THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 6, Issue of February 6, pp. 3461–3469, 1998

Printed in U.S.A.

A Biochemical and Genetic Model for Parasite Resistance to Antifolates

TOXOPLASMA GONDII PROVIDES INSIGHTS INTO PYRIMETHAMINE AND CYCLOGUANIL RESISTANCE IN *PLASMODIUM FALCIPARUM**

(Received for publication, July 29, 1997, and in revised form, November 6, 1997)

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We have exploited the experimental accessibility of the protozoan parasite *Toxoplasma gondii* and its similarity to *Plasmodium falciparum* to investigate the influence of specific dihydrofolate reductase polymorphisms known from field isolates of drug-resistant malaria. By engineering appropriate recombinant shuttle vectors, it is feasible to examine mutations by transient or stable transformation of *T. gondii* parasites, in bacterial and yeast complementation assays, and through biochemical analysis of purified enzyme. A series of mutant alleles that mirror *P. falciparum* variants reveals that the key mutation Asn-108 (Asn-83 in *T. gondii*) probably confers resistance to pyrimethamine by affecting critical interactions in the ternary complex. Mutations such as Arg-59 (*T. gondii* 36) have limited effect in isolation, but in combination with other mutations they enhance the competitive ability of folate by increasing the speed of product turnover. Val-16 (*T. gondii* 10) confers low level resistance to cycloguanil but hypersensitivity to pyrimethamine. This mutation precludes Asn-108, probably because compression of the folate binding pocket introduced by this combination is incompatible with enzyme function. These studies permit detailed biochemical, kinetic, and structural analysis of drug resistance mutations and reconstruction of the probable phylogeny of antifolate resistance in malaria.

Among protozoan pathogens of clinical significance, *Toxoplasma gondii* and *Plasmodium* sp. share a common evolutionary history (as members of the phylum Apicomplexa) (1) and a vulnerability in folate metabolism, which forms the basis for effective drug treatment (2–4). These parasites lack the ability to salvage pyrimidine nucleosides from the host cell and are capable of only limited salvage (of uracil) from their own pool of metabolites (5–7). They therefore depend heavily on *de novo* biosynthetic pathways that consume reduced folate molecules as methyl donors in reactions involving 1-carbon transfer. This necessitates that rapidly dividing parasites maintain an abundant folate pool.

A key enzymatic step in folate metabolism is the NADPH-dependent reduction of folate to H_2-folate and further reduction of H_2-folate to H_4-folate. These reactions are catalyzed by the enzyme dihydrofolate reductase (DHFR)[1] (4). As in other protozoa (and plants), DHFR occupies the NH_2-terminal portion of a homodimeric bifunctional protein in both *Toxoplasma* and *Plasmodium*, fused at the COOH terminus to thymidylate synthase (TS) (8–10). TS and DHFR catalyze sequential steps in the regeneration of methyl-charged folate molecules, and coupling of these two activities within a single protein is thought to facilitate channeling of H_2-folate from TS to DHFR (10, 11).

Antifolate chemotherapy for the treatment of malaria, toxoplasmosis, and many other infectious diseases relies on the combination of folate analogs to inhibit DHFR (pyrimethamine, trimethoprim, cycloguanil, etc.) with sulfonamides to inhibit a prior step in folate biosynthesis (2–4). When used in combination, these two classes of drugs exhibit a pronounced synergistic effect (12–14). By virtue of its effectiveness, however, antifolate therapy establishes a robust selection for resistant microbes. Mechanisms of acquired resistance, and the metabolic cost of resistance, are issues of considerable importance in medical and biological science.

A close correlation has been noted between resistance to pyrimethamine (and/or cycloguanil) and specific point mutations in the malarial DHFR gene (15–28). Because malaria parasites may be spread from one individual to another via the mosquito vector, drug resistance mutations are disseminated rapidly. Widespread resistance to pyrimethamine threatens efforts to manage cerebral malaria worldwide (29, 30). Reports of clinical treatment failures caused by acquired pyrimethamine resistance in *Toxoplasma* are rare, probably because the drug-resistant mutations in humans are unlikely to be transmitted from one individual to another. Should antifolate-resistant *T. gondii* emerge, however, it would threaten the most common treatment for acute toxoplasmosis and the sole effective chemoprophylaxis in immunocompromised patients (3, 31–33).

The precise role of DHFR sequence polymorphisms in drug-resistant malaria has been difficult to ascertain for several reasons including: (i) experimental difficulties in working with *Plasmodium* parasites; (ii) the difficulty of maintaining stable bacterial clones spanning the highly A/T-rich *Plasmodium* DHFR-TS gene; (iii) the difficulty of expressing bifunctional *Plasmodium* DHFR-TS in *Escherichia coli* (even using synthetic clones that optimize for *E. coli* codon usage) (34–40); and (iv) the inefficiency of molecular transformation systems for *Plasmodium* (41, 42). We have demonstrated previously that certain DHFR polymorphisms observed in Fansidar-resistant *Plasmodium falciparum* confer pyrimethamine resistance in

* This work was supported by Grant AI-28724 and a training grant in cell and molecular biology from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: DHFR, dihydrofolate reductase; TS, thymidylate synthase.

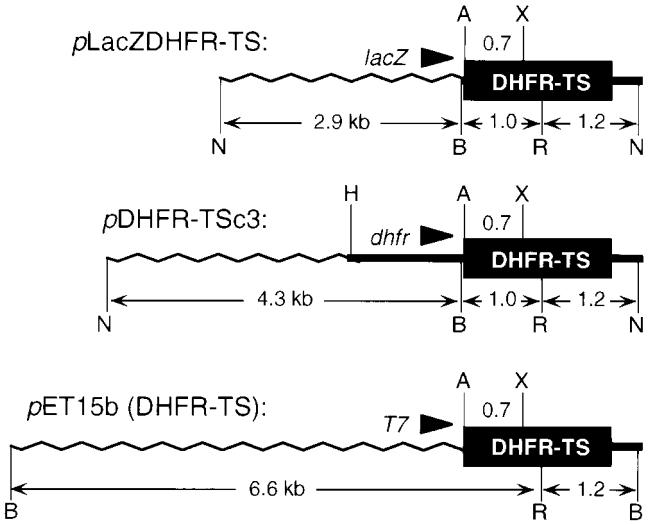

FIG. 1. **Recombinant plasmids for expression of *T. gondii* DHFR-TS.** Plasmid *placZ*DHFR-TS was used for bacterial complementation studies; minigene *p*DHFR-TSc3 (containing flanking sequences derived from the *T. gondii* genomic DHFR-TS locus; 43) was used for transient expression assays in *T. gondii* parasites; and *p*ET15b(DHFR-TS) (11) was used for expression of recombinant enzyme. The DHFR-TS protein coding sequence is indicated by *black boxes*, *T. gondii* flanking sequences are indicated by *heavy black lines*, and *zig-zag lines* indicate plasmid vector sequences (containing an ampicillin resistance gene and bacterial origin of replication); promoters used for expression are shown in *lowercase letters*, with *arrows* indicating orientation. These circular plasmids are drawn to scale but are shown in linearized form for convenience. *kb*, kilobases. Restriction sites used in cloning and for the transfer of mutated DHFR cassettes are indicated; *A*, *Age*I; *B*, *Bam*HI; *H*, *Hin*dIII; *N*, *Not*I; *R*, *Eco*RI; *X*, *Xho*I.

transgenic *T. gondii* parasites (43, 44), and we have therefore chosen to examine a wider variety of such alleles in this experimentally accessible system. *Plasmodium* DHFR-TS shares greater primary amino acid sequence identity with *Toxoplasma* than it does with any other species whose DHFR or TS genes have been examined to date (8). The objectives of this study are to clarify the mechanistic effects of DHFR point mutations in drug-resistant malaria and to gain insights into the biochemical characteristics of *T. gondii* DHFR and its role in drug susceptibility and resistance.

By engineering a suitable set of recombinant shuttle vectors, it has proven feasible to examine *in vivo* and *in vitro* the phenotypes associated with a variety of DHFR-TS mutations using transient or stable transformation of *T. gondii* parasites, bacterial (and yeast) complementation assays, and biochemical analysis of purified enzyme. These studies extend previous findings on the suitability of *Toxoplasma* as a model for certain aspects of *Plasmodium* biology, revealing how the interaction of drug and enzyme is disrupted by naturally occurring mutations.

