Published in final edited form as:

Mol Biochem Parasitol. 2007 July ; 154(1): 1–5.

Pyrimethamine-resistant dihydrofolate reductase enzymes of *P. falciparum* are not enzymatically compromised *in vitro*

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Abstract

Plasmodium falciparum, the protozoan that causes the most lethal form of human malaria, has been controlled principally by two safe, affordable drugs, chloroquine and sulfadoxine-pyrimethamine (SP). Studies in the laboratory and in the field have demonstrated that resistance to SP depends on non-synonymous point mutations in the dihydrofolate reductase (DHFR), and dihydropteroate synthase (DHPS) coding regions. Parasites that carry *dhfr* genes with 3 or 4 point mutations (51I/59R/108N triple mutation or 51I/59R/108N/164L quadruple mutation) are resistant to pyrimethamine *in vitro* and patients infected with these parasites respond poorly to SP treatment.

The wide spread of these pyrimethamine-resistant alleles demonstrates the increased fitness over drug-sensitive alleles in the presence of the drug. However, it is not clear whether these alleles might reduce the fitness of parasites in the absence of drug pressure. As a first step, we compared the kinetic properties of the wild type, and three mutant alleles to determine whether the native DHFR-thymidylate synthase form of the mutant proteins showed compromised activity *in vitro*. The mutant enzymes had K_m values for their substrate, dihydrofolate that were significantly lower than the wild type, k_{cat} values in the same range as the wild type enzyme, and k_{cat}/K_m values higher than wild type. In contrast, the K_m values for the NADPH cofactor were higher than wild type for the mutant enzymes. These observations suggest that the fitness of these parasites may not be compromised relative to those that carry the wild type allele, even without sustained SP drug pressure.

Keywords

Drug resistance; Antifolate; Relative fitness; Selection

Introduction

HIV, TB, malaria and many bacterial infections can be controlled mainly by drug treatment, and the development of resistance to the drugs is a major clinical problem world wide. To slow the rise in resistant pathogens, it is important to understand the mechanisms by which drug resistance is selected and sustained. *Plasmodium falciparum*, the protozoan that causes the

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most lethal form of human malaria, has been controlled principally by two safe, affordable drugs, i.e. chloroquine and sulfadoxine-pyrimethamine (SP). Although overall child mortality due to malaria has declined in many areas of Africa, the development of parasites resistant to chloroquine has been associated with increased mortality in recent years [1,2]. Resistance to each drug is strongly associated with point mutations in specific parasite genes [3-5]. Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (DHFR) and sulfadoxine targets the enzyme dihydropteroate synthase (DHPS). Studies in the laboratory and in the field have demonstrated that resistance to SP depends on non-synonymous point mutations in the DHFR, and DHPS coding regions. Parasites that carry *dhfr* genes with 3 or 4 point mutations, i.e. 511/59R/108N triple mutant or 511/59R/108N/164L quadruple mutant) are resistant to pyrimethamine *in vitro* and patients infected with these parasites respond poorly or not at all to SP treatment [4,6,7] (See [5] for a comprehensive review).

The completion of the *P. falciparum* genome made it possible to use microsatellite markers linked to *dhfr* and *dhps* to trace the ancestry of SP resistant strains. Using this approach, recent work has demonstrated that highly resistant alleles have not arisen locally by selection to high prevalence *in situ*. Rather, only a very few SP resistant strains that carry the triple or quadruple mutant allele of *dhfr* have been selected and these strains have “swept” the *P. falciparum* populations in low and moderate transmission regions of Southeast Asia, Eastern Africa and South America [8-11]. This may not be the case in higher transmission settings, and there is a need to examine population histories in these areas [12]. These new techniques have changed radically our capacity to understand the selective process, and provide new approaches for surveillance of drug resistant strains.

