) and φ (−76°) angles of the peptide plane reveals no unusual dihedral angles for Gly-15.

wild-type species at neutral pH, and an increased expression of the Gly to Trp DHFR may be necessary to offset the intrinsic instability of the mutant protein. This would ensure sufficient catalytic flux of the enzyme pool and maintain cellular requirements for reduced folates on exposure to MTX. Preliminary experiments to determine the turnover half-life of the Gly-15 to Trp mutant DHFR *in vivo* indeed indicate that the mutant is far more labile compared to wild-type enzyme. This is consistent with the stability data presented in Fig. 3 for purified enzyme from both sources.

The Gly-15 residue is conserved in all vertebrate DHFRs sequenced to date (2), but in the wild-type enzyme it is not known to interact with folate, antifolates (including MTX), NADPH, or indirectly via interaction with an active site residue (30). Ala-9 and Leu-22 for vertebrate DHFR are known to contribute important active site residues with roles in substrate and inhibitor binding but both are ≈8 Å from Gly-15. Despite this lack of known interaction(s) for Gly-15, its replacement has produced substantial changes in affinity of substrate and cofactor (i.e., K_m) possibly by an indirect effect involving perturbation of local secondary or tertiary structure. Of interest is that the binding energy for substrate (and cofactor) appears to be far less affected by the Gly-15 to Trp substitution by comparison with inhibitor compounds, particularly for the *in vivo* resistance selecting agent MTX. This phenomenon is also found for active-site mutants of DHFR identified in MTX-resistant cultured cells and is understandably a desirable feature for these mutants in providing the resistance phenotype.

The basis for reduced MTX binding with this non-active site mutant may result from impaired movement of loop or secondary structure regions involved in ligand binding and catalysis. Gly-15 is located in a loop region that connects elements of protein secondary structure, a β-sheet near the N terminus (βA, residues 4–10) and an α-helical region (αB, residues 27–40) (30). All except one residue of this loop (residue 11 of region 11–26) is conserved identically in all vertebrate species of DHFR (2). The loop has high sequence homology with, and is analogous to, the flexible Met-20 loop described for *Escherichia coli* DHFR (31). Combined data based on x-ray crystal structures of *E. coli* DHFR and mutagenesis of *E. coli* DHFR have highlighted the importance of this mobile and flexible region in permitting a substrate-induced conformational distortion that closes the active site and possibly facilitates hydride transfer through substrate alignment (32, 33). We speculate that this conformational change or closing (33) of the DHFR active site is also instrumental in the binding of antifolates. The bulky substitution in this loop region for the mutant enzyme has not compromised the catalytic function of this loop movement but prevents formation of a tight ternary complex for DHFR, cofactor, and MTX. Conformational changes or isomerizations subsequent to MTX binding have been well documented for essentially all wild-type species of DHFR studied by enzyme kinetic means (2, 16, 34, 35); however, the molecular basis of these changes remains unknown and direct studies of the interplay between loop mobility and the tight-binding process that characterize these interactions are yet to be undertaken.

Relatively little is known of the mechanisms underlying resistance to antifolates (or the frequency of such mechanisms) in the clinic. DHFR mutations have not been identified as yet from clinical human neoplasms refractory to MTX; however, there appears to have been little effort to address this question (36). This is partially due to inherent difficulties in acquiring samples from patients and tumor heterogeneity. The fact that the mutation described in the current study was obtained after MTX treatment of an *in vivo* tumor has prompted us to examine tumor samples from patients with clinical indications of MTX resistance. While a Gly to Trp substitution at amino acid 15 for human DHFR is only possible by a double-nucleotide base change at the corresponding codon [due to a codon difference for mouse Gly-15 (GGG) and human Gly-15 (GGC)], similar or even more dramatic changes in antifolate/substrate binding may possibly be obtained for human DHFR by an alternative single base change at this or adjacent codons. Importantly, this study indicates that functional DHFR mutants providing antifolate resistance need not be restricted to those affecting residues that directly participate in inhibitor binding.

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