EXPERIMENTAL PROCEDURES

DNA Manipulations and Plasmid Construction—Vectors used in this study are shown in Fig. 1. Manipulation of recombinant DNA and bacterial transformation was accomplished according to standard protocols. *E. coli* strain DH5α was used for routine cloning and plasmid propagation. A unique *Bam*HI site was introduced slightly upstream of the ATG initiation codon in a bacterial expression vector containing *T. gondii* DHFR-TS (43), to produce plasmid *placZ*DHFR-TS. A 1-kilobase *Bam*HI-*Eco*RI fragment harboring the complete DHFR coding sequence was excised from this plasmid and introduced into the *p*KS(−) vector (Stratagene) for site-directed mutagenesis (45) using the following synthetic oligonucleotides (underlined nucleotides indicate introduced mutations, and mutated codons are shown in bold): 5′-tctacggggcgttgtcggatccgggaagATGCAGAAACC-3′ (upstream *Bam*HI site; coding sequence shown in uppercase); 5′-GTGTCTGGTCGTC**GT**<u>**G**</u>ATGACCCCCAAGA-3′ (Val-10); 5′-GATTTCAAACACTTT<u>**CG**</u>TCGTGTGACAAAAACGGAC-3′ (Arg-36); 5′-ATGGGACGGAAA<u>**AA**</u>CTGGGAAAGCATGCCT-3′ (Asn-83); 5′-ATGGGACGGAAA<u>**AG**</u>CTGGGAAAGCATGCCT-3′ (Ser-83); 5′-GGTACCCTACGAC<u>**TCC**</u>GTGGTTCTCGAGAAG-3′ (Ser-245). Polymerase chain reaction-based methods were avoided to minimize the possibility of introducing unintended mutations, and all mutants were confirmed by sequencing across the entire targeted region. DHFR sequences, mutated as described in the text, were then returned to the *placZ*DHFR-TS vector for expression as transcriptional fusions in *E. coli* or introduced in place of the wild-type DHFR locus in plasmid *p*DHFR-TSc3 (43) for expression in transgenic *T. gondii* parasites. For overexpression in bacteria and purification of recombinant enzyme, DHFR domain mutants (produced as described above) were introduced into a *p*ET15b-based vector (11) using a slightly smaller cassette (*Age*I-*Xho*I).

Bacterial Complementation—E. coli strain LH-18 (Δ*lac-pro supE hsdR5 thyA* Δ*fol::Kan F′lac [Iq-ZΔM15]*$^+$ *pro*$^+$) (46) was used for complementation analysis of the various *T. gondii* DHFR-TS alleles (in plasmid *placZ*DHFR-TS). The capacity of mutagenized alleles to complement the *fol*$^-$ phenotype was determined on minimal media in the absence of folate supplements (methionine, glycine, thymidine, adenine, and pantothenic acid) (46, 47).

Transient Expression in T. gondii Parasites—To determine the effect of DHFR-TS mutations on drug resistance in tissue culture, wild-type RH-strain tachyzoites (14) were transfected with *p*DHFR-TSc3 vectors harboring various mutant alleles of interest. Two $\times$ 10^7 parasites + 30 μg of CsCl-purified plasmid DNA were mixed in 2-mm gap electroporation cuvettes (BTX), subjected to a ~0.25-msec pulse (1.5 kEV, 24 ohms) and inoculated into confluent monolayers of human foreskin fibroblast cells in 24-well plates in modified Eagle's medium + 1% heat-inactivated, dialyzed fetal bovine serum (14, 43). The viability and proliferation of transiently transfected parasites grown in various concentrations of pyrimethamine were measured 24–36 h post-transfection by [^{3}H]uracil uptake assay (14, 48). These data were used for calculation of IC_{50} values. Wild-type *p*DHFR-TSc3 and a null allele in which the ATG initiation codon was mutated to TTG (43) served as controls for transfection studies.

Expression and Purification of Recombinant T. gondii DHFR-TS—Plasmid vector *p*ET15b and *E. coli* host strain BL21(DE3)PLysS (Novagen) were employed for recombinant enzyme expression in 2-liter cultures. Induction with isopropyl-1-thio-β-D-galactopyranoside and purification were carried out essentially as described (11), with the following modifications: only soluble DHFR-TS protein was purified after induction; both affinity chromatography (on methotrexate-agarose; Sigma) and anion exchange chromatography (DE52; Whatman) were accomplished by gravity flow; and protein concentrations in crude and purified enzyme preparations were determined by Bradford assay. Enzyme purity was confirmed by SDS-polyacrylamide gel electrophoresis, and lack of contamination with folate derivatives was confirmed by assaying $A_{280/320}$ ratios. Purified enzyme was stored at −20 °C in 10% glycerol; no loss of enzymatic activity was detected during the course of this study.

Enzymatic Assays—Kinetic parameters of enzyme produced from mutant *T. gondii* DHFR-TS alleles were determined spectrophotometrically at 25 °C under the steady-state conditions established previously for the wild-type enzyme (11). DHFR activity was measured as a decrease in absorbance at 340 nm in a 1.0-cm cuvette during a 2–3-min interval (results calculated using a molar extinction coefficient for NADPH of 12,000 M^{-1} cm^{-1} at 340 nm). One unit of DHFR activity is defined as the amount of enzyme necessary to produce 1 μmol of product/min at 25 °C. Purified DHFR-TS fractions from different mutants were assumed to retain a uniform percentage of active enzyme (~90%).

Apparent K_m values for H_2-folate and for NADPH were determined while keeping the concentration of one substrate constant at 100 μM and varying the other, initially between 0.5 and 50 times the K_m of wild-type enzyme and subsequently over a range encompassing concentrations 0.5–50 times the estimated K_m of the mutant. All reactions were initiated by the addition of enzyme at $t = 0$. Kinetic values for H_2-folate and for NADPH were calculated on the basis of three to six independent experiments (observed variation between experiments was always $\leq$ 15%).

K_i values for the binding of pyrimethamine (Sigma) and cycloguanil (Jacobus Pharmaceutical Co., Princeton NJ) to wild-type *T. gondii* DHFR-TS are consistent with expectations for tight binding inhibitors, complicating the calculation of inhibition constants (49–51). In contrast to certain antifolates (*e.g.* methotrexate; 51), pyrimethamine does not typically behave as a slow tight binding inhibitor (52, 53); progress curves following inhibition of the *T. gondii* enzyme show that steady-

TABLE I
DHFR-TS polymorphisms in P. falciparum
Only polymorphisms considered in this report are shown. For further discussion of these and other polymorphisms, see Refs. 15–28.

Polymorphic locus	Amino acid residue	Ref.	Characteristics
16	Ala	9, 16	Most common wild-type allele, sensitive to pyrimethamine and cycloguanil. Invariably associated with Ser-108. Representive *P. falciparum* isolate: 3D7.
	Val	16	Rare variant, correlated with low level resistance to cycloguanil. Always associated with Thr-108. Representive isolate: FCR3.
59	Ser	9, 16	Wild-type (3D7, FCR3).
	Arg	15, 16	Only observed in association with pyrimethamine resistance mutation Asn-108 (K1).
108	Ser	9, 16	Most common wild-type allele (3D7). Associated with Ala-16. All known wild-type DHFR enzymes from other species contain a Thr at this position.
	Thr	16	Rare variant (FCR3). Associated with Val-16.
	Asn	15, 16	Most common mutation in pyrimethamine-resistant malaria (HB3). Role in drug resistance confirmed by genetic and biochemical studies (16, 35, 37, 38, 40). Only found in association with Ala-16.
223	Phe	15, 31	Wild-type (3D7, FCR3).
	Ser	19	Only observed in laboratory mutants resistant to pyrimethamine (FCR3/D4). Associated with Val-16/Thr-108.

state levels of inhibition are achieved rapidly and that initial velocities are proportional to inhibitor concentration over two logs. Inhibition of *T. gondii* DHFR by pyrimethamine and cycloguanil meets established criteria for the use of transient-state kinetics to calculate inhibition constants for tight binding inhibitors (50), and K_i values were therefore calculated from IC_{50} values under quasi-steady-state conditions (3–30 nM enzyme, 100 μM NADPH, 50 μM H_2-folate, and various inhibitor concentrations) (Equations 6–9 in Ref. 50).

Lineweaver-Burk plots were used to determine the mode of inhibition by pyrimethamine, examining ligand competition under conditions where [inhibitor] $\ll$ [enzyme] and [H_2-folate] = 0.5–10 $\times$ K_m. These were the only conditions under which double-reciprocal plots displayed linearity, supporting the conclusion that such plots are inappropriate for computation of K_i values.