The wide spread of these pyrimethamine-resistant alleles demonstrates their increased fitness over drug-sensitive alleles in the presence of the drug, but the model raises a key issue. Do these mutations that confer drug resistance reduce the fitness of parasites that carry them if the drugs are withdrawn? If that is the case, then resistant parasite populations might be reduced or even disappear. This reduction in prevalence of a drug resistant parasite has occurred in at least one very well documented case. In Malawi, SP replaced chloroquine as the recommended treatment for falciparum malaria in 1993. This replacement was followed by a rapid replacement of the K76T bearing allele of *pfert* that is the hallmark of chloroquine-resistant strains, with a K76 chloroquine-sensitive allele and a return of clinical chloroquine sensitivity [13,14]. This observation raised the hope that the pyrimethamine-resistant alleles of *dhfr* might be similarly compromised, and recede from prevalence if SP drug pressure were removed.

The prediction of decreased fitness of parasites that carried the triple or quadruple mutant allele of *dhfr* was supported by measurement of the DHFR enzyme activity *in vitro*. Early work by Sirawaraporn and colleagues showed that the catalytic activity (k_{cat}) of the DHFR domain of the triple and quadruple mutant enzymes was severely compromised *in vitro* compared with the wild type enzyme [15]. However, this deficiency was hard to reconcile with the persistence of the parasites that carried these alleles. In Southeast Asia, SP has not been used extensively for almost 30 years, but parasites that carry the quadruple mutant genotype are still common [16-19], and parasites that carry the triple mutant allele have persisted in Ghana despite very low usage of SP [20]. These observations suggest that the fitness of these parasites may not be compromised relative to those that carry the wild type allele, even without sustained SP drug pressure.

In protozoan parasites, the DHFR enzyme is one domain of a bifunctional protein with thymidylate synthase (TS) [21-23]. Because of the poor expression of *P. falciparum* proteins in heterologous systems [24], attempts to express the DHFR domain alone were made and the DHFR was purified after denaturation and refolding from inclusion bodies within the *E. coli* [15]. Since the structure of the native DHFR-TS protein was recently published [23], and it

was shown that the two domains could be co-expressed as a DHFR-TS complex [25], we have now reexamined the kinetic parameters of the whole DHFR-TS protein complex that contain the wild-type, triple and quadruple mutant DHFR alleles purified only from the soluble fraction of the *E. coli*. In addition, we had previously identified a novel 51I/59R/108N/213A allele found at low frequency in field isolates from 4 different locations in Africa, and the DHFR domain of this protein conferred substantial resistance to pyrimethamine when tested in a heterologous yeast system [25]29]. We also compared the kinetic properties of this DHFR-TS protein with other DHFR enzymes that are commonly found in *P. falciparum* populations.

Materials and Methods

Constructs

A synthetic DHFR construct (codons 1- 231) with the *E. coli* codon bias [15] was cloned into the carbenicillin resistant pET-17b vector with *Nde*I and *Hind*III sites (Novagen, Madison, WI) and an additional N-terminal 6XHisTag. A second construct that contained the joining region (codons 232-324) connected with the TS region (codons 325-607) was cloned into the kanamycin resistant pET-24d (Novagen, Madison, WI) with a C-terminal 6XHisTag. The three mutant constructs (51I/59R/108N, 51I/59R/108N/164L and 51I/59R/108N/213A) were created using QuikChange II with the wild type synthetic DHFR construct as a template (Stratagene, Cedar Creek, TX).

Growth in *E. coli*

The constructs harboring the *dhfr* and *jr-ts* sequences were co-transformed into BL21(DE3) pLysS *E. coli* cells (Novagen, Madison, WI) and grown overnight at 37°C in 50 mL Luria-Bertani broth (LB) supplemented with 60 µg/mL carbenicillin, 30 µg/mL kanamycin and 50 µg/mL chloramphenicol. Four liters of LB supplemented with the same concentrations of antibiotics were inoculated with 1:100 dilution of the saturated culture and grown at 37°C to an OD₆₀₀ of 0.6 – 0.8.

Protein expression was induced by addition of 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) and the culture was allowed to grow at 22°C for 16 hours. The induced cells were harvested, washed in 20 mM Tris buffer, pH 8.0 and the pelleted cells were stored at –80° C until purification.