Development of Three-dimensional Model of the T. gondii DHFR-TS—The structure of *T. gondii* DHFR-TS was modeled on an IRIS Indigo XZ4000 using the Homology module of the molecular graphics package Insight II (Biosym Technologies). The primary structures of *T. gondii* and *Leishmania major* DHFR-TS proteins were aligned using a PAM250 matrix to identify possible conserved regions. Coordinates were assigned to *T. gondii* residues using the 2.9 Å *L. major* x-ray structure (generously provided by Dr. D. A. Matthews) (54), and loop structure between conserved domains was assigned by root mean square distance minimization. Energy minimization for the *T. gondii* enzyme and pyrimethamine inhibitor was performed to convergence using the Insight II Discover module.

RESULTS

Construction of Allelic Series in T. gondii DHFR-TS Using Site-directed Mutagenesis—As noted above, there is a strong correlation between resistance to pyrimethamine (and cycloguanil) in malaria and specific amino acid substitutions in the parasite's DHFR protein sequence (15–28). A subset of the polymorphisms observed in *P. falciparum* DHFR-TS is shown in Table I. To investigate the effects of these amino acid substitutions on antifolate resistance, we constructed a large series of mutant *T. gondii* DHFR-TS alleles, including mutants analogous to wild-type and drug-resistant *P. falciparum* strains (Table II). Mutagenesis was carried out using a bacterial plasmid containing *T. gondii* DHFR and junctional domain coding sequence, which could be excised conveniently as a cassette for insertion into bacterial complementation and parasite transformation vectors encoding the complete DHFR-TS enzyme (Fig. 1).

Complementation of DHFR-TS-deficient E. coli LH-18—Wild-type *T. gondii* DHFR-TS has been shown previously to be able to complement the *fol*$^-$ phenotype of *E. coli* strain LH-18, which is deficient in both DHFR and TS activity (46). Conversion of *E. coli* LH-18 to prototrophy (ability to grow on minimal media without folate supplements) was therefore used as an assay for functional expression of *T. gondii* DHFR-TS alleles that harbor mutations associated with drug resistance in *P. falciparum* (Table II). Most of the single point mutations under investigation (and many of the multiply substituted alleles) yielded viable enzyme by this assay. Failure to complement the Δ*fol* phenotype was presumed to indicate inactivation of the *T. gondii* DHFR-TS enzyme or severely compromised enzyme function (later confirmed by enzymatic assay of purified protein; see below).

As noted in the summary of DHFR-TS polymorphisms in *Plasmodium* (Table I), Asn-108 is the hallmark of pyrimethamine resistance in the field, but this mutation has only been reported in association with alanine at position 16, never with Val-16. Combining the analogous mutations in *Toxoplasma* (Asn-83 + Val-10; line (i) in Table II) yields a DHFR-TS allele that fails to complement the Δ*fol* lesion in *E. coli*, suggesting that this combination is fundamentally incompatible (confirmed by examination of purified recombinant enzyme; see below).

Serine at *P. falciparum* position 223 has only been observed in laboratory attempts to isolate pyrimethamine-resistant parasites from the Val-16 + Thr-108 background (19). We were unable to complement *E. coli* with Ser-245 (the *T. gondii* homolog of Ser-223 in *P. falciparum*) either as a single mutation (line (f)) or in combination with Arg-36 or other mutations (lines (j), (m), etc). In a previous study, however, bacterial complementation was observed under less stringent growth conditions (43), suggesting that these alleles are functional but inefficient. Several Ser-245 alleles were also functional in transiently transfected parasites (see below), supporting this interpretation.

Transient Expression of Mutant Alleles in T. gondii—To examine the contribution of individual amino acid substitutions (alone or in combination) to drug resistance *in vivo*, the various DHFR-TS alleles shown in Table II were placed under control of the *T. gondii* DHFR-TS promoter in plasmid *p*DHFR-TSc3 (Fig. 1) and assayed for the ability to protect parasites from pyrimethamine toxicity 24 h after transfection. Four general classes of pyrimethamine sensitivity were defined based on IC_{50} values (Table II).

Incorporation of [^{3}H]uracil (a parasite-specific metabolic marker (48)) was strongly inhibited by pyrimethamine, and

TABLE II

Mutations in the DHFR-TS coding sequence of T. gondii and P. falciparum

The ability of various polymorphisms in the DHFR domain of *T. gondii* DHFR-TS to complement DHFR- and TS-deficient *E. coli* and to confer pyrimethamine resistance upon transfected *T. gondii* parasites is shown. The wild-type *T. gondii* sequence is shown for the four amino acid residues investigated (along with the corresponding positions in the *P. falciparum* enzyme (9)). Unless otherwise indicated the sequence for the various alleles is identical to that of the wild-type. Letters at the left are used to designate alleles discussed in the text. An annotation after the parenthetical letter indicates convenient designations for several point mutations; *P. falciparum* strains harboring comparable alleles are noted.

		Amino acid residue				Complementation of *fol*⁻ *E. coli*[a]	Pyrimethamine resistance in *T. gondii*[b]
	P. falciparum DHFR-TS: / *T. gondii* DHFR-TS:	16 / 10	59 / 36	108 / 83	223 / 245		
(a)	*T. gondii* wild-type	Ala	Ser	Thr	Phe	+	+/− (0.58 ± 0.04)
(b)	Cofactor (FCR3)	Val				+	+/− (0.56 ± 0.04)
(c)	Folate pocket		Arg			+	+/− (0.58 ± 0.06)
(d)	Malaria only (3D7)			Ser		+	+/− (0.58 ± 0.02)
(e)	Drug hallmark (HB3)			Asn		+	+ (0.71 ± 0.02)
(f)	Laboratory (FCR3/D4)				Ser	(−)	+ (0.83 ± 0.16)
(g)		Val	Arg			+	+/− (0.59 ± 0.03)
(h)		Val		Ser		+	− (0.52 ± 0.05)
(i)		Val		Asn		−	− (0.50 ± 0.05)
(j)		Val			Ser	−	− (0.55 ± 0.04)
(k)			Arg	Ser		+	+/− (0.63 ± 0.06)
(l)	(K1)		Arg	Asn		+	++ (1.12 ± 0.22)
(m)			Arg		Ser	(−)	++ (0.92 ± 0.11)
(n)				Asn	Ser	−	− (ND)
(o)		Val	Arg	Ser		+	− (0.54 ± 0.02)
(p)		Val	Arg	Asn		−	ND
(q)		Val	Arg		Ser	−	− (0.55 ± 0.02)
(r)		Val		Ser	Ser	−	+/− (0.61 ± 0.12)
(s)		Val		Asn	Ser	−	ND
(t)			Arg	Ser	Ser	−	+ (0.70 ± 0.16)
(u)		Val	Arg	Ser	Ser	−	+ (0.70 ± 0.04)
(v)	Null mutant					−	− (0.52 ± 0.02)

[a] Complementation was determined by assessing the ability of *E. coli* LH-18 to grow on minimal medium after transformation with *placZ*DHFR-TS plasmids harboring the indicated mutations. Parentheses indicate that although complementation was not observed for any allele containing Ser-245, certain of these alleles have been observed previously to complement DHFR− bacteria (43).

[b] Pyrimethamine resistance was determined by uracil uptake assay in 24-well plates infected 24 h previously with transiently transfected parasites. IC_{50} values (in μM ± S.D.) are provided in parentheses, and resistance profiles are grouped into the following categories: +/− indicates inhibition comparable to parasites transfected with wild-type DHFR-TS plasmid (<50% inhibition at 0.6 μM pyrimethamine); − indicates ≥50% inhibition (comparable to parasites transfected with null allele of DHFR-TS); + indicates <35% inhibition (moderate resistance); ++ ≤25% inhibition (high level resistance); ND = not determined. All data reflect results from at least two experiments.

this inhibition was not affected by transfection with an untranslatable (null) DHFR-TS gene. Parasites electroporated with wild-type DHFR were slightly less inhibited by pyrimethamine, presumably because of overexpression of the wild-type enzyme, allowing subtle distinctions to be made between pyrimethamine-sensitive alleles (*e.g.* wild-type) and DHFR-TS null alleles. The Val-10 + Asn-83 double mutant, which failed to complement *E. coli*, behaved like the null mutant in transfected *T. gondii* tachyzoites (line (i) in Table II). Mutants harboring Val-10 + Ser-83 also failed to confer even low level resistance (line (h)). Because these alleles are capable of bacterial complementation, we presume that they function enzymatically in *T. gondii* as well but that their expression, stability, and/or activity in intracellular parasites is not sufficient to provide even the low level resistance observed with wild-type DHFR-TS (discussed further below). Various single site mutations (Val-10, Arg-36, Ser-83; lines (b) through (d)) behaved comparably to wild-type DHFR-TS in transfected parasites, as did the Val-10 + Arg-36 and Arg-36 + Ser-83 double mutants (lines (g) and (k), respectively).

Moderate level pyrimethamine resistance was observed in several mutants: Asn-83 (line (e)), Ser-245 (f), Arg-36 + Ser-83 + Ser-245 (t), and Val-16 + Arg-36 + Ser-83 + Ser-245 (u). It is notable that several alleles containing Ser-245 exhibit pyrimethamine resistance (lines (f), (m), (t), and (u)), despite their inability to complement the Δ*fol* phenotype of LH-18 bacteria. These results were reproducibly observed and probably reflect the efficiency of functional enzyme expression in the two assay systems. In agreement with previous findings (40), high level pyrimethamine resistance was only observed using mutants Arg-36 + Asn-83 (line (l)) and Arg-36 + Ser-245 (line (m)). The former double mutant is commonly found in Fansidar-resistant malaria (15, 16), whereas the latter is a novel combination. Note that the folate pocket mutation (Arg-36; line (c)) appears to confer no pyrimethamine resistance benefits in isolation but can enhance significantly the resistance of moderately resistant alleles.

We were unable to characterize cycloguanil sensitivity in these experiments, as wild-type *T. gondii* parasites are inherently resistant to the drug (data not shown). Alternative approaches to this problem are described below.

Expression and Purification of Recombinant DHFR-TS Enzyme for Enzymology—Wild-type and mutant *T. gondii* DHFR-TS alleles were expressed in *E. coli* as described previously (11). All alleles examined showed comparable levels of expression and protein stability, and all bound to methotrexate-agarose as effectively as wild-type protein, despite the presence of amino acid substitutions within the predicted substrate binding site. Recombinant enzyme was purified to apparent homogeneity by affinity chromatography and anion exchange chromatography, as shown in Fig. 2. Typical enzyme recovery was ~17%, and activity was ~10 units/mg.

Kinetic analyses of apparent K_m and k_{cat} values for H_2-folate and NADPH were performed using spectrophotometric assays that monitor the progressive oxidation of NADPH (55). IC_{50} values for pyrimethamine and cycloguanil were determined for the various DHFR-TS alleles and were used to calculate inhibition constants (K_i values), according to equations appropriate for fast tight binding inhibitors (11, 50). The formula employed for K_i determination (50) takes into account both affinity for the competing molecule (H_2-folate) and total enzyme concentration, permitting meaningful comparison of the various DHFR-TS alleles examined in different experiments.

Antifolate Sensitivity—The K_i determined for wild-type *T.*

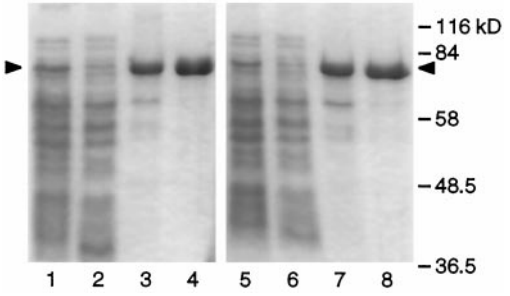

FIG. 2. **Two-step purification of recombinant DHFR-TS.** 10-μg aliquots of protein fractions from each purification step (see "Experimental Procedures") were examined by SDS-polyacrylamide gel electrophoresis after staining with Coomassie Blue. *Lanes 1–4*, wild-type enzyme; *lanes 5–8*, Asn-83 mutant. *Lanes 1* and *5*, crude lysate from isopropyl-1-thio-β-D-galactopyranoside-induced BL21(DE3)pLysS *E. coli*; *lanes 2* and *6*, pooled flow-through from methotrexate affinity columns; *lanes 3* and *7*, protein eluted from methotrexate affinity columns with 2 μM H_2-folate; *lanes 4* and *8*, highly purified enzyme recovered after anion exchange chromatography (DE52 Sepharose). Molecular mass standards are indicated at the *right*; the major band at ~79,000 kDa (*arrowhead*) corresponds to the predicted size for the *T. gondii* DHFR-TS monomer (8, 11).

gondii DHFR-TS for pyrimethamine (7.6 nM; line (a) in Table III) is comparable to values reported previously (11). Mutant alleles Arg-36 + Asn-83 and Arg-36 + Ser-245 (lines (l) and (m)) provided the highest K_i values observed, ~250–1,000-fold above wild-type, in keeping with the *in vivo* data from transient expression assays (Table II). In general, most other alleles also exhibit K_i values for pyrimethamine which correspond well with *in vivo* phenotypes: the Ser-245 allele (f) provided moderate levels of resistance and ~40-fold elevated K_i levels, whereas Arg-36 in isolation (c) showed neither pyrimethamine resistance nor an increased dissociation constant.

Despite the moderate level resistance observed in the Asn-83 mutant (Table II), this enzyme exhibited only a slightly elevated K_i for pyrimethamine (line (e) in Table III). The K_i of Asn-83 was lower, in fact, than that observed for Ser-83 (d), an allele that was drug-sensitive *in vivo* (Table II). Thus K_i values alone are not sufficient to explain *in vivo* activity completely.

Given the complete inability of cycloguanil to inhibit parasite replication (56), it is surprising to note that this compound is an effective inhibitor of the purified *T. gondii* DHFR-TS enzyme (K_i = 2.1 nM). For most of the mutant alleles examined, sensitivity to cycloguanil and pyrimethamine was comparable. The Val-10 mutant (line (b)), however, exhibits a K_i for pyrimethamine which is roughly half that of the wild-type (results confirmed in yeast expression studies; not shown) but a K_i for cycloguanil which is ~10 times higher. These data support suggestions that the analogous mutation in *Plasmodium* (Val-16) is responsible for differential resistance to cycloguanil (Paludrine) *versus* pyrimethamine (20, 21, 40).

Kinetics of Dihydrofolate Utilization—Table IV presents the kinetic parameters of H_2-folate utilization for the various *T. gondii* DHFR-TS enzymes examined. Considered alone, Arg-36 (line (c)) has no detectable effect on enzyme kinetics. Asn-83 (e) produces a moderate increase in the K_m for H_2-folate but demonstrates no alteration in k_{cat}. Val-10 (b) increases K_m and decreases in k_{cat}, yielding an enzyme with substantially decreased overall efficiency. The Ser-245 mutation (f) decreases the enzyme catalytic rate dramatically; this low turnover number may explain why Ser-245 has never been selected in the field. Ser-245 exerts only a small effect on substrate affinity, however (possibly because its predicted position within the protein does not fall within the substrate binding pocket; see below). Enzymes carrying the Ser-83 substitution, an anomaly that is restricted in nature to malaria parasites, exhibit substantially *increased* reaction velocities (Ser-83, Ser-83 + Arg-36; lines (d) and (k)). These gains are effectively erased by the addition of Val-10 or Ser-245 (lines (h) and (t)), mutations that are themselves associated with decreases in k_{cat} relative to the wild-type enzyme.

The kinetic characteristics of multiply-substituted alleles do not necessarily reflect the additive properties of individual mutations; combined mutations generate enzymes with distinct identities. For example, the affinity of highly pyrimethamine-resistant alleles for H_2-folate is decreased far beyond what would have been expected given the values for individual substitutions: K_m values increased 20- and 10-fold, respectively, for Arg-36 + Asn-83 and Arg-36 + Ser-245 (lines (l) and (m)). Significantly, these decreases in affinity are compensated for, in part, by higher rates of substrate turnover.

NADPH Kinetics—Models of the *T. gondii* DHFR enzyme place amino acids 10 and 83 within the catalytic domain, in close proximity to both the folate substrate and NADPH cofactor (Fig. 3). These residues are predicted to lie on opposite sides of the NADPH binding cleft, and the analogous amino acids in bacterial and mammalian DHFRs have been shown to participate in cofactor binding and catalysis (57). To evaluate the possibility that changes in cofactor utilization might contribute to parasite drug resistance, NADPH utilization was examined for alleles harboring mutations at these positions (Table IV). Both Ser-83 (line (d)) and Asn-83 (e) show marginally lower binding constants than the wild-type enzyme (a), whereas the binding constant for Val-10 (b) is roughly twice that of wild-type. These differences suggest that mutations at positions 10 and 83 may influence inhibitor/cofactor interactions in the enzyme ternary complex.

Inhibition by Antifolates—Lineweaver-Burk double-reciprocal plots were employed to examine the character of ligand competition between H_2-folate and pyrimethamine for wild-type and mutant enzymes. The limitations of this technique for studying tight binding inhibitors in multisubstrate reactions have been discussed in detail (49, 50). By using low inhibitor concentrations relative to enzyme, however, we were able to identify a narrow window of conditions for each of the mutant DHFR-TS enzymes within which steady-state velocities could be achieved before appreciable reactant depletion. Under these conditions it is possible to compare reliably the effects of pyrimethamine on enzyme kinetics for the various alleles under study. Representative data are shown in Fig. 4.

At very low concentrations of pyrimethamine, drug-sensitive enzymes yield curves characteristic of uncompetitive mixed inhibition (see wild-type and Val-10 mutant; *top panels*). This observation is consistent with the complex, multiphasic binding kinetics of a tight binding inhibitor in a multireactant system (49, 50, 53). It is interesting to note that increasingly pyrimethamine-resistant alleles exhibit more purely competitive inhibition patterns: moderately pyrimethamine-resistant alleles demonstrate noncompetitive inhibition (Asn-83 and Arg-36 + Ser-83 + Ser-245; *central panels*), whereas highly pyrimethamine-resistant alleles display a classic pattern of competitive inhibition (Arg-36 + Asn-83 and Arg-36 + Ser-245; *bottom panels*). Similar patterns have been observed consistently for all of the mutant alleles in Table III for which double-reciprocal plots could be reproducibly generated. This phenomenon illustrates both the magnitude of the resistance achieved through naturally occurring mutations and demonstrates that minor changes at the enzyme active site can substantially influence the mechanism of inhibition (49, 53, 58).

DISCUSSION

We have capitalized on the relative ease with which *T. gondii* DHFR-TS protein is expressed in several systems (*E. coli,*

TABLE III
Inhibition constants (K_i) for T. gondii DHFR-TS alleles

K_i values (±S.D.) were calculated from IC_{50} measurements determined under quasi-steady-state conditions as described in text. Letter designations correspond to those used in Table II.

		Amino acid residue				K_i	
	P. falciparum DHFR-TS: / *T. gondii* DHFR-TS:	16 / 10	59 / 36	108 / 83	223 / 245	Pyrimethamine	Cycloguanil
						nM	*nM*
(a)	*T. gondii* wild-type	Ala	Ser	Thr	Phe	7.4 ± 0.1	2.1 ± 0.8
(b)	Cofactor (FCR3)	Val				3.5 ± 0.4	24.1 ± 0.7
(c)	Folate pocket		Arg			7.8 ± 0.3	1.9 ± 0.1
(d)	Malaria only (3D7)			Ser		40.7 ± 0.2	58.8 ± 3.7
(e)	Drug hallmark (HB3)			Asn		20.3 ± 2.3	7.2 ± 0.8
(f)	Laboratory (FCR3/D4)				Ser	360.0 ± 7.0	38.2 ± 4.8
(h)		Val		Ser		9.6 ± 0.1	2.4 ± 0.4
(k)			Arg	Ser		1.8 ± 0.6	1.2 ± 0.4
(l)	(K1)		Arg	Asn		7,718.0 ± 42	436.0 ± 10
(m)			Arg		Ser	1,704.0 ± 28	457.0 ± 17
(t)			Arg	Ser	Ser	209.0 ± 11	21.6 ± 6.6

TABLE IV
Kinetic parameters of dihydrofolate and NADPH utilization for T. gondii DHFR-TS alleles

Letter designations correspond to those used in Table II.

		Amino acid residue				$K_{m(app)}$ H_2-folate	K_{cat}	Efficiency	$K_{m(app)}$ NADPH
	P. falciparum DHFR-TS: / *T. gondii* DHFR-TS:	16 / 10	59 / 36	108 / 83	223 / 245				
						μM	s^{-1}	k_{cat}/K_m	*μM*
(a)	*T. gondii* wild-type	Ala	Ser	Thr	Phe	1.1 ± 0.2	11.4 ± 0.6	10.4	5.7 ± 1.6
(b)	Cofactor (FCR3)	Val				6.6 ± 0.1	4.5 ± 0.3	0.7	12.1 ± 3.1
(c)	Folate pocket		Arg			1.8 ± 0.4	9.2 ± 0.7	5.1	ND
(d)	Malaria only (3D7)			Ser		6.0 ± 1.8	23.6 ± 2.1	4.4	3.9 ± 0.2
(e)	Drug hallmark (HB3)			Asn		7.8 ± 0.9	12.1 ± 0.8	1.6	3.9 ± 0.1
(f)	Laboratory (FCR3/D4)				Ser	3.9 ± 0.2	2.7 ± 0.5	0.7	ND
(h)		Val		Ser		0.7 ± 0.4	5.3 ± 0.8	7.6	ND
(i)		Val		Asn		>100.0[a]	<0.01[a]	ND[a]	ND
(j)		Val			Ser	28.5[a]	0.04[a]	ND[a]	ND
(k)			Arg	Ser		3.0 ± 0.3	20.5 ± 0.9	6.8	ND
(l)	(K1)		Arg	Asn		20.1 ± 0.7	16.1 ± 0.3	0.8	3.5 ± 0.2
(m)			Arg		Ser	11.7 ± 0.9	15.9 ± 0.3	1.4	ND
(t)			Arg	Ser	Ser	4.9 ± 0.1	5.4 ± 0.3	1.1	ND

[a] Beyond limits of detection. ND, not determined.

Saccharomyces cerevisiae, T. gondii) to investigate the influence of various point mutations observed in malaria isolates on enzyme function. Comparison of parasites expressing transgenic DHFR-TS alleles with kinetic analyses on purified recombinant enzyme reveals consistent susceptibility profiles for pyrimethamine, the drug of choice in clinical management of acute toxoplasmosis (2–4). Antifolate sensitivity/resistance profiles in *Toxoplasma* are also consistent with laboratory and field studies on drug-resistant *Plasmodium* (15–28), validating the use of *T. gondii* as a model for examining questions relevant to antifolate resistance in malaria. The suitability of this model is supported further by the viability of pyrimethamine-resistant *T. gondii* alleles in transgenic *Plasmodium* (41, 42) and *vice versa*.[2]

Parasite Drug Resistance Phenotypes in Toxoplasma and Plasmodium—Two pyrimethamine-sensitive variants are observed in field isolates of *Plasmodium:* Ala-16 + Ser-108 and Val-16 + Thr-108 (exemplified by strains 3D7 and HB3, respectively; Table I). The analogous *T. gondii* alleles (Ala-10 + Ser-83 and Val-10 + Thr-83) both complement DHFR-deficient *E. coli* and behave comparably to the wild-type *T. gondii* allele (Ala-10 + Thr-83) in *in vivo* expression assays (Table II). A third pyrimethamine-sensitive *T. gondii* variant, Val-10 + Ser-83 (which has never been observed in malaria), exhibits fitness on par with wild-type enzyme in *in vitro* kinetic assays (Table IV), but it behaves like a null allele in transfected parasites (Table II). This incongruence may direct our attention to biological features beyond kinetic fitness which could affect DHFR-TS function *in vivo* (*e.g.* translational efficiency, protein stability).

Considered individually, only two amino acid mutations conferred resistance to pyrimethamine in this study (Asn-83 and Ser-245), and these are the only single mutations that have been reported in association with pyrimethamine-resistant malaria. *Plasmodium* Asn-108 (analogous to *T. gondii* Asn-83) is known from both field and laboratory studies (15–18, 20–28), where it is invariably linked with an alanine (rather than valine) at position 16. *Plasmodium* mutation Ser-223 (analogous to *T. gondii* Ser-245) has only been observed in the laboratory and was derived from a Val-16 genetic background (19). A rationale for these correlations is suggested by the *T. gondii* model: the Ala-10 + Asn-83 combination yields an active enzyme that is resistant to pyrimethamine, but the Val-10 + Asn-83 mutant fails to complement *E. coli*, behaves like a null mutation *in vivo* (Table II), and produces protein without measurable kinetic activity *in vitro* (Table IV). For malaria parasites harboring Val-16, mutation Ser-223 provides an alternative route for acquiring drug resistance. The analogous Ser-245 mutation in *Toxoplasma* yields and enzyme that is resistant to pyrimethamine both *in vivo* and *in vitro* (Tables II and III), albeit one with greatly diminished catalytic capacity (see below).

The sensitivity of wild-type *T. gondii* DHFR-TS (Ala-10 + Thr-83) to cycloguanil was somewhat unexpected, as *Toxo-*

[2] M. G. Reynolds and D. S. Roos, unpublished data.

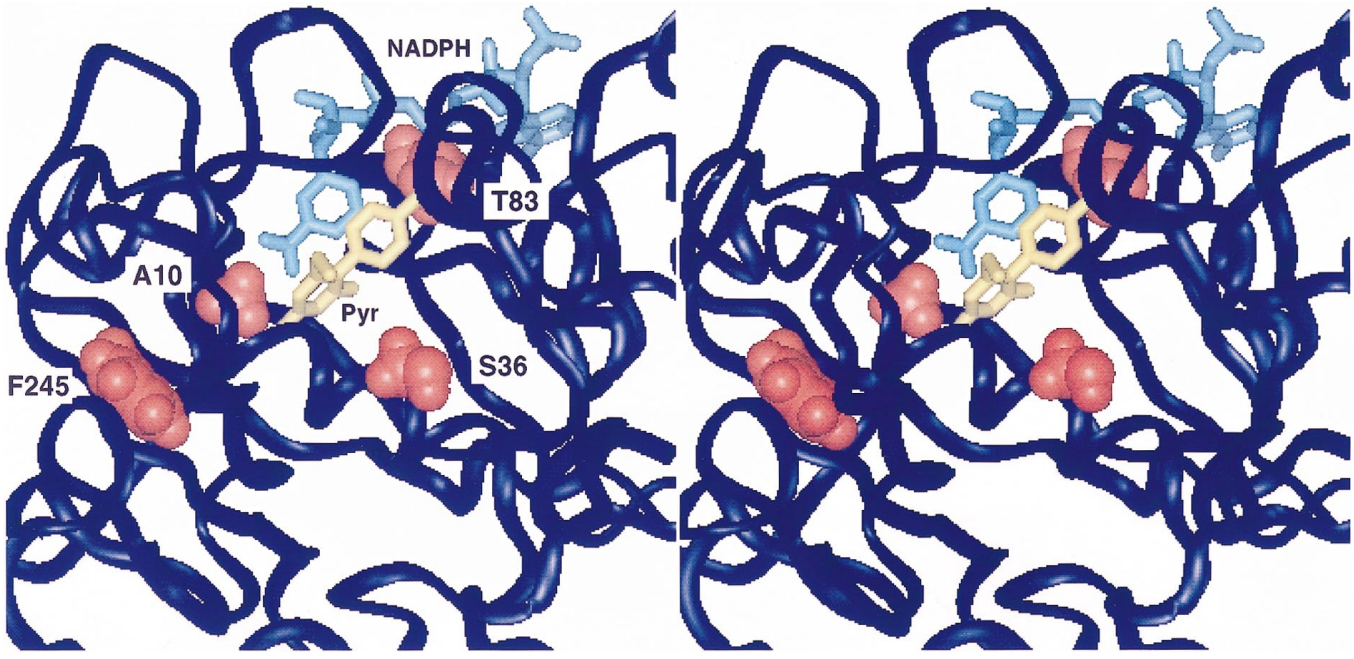

FIG. 3. **Three-dimensional model of inhibitor and cofactor binding within the *T. gondii* DHFR-TS active site pocket.** A molecular model for *T. gondii* DHFR-TS was constructed by energy minimization using coordinates for the *Leishmania* DHFR-TS·cofactor-inhibitor complex (54) and pyrimethamine was docked into the folate binding site as described under "Experimental Procedures." The DHFR-TS α-carbon ribbon structure is shown in *purple*; amino acid side chains mutated in this study are shown as space-filling molecules in *red*; NADPH cofactor, *light blue*; pyrimethamine, *yellow*. Note the proximity of amino acid 83 to both pyrimethamine and NADPH; resistance mutation Asn-83 may drive the inhibitor deeper into the active site pocket, disrupting planar interactions with the nicotinamide ring of NADPH. Val-10 confers resistance to cycloguanil (Table III): projection of the bulkier Val side chain into the active site prevents the binding of cycloguanil, which has two methyl groups at C_6 of its diaminopyrimidine ring. The ethyl group at the analogous position in pyrimethamine (pointing out toward the observer in this image) would not be similarly constrained. Combining the Asn-83 and Val-10 mutations destroys enzyme function, helping to explain the evolution of drug resistance in malaria. For further discussion, see "Results."

plasma parasites are not susceptible to cycloguanil treatment either *in vitro* or *in vivo* (53). Cycloguanil resistance in *Toxoplasma* must therefore be attributed to something other than poor affinity for the enzyme target. In malaria, the Val-16 + Thr-108 variant has been suggested as a source of resistance to cycloguanil (20, 21, 40), and the analogous *T. gondii* allele (Val-10 + Thr-83) demonstrates enhanced cycloguanil resistance *in vitro* (Table III). The differential drug sensitivity of valine-substituted alleles can be understood through three-dimensional modeling of the folate binding pocket (see below). Identification of a pyrimethamine-sensitive/cycloguanil-resistant allele also suggests the possibility of cycloguanil-specific selectable markers for molecular genetic studies in *Plasmodium*, where drug-resistant *T. gondii* DHFR-TS alleles have proven very useful (41, 42).

Several mutations have been detected only in association with Asn-108 in *Plasmodium* (15–28). Some of these secondary mutations have no obvious homolog in *Toxoplasma*, but the *Plasmodium* wild-type residue Cys-59 is probably comparable to *T. gondii* Ser-36. In isolation, Arg-36 (*Plasmodium* Arg-59) has no detectable effect in any of the assays conducted here, but combining Arg-36 with Asn-83 yields highly pyrimethamine-resistant enzyme and confers high levels of resistance to *T. gondii* parasites (Tables II and III). Arg-36 yields a high level of pyrimethamine resistance in *T. gondii* when combined with Ser-245, and the Arg-36 + Ser-83 + Ser-245 triple mutant provided low level resistance; these last two combinations have never been observed in either laboratory or field studies of pyrimethamine-resistant *Plasmodium*.

Evolution of Drug Resistance Mutations—These data provide new insights into the evolution of drug resistance in malaria, where at least two mutations appear to be required for high level drug resistance. Note that the order in which these mutations are acquired is critical and can be reconstructed with a high degree of confidence by looking at the resistance characteristics of singly substituted alleles. All pyrimethamine-resistant malaria appears to have arisen by a stepwise process, first acquiring the Asn-108 mutation, followed by subsequent mutations such as Arg-59 (which augments resistance levels). The reverse order of acquisition is unlikely, as Arg-59 confers no resistance on its own.

In *Toxoplasma*, mutation Ser-245 (analogous to *Plasmodium* Ser-223) produces even higher levels of drug resistance than Asn-83 (*Plasmodium* Asn-108), and subsequent addition of Arg-36 (*Plasmodium* Arg-59) yields an extremely pyrimethamine-resistant allele. Given that *Plasmodium* Ser-223 produces low level pyrimethamine resistance in the laboratory (in the Val-16 + Thr-108 background; 32), why does the evolution of drug-resistant malaria not follow the wild-type to Ser-245 to Arg-59 + Ser-245 progression in the field? It is likely that the explanation for this lies in its evolutionary improbability: while the *T. gondii* Arg-36 + Ser-245 double mutant (Table IV, line (m)) exhibits kinetics similar to that of the pyrimethamine-resistant mutant Arg-36 + Asn-83 (line (l)), the necessary first mutation (Ser-245) may not survive in the field because of its poor kinetic fitness.

Kinetic Characteristics of Enzyme Fitness—Kinetic analyses consistently indicate that amino acid substitutions that produce drug resistance (*e.g.* Asn-83, Ser-245, Arg-36 + Asn-83) simultaneously damage catalytic function of the DHFR-TS enzyme (Table IV), suggesting a tradeoff between the survival benefits achieved through drug resistance and loss of enzyme function. This is not simply a natural consequence of mutations in an enzyme whose activity has been optimized through evolutionary selection, as other mutations that do not confer pyrimethamine resistance do not necessarily diminish catalytic efficiency (see Arg-36 or Ser-83; Table IV, lines (c) and (d)).

Detailed kinetic mechanisms have been proposed for the

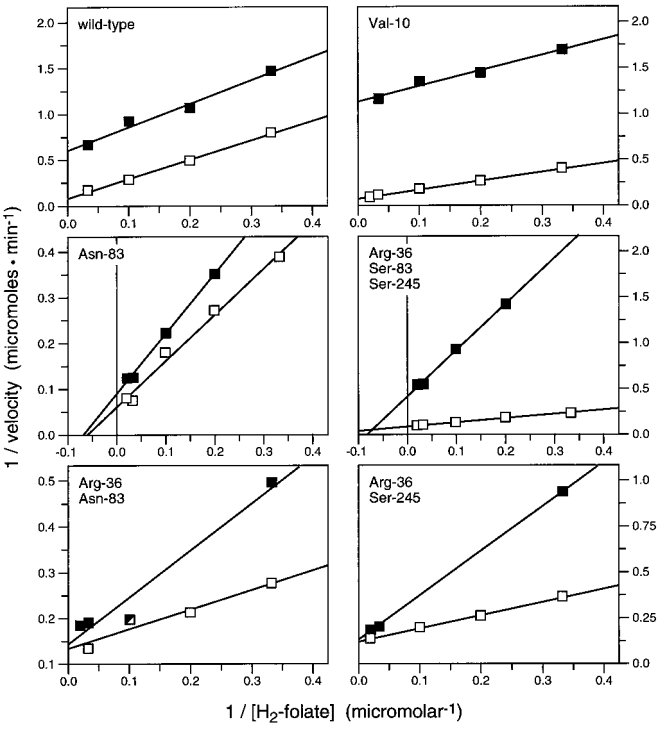

FIG. 4. **Lineweaver-Burk plots for pyrimethamine inhibition of various DHFR-TS alleles.** *Open squares* indicate uninhibited enzyme; *filled squares* indicate assays in the presence of pyrimethamine. Drug-sensitive alleles (wild-type and Val-10; *top panels*) exhibit uncompetitive mixed type inhibition, indicating that pyrimethamine binds primarily to cofactor-liganded DHFR-TS rather than the naked enzyme. Increasingly pyrimethamine-resistant alleles demonstrate increasingly competitive inhibition profiles: Asn-83 and Arg-36 + Ser-83 + Ser-245 (*middle panels*) are moderately drug-resistant, whereas Ser-36 + Asn-83 and Ser-36 + Ser-245 (*bottom panels*) are highly drug-resistant (Tables II and III). For conditions employed and discussion of the limitations of this technique for examining tight binding inhibitors, see "Experimental Procedures" (49, 50).

monofunctional DHFR enzyme from many species (59, 60), and it is probably reasonable to assume that the salient features of the kinetic mechanism of *T. gondii* and *P. falciparum* DHFR-TS are similar (the random bi-bi order of substrate binding and product leaving, organochemical mechanism of hydride ion transfer, occurrence of the rate-limiting step subsequent to chemical transformation, etc.). Folate handling in the bifunctional enzyme is undoubtedly distinct from monofunctional DHFR, however, as H_2-folate produced by TS is channeled to DHFR directly (10, 11). Assuming that H_2-folate is transferred to DHFR faster than it is released into solution, substrate availability is unlikely to be limiting in the bifunctional enzyme, and mutations that lower affinity of the DHFR domain for H_2-folate should be less deleterious than mutations that affect rate-limiting steps (such as product release). Thus, a high k_{cat} is probably a more important requirement for enzyme activity than a low K_m. Indeed, those *T. gondii* alleles analogous to pyrimethamine-resistant *Plasmodium* isolates commonly observed in the field exhibit k_{cat} values comparable to wild-type (*e.g.* Asn-83 and Arg-36 + Asn-83; Table IV, lines (e) and (l)). For these mutants, the tradeoff between drug resistance and kinetic fitness is probably minimal. Conversely, despite an overall catalytic efficiency (k_{cat}/K_m) comparable to these alleles, mutation Ser-245 (f) exhibits poor k_{cat} values, possibly explaining why *Plasmodium* mutation Ser-223 has only been observed in the laboratory.

Kinetic Components of the Drug Resistance Mechanism—How does a substrate analog such as pyrimethamine compete effectively for active site binding when folate is directly provided to DHFR by the TS domain (11)? Inhibition is feasible under such circumstances if a drug can block substrate access by binding tightly to the active site, yielding very low inhibitor dissociation rates. Pyrimethamine binds tightly to the drug-sensitive *T. gondii* enzyme, but not by a slow binding mechanism (51): there is no apparent barrier to enzyme-inhibitor association when cofactor is abundant and no suggestion of inhibitor isomerization. Pyrimethamine may achieve its tight binding characteristics via binding synergism with the NADPH cofactor as shown for trimethoprim interaction with bacterial DHFR (57, 61) and suggested by structural predictions (see below). If so, changes in the enzyme ternary complex might be expected to influence inhibitor potency.

Encounters between NADPH and product-bound *E. coli* DHFR have been shown to enhance the rate of product leaving, thereby affecting the overall rate constant (60). Similarly, it may be that changes in NAPDH affinity could produce drug resistance by enhancing inhibitor dissociation. Experimental observations indicate that pyrimethamine resistance (see. Asn-83) is indeed associated with increased affinity for NADPH (Table IV); conversely, the pyrimethamine-hypersensitive mutation Val-10 exhibits an increased K_m for NADPH.

Structural Components of Resistance Mechanism—Fig. 3 presents a three-dimensional model of *T. gondii* DHFR-TS liganded with NADPH and pyrimethamine, based on the crystal structure of *L. major* DHFR-TS (54). Pyrimethamine placement assumes similar binding geometry for the pyrimidine ring amino groups and the analogous groups of methotrexate in the *L. major* structure. Energy minimization argues for a nonplanar organization of the inhibitor, rotating the chlorobenzyl ring ~90° with respect to the pyrimidine ring, resulting in parallel planar organization with the nicotinamide ring of NADPH (with partially overlapping van der Waals radii). This conformation is predicted to promote advantageous hydrophobic interactions between inhibitor and cofactor and to be entropically favored, excluding water from the intervening space in the binding pocket. The model therefore supports arguments for tight binding of pyrimethamine to the DHFR·NADPH complex (see above).

This structural model also permits exploration of the predicted effects of drug resistance mutations.

· Substituting Asn for Thr (or Ser) at position 83 is expected to force the inhibitor deeper into the binding crevice, where it could no longer sustain a favorable binding geometry with the NADP(H) cofactor. This would have the effect of uncoupling inhibitor and cofactor binding, precisely as observed in drug-resistant mutants (Fig. 4).

· Arg-36, which augments the drug resistance phenotype of Asn-83, is predicted to lie in close proximity to the glutamate moiety of bound folate. The function of this substitution is not immediately obvious, but it may contribute to the resistance phenotype by increasing H_2-folate affinity (or channeling efficiency) of the mutant enzyme through enhanced ionic interactions. Alternatively (or in addition), Arg-36 may increase the speed of chemical transformation by expediting the shift in electron density of bound folate toward the glutamate portion of the molecule, a process driven by positively charged residues nearby (62).

· Replacing Ala with Val at position 10 is expected to restrict the rotational freedom of substituents extending from C6 of the inhibitors' amino-pyrimidine ring. The ethyl group found in pyrimethamine can be rotated freely, but the analogous carbon in cycloguanil is bonded tetrahedrally to two methyl substituents and thus sterically constrained. One would therefore predict that the Val-10 mutant might retain sensitivity to py-

rimethamine but should not be able to bind cycloguanil, as is indeed the case.

· The combination of a Val at position 10 and an Asn at 83 would probably compress the binding pocket to such an extent that substrate access is severely restricted, accounting for the extremely poor enzyme activity of this mutant.

In summary, the experimental accessibility of *T. gondii* DHFR-TS permits a concerted biochemical, structural, and functional examination of antifolate sensitivity and resistance in the closely related parasites *T. gondii* and *P. falciparum*. These studies provide new insights into the biochemistry of pyrimethamine inhibition and the evolution of resistance genes and resistance phenotypes in malaria and should be useful for investigating the role of DHFR-TS in antifolate resistance in *T. gondii* as well.

Acknowledgments—We thank Darrick Carter and Buddy Ullman (Oregon Health Sciences University) for helpful discussions and assistance with structural modeling; D. A. Matthews (Agaron) for the *Leishmania* DHFR-TS crystal structure coordinates; D. P. Jacobus (Jacobus Pharmaceuticals) for cycloguanil and Monica Trujillo and Dan Santi (University of California, San Francisco) for plasmid *p*ET15b (DHFR-TS) and advice on protein purification strategies; Jason Wooden and Carol Sibley (University of Washington) for host strains and vectors suitable for DHFR expression in yeast; and Robert Donald, Jung Oh, and other members of the Roos laboratory for critical reading of the manuscript.

REFERENCES

1. Levine, N. D. (1988) *J. Protozool.* **35,** 518–520
2. Brooks, R. G., Remington, J. S., and Luft, B. (1987) *Antimicrob. Agents Annu.* **2,** 297–306
3. Dannemann, B., McCutchan, J. A., Israelski, D., Antoniskis, D., Leport, C., Luft, B., Nussbaum, J., Clumeck, N., Morlat, P., and Chiu, J. (1992) *Ann. Int. Med.* **116,** 33–43
4. Hitchings, G. H. (1983) *Inhibition of Folate Metabolism in Chemotherapy* (Hitchings, G. H., ed) pp. 1–20, Springer-Verlag, Berlin
5. Schwartzman, J. D., and Pfefferkorn, E. R. (1981) *J. Parasitol.* **67,** 150–158
6. Reyes, P., Rathod, P. K., Sanchez, D. J., Mrema, J. E., Rieckmann, K. H., and Heidrich, H. G. (1982) *Mol. Biochem. Parasitol.* **5,** 275–290
7. Iltzsch, M. H. (1993) *J. Euk. Microbiol.* **40,** 24–28
8. Roos, D. S. (1993) *J. Biol. Chem.* **268,** 6269–6280
9. Bzik, D. J., Li, W. B., Horii, T., and Inselburg, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8360–8364
10. Ivanetich, K. M., and Santi, D. V. (1990) *Exp. Parasitol.* **70,** 367–371
11. Trujillo, M., Donald, R. G. K., Roos, D. S., Greene, P. J., and Santi, D. V. (1996) *Biochemistry* **35,** 6366–6374
12. Eyles, D. E., and Coleman, M. (1955) *Antibiot. Chemother.* **5,** 529–539
13. Eyles, D. E., and Jones, F. E. (1955) *Antibiot. Chemother.* **5,** 731–734
14. Roos, D. S., Donald, R. G. K., Morrissette, N. S., and Moulton, A. L. C. (1994) *Methods Cell Biol.* **45,** 27–63
15. Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. M., and Foote, S. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9109–9113
16. Peterson, D. S., Walliker, D., and Wellems, T. E. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9114–9118
17. Snewin, V. A., England, S. M., Sims, P. F., and Hyde, J. E. (1989) *Gene* (*Amst.*) **76,** 41–52
18. Zolg, J. W., Plitt, J. R., Chen, G. X., and Palmer, S. (1989) *Mol. Biochem. Parasitol.* **36,** 253–263
19. Tanaka, M., Gu, H., Bzik, D. J., Li, W., and Inselburg, J. W. (1990) *Mol. Biochem. Parasitol.* **39,** 127–134
20. Foote, S. J., Galatis, D., and Cowman, A. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3014–3017
21. Peterson, D. S., Milhous, W. K., and Wellems, T. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3018–3022
22. Hyde, J. E. (1990) *Pharmacol. Ther.* **48,** 45–59
23. Thaithong, S., Chan, S. W., Songsomboon, S., Wilairat, P., Seesod, N., Sueblinwong, T., Goman, M., Ridley, R., and Beale, G. (1992) *Mol. Biochem. Parasitol.* **52,** 149–158
24. Cheng, Q., and Saul, A. (1994) *Mol. Biochem. Parasitol.* **65,** 361–363
25. Basco, L. K., Ramiliar, O., and Le Bas, J. (1994) *Am. J. Trop. Med.* **10,** 193–199
26. Basco, L. K., Pecoulas, P. E., Wilson, C. M., Le bas, J., and Mazabraud, A. (1995) *Mol. Biochem. Parasitol.* **69,** 135–138
27. Reeder, J. C., Rieckmann, K. H., Genton, B., Lorry, K., Wines, B., and Cowman, A. F. (1996) *Am. J. Trop. Med. Hyg.* **55,** 209–213
28. Zindrou, S., Nguyen, P. D., Nguyen, D. S., Skold, O., and Swedberg, G. (1996) *Exp. Parasitol.* **84,** 56–64
29. Cowman, A. F. (1995) *Austr. N. Z. J. Med.* **25,** 837–844
30. Le bras, J., Basco, L. K., and dePecoulas, P. E. (1996) *C. R. Seances Soc. Biol. Fil.* **190,** 471–485
31. Leport, C., Raffi, F., Matheron, S., Katlama, C., Regnier, B., Saimot, A. G., Marche, C., Vedrenne, C., and Vilde, J. L. (1988) *Am. J. Med.* **84,** 94–100
32. Luft, B. J., Hafner, R., Korzan, A. H., Leport, C., Antoniski, D., Bosler, E. Bourland, D. D., Uttamchandani, R., Fuhrer, J., and Jacobsin, J. (1993) *N. Engl. J. Med.* **529,** 995–1000
33. Tenant-Flowers, M., Boyle, M. J., Carey, D., Marriott, D. J., Harkness, J. L., Penny, R., and Cooper, D. (1991) *AIDS* **5,** 311–315
34. Sirawaraporn, W., Sirawaraporn, R., Cowman, A. F., Yuthavong, Y., and Santi, D. V. (1990) *Biochemistry* **29,** 10779–10785
35. Hall, S. J., Sims, P. F., and Hyde, J. E. (1991) *Mol. Biochem. Parasitol.* **45,** 317–330
36. Sirawaraporn, W., Prapunwattana, P., Sirawaraporn, R., Yuthavong, Y., and Santi, D. V. (1993) *J. Biol. Chem.* **268,** 21637–21644
37. Sano, G., Morimatsu, K., and Horii, T. (1994) *Mol. Biochem. Parasitol.* **63,** 265–273
38. Prapunwattana, P., Sirawaraporn, W., Yuthavong, Y., and Santi, D. V. (1996) *Mol. Biochem. Parasitol.* **83,** 93–106
39. Hekmat-Nejad, M., Lee, P. C., and Rathod, P. K. (1997) *Exp. Parasitol.* **85,** 303–305
40. Sirawaraporn, W., Sathitkul, T., Sirawaraporn, R., Yuthavong, Y., and Santi, D. V. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1124–1129
41. Wu, Y., Kirkman, L. A., and Wellems, T. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1130–1134
42. van der Wel, A. M., Tomas, A. M., Kocken, C. H., Malhotra, P., Janse, C. J., Waters, A. P., and Thomas, A. W. (1997) *J. Exp. Med.* **185,** 1499–1503
43. Donald, R. G. K., and Roos, D. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11703–11707
44. Donald, R. G. K., and Roos, D. S. (1994) *Mol. Biochem. Parasitol.* **63,** 243–253
45. McClary, J. A., Whitney, F., and Geisselsoder, J. (1989) *BioTechniques* **7,** 282–289
46. Howell, E. E., Forster, P. G., and Forster, L. M. (1988) *J. Bacteriol.* **170,** 3040–3045
47. Singer, S., Ferone, R., Walton, L., and Elwell, L. (1985) *J. Bacteriol.* **164,** 470–472
48. Pfefferkorn, E. R., and Pfefferkorn, L. C. (1977) *J. Protozool.* **24,** 449–453
49. Morrison, J. F. (1969) *Biochim. Biophys. Acta* **185,** 269–286
50. Cha, S. (1975) *Biochem. Pharmacol.* **24,** 2177–2185
51. Morrison, J. F. (1982) *Trends Biochem. Sci.* **7,** 102–105
52. Baccanari, D. P., and Joyner, S. S. (1981) *Biochemistry* **20,** 1710–1716
53. Stone, S. R., and Morrison, J. F. (1986) *Biochim. Biophys. Acta* **869,** 275–285
54. Knight, D. R., Kan, C. C., Howland, E., Janson, C. A., Hostomska, Z., Welsh, K. M., and Matthews, D. A. (1994) *Nat. Struct. Biol.* **1,** 186–194
55. Hillcot, B. L., Nixon, N. F., and Blakley, R. L. (1967) *Anal. Biochem.* **21,** 178–189
56. Holfels, E., McAuley, J., Mack, D., Milhous, W. K., and McLeod, R. (1994) *Antimicrob. Agents Chemother.* **38,** 1392–1396
57. Filman, D. J., Bolin, J. T., Matthews, D. A., and Kraut, J. (1982) *J. Biol. Chem.* **257,** 13663–13672
58. Chen, G., Mueller, C., Wendlinger, M., and Zolg, W. (1987) *Mol. Pharmacol.* **31,** 430–437
59. Thillet, J., Adams, J. A., Benkovic, S. J. (1990) *Biochemistry* **29,** 5195–5202
60. Penner, M. H., and Frieden, C. (1987) *J. Biol. Chem.* **262,** 15908–15914
61. Baccanari, D. P., Daluge, S., and King, R. W. (1982) *Biochemistry* **21,** 5068–5075
62. Brown, K. A., and Kraut, J. (1992) *Faraday Discuss. Chem. Soc.* **93,** 217–224