Protein purification

Protein purification was based on Shallom *et al* [26]. Cells were lysed with (per half liter) 20 mL BugBuster Protein Extraction Reagent (Novagen, Madison, WI), 500 units benzonase nuclease, > 99% Purity (Novagen, Madison, WI), aprotinin (1 µg/mL), leupeptin (1 µg/mL), pepstatin A (1 µg/mL) and 50 mM phenylmethanesulfonyl fluoride (PMSF). Cell debris was pelleted and the soluble fraction was adjusted to 20% glycerol and 20 mM imidazole. The column (Econo-10 columns) containing Ni-NTA resin (Qiagen, Valencia, CA) was packed (50 mM NaH₂PO₄ / 300 mM NaCl) and shielded (50 mM NaH₂PO₄ / 300 mM NaCl / 20% glycerol / 10 mM imidazole). The adjusted lysate was passed through the column in a cold room at a rate of 1 mL/min. The column was washed (50 mM NaH₂PO₄ / 300 mM NaCl / 20% glycerol / 50 mM imidazole) and bound protein was eluted with 10 mL elution buffer (50 mM NaH₂PO₄ / 300 mM NaCl / 20% glycerol / 250 mM imidazole). For storage, 10 mM dithiothreitol (DTT) was added and purified protein was stored at 4° C. Protein concentrations of the fractions were determined by the Bradford assay [27]. A sample of each fraction was analyzed before addition of DTT on a non-reducing 4-15% polyacrylamide gel, and after reduction, on a 12% polyacrylamide gel using standard protocols. The average yield of DHFR-TS protein was 800 micrograms from 4L culture.

Enzyme Assay

The activity of DHFR was determined spectrophotometrically according to the method of Sirawaraporn [28] by measuring the oxidation of NADPH to NADP⁺ at 340 nm using a microplate reader (Thermoelectron, LabSystems Multiskan MCC/240). The activity assay contained 100 μ M NADPH, 50 mM TES, pH 7.0, 1 mM EDTA, pH 8.0, 75 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin and $\sim$ 0.005 unit of enzyme. The reaction was initiated with 100 μ M dihydrofolate (DHF). One unit of DHFR enzyme activity is defined as the amount of enzyme required to produce 1 μ mole product/min at 25°C.

Steady-state kinetic parameters (K_m and V_{max}) were determined by varying the concentrations of DHF or NADPH between 3.5 μ M and 225 μ M with the other (NADPH or DHF, respectively) remaining constant at 100 μ M and the enzyme was added to initiate the reaction. Absorbance values were converted to concentrations using Beer-Lambert's law. The rates of reactions were plotted in Kaleidograph (Synergy Software, Reading, PA) and kinetic parameters were calculated using the Michaelis-Menten curve fit.

Results

The protein is purified as a DHFR-TS complex

Because of the difficulties of expressing large quantities of native DHFR-TS protein in *E. coli* [24], previous studies on the kinetic properties of the *P. falciparum* DHFR enzyme have focused mainly on using partially purified enzyme preparations [29] or DHFR domain alone purified and refolded from inclusion bodies from *E. coli* [15]. To circumvent this problem, we capitalized on an observation from the laboratory of Pradipsingh Rathod: when the DHFR and TS domains are coexpressed in *E. coli*, the two domains form a tight complex of 2 TS and 2 DHFR domains, like the native protein [26]. We transformed into BL21(DE3)pLysS *E. coli* separate plasmids that encoded the DHFR domain and the joining region/TS domains, and purified the complex from the soluble fraction using 6XHis tags on the proteins. Figure 1A shows that the protein purified from *E. coli* had the expected molecular size for this complex, 132 kDa. Upon reduction, two components of the appropriate sizes, 27 kDa for the DHFR domain and 40 kDa for the bridge/TS domain were observed (Figure 1B).

The activities of wild type and mutant enzymes are indistinguishable

Our goal was to compare the kinetic properties of the wild type, and three mutant alleles to determine whether the native form of the mutant proteins also showed compromised activity *in vitro*. Each DHFR domain and the bridge-TS domain were co-expressed in *E. coli* and the complex purified from the soluble fraction. We determined the K_m and V_{max} for both the substrate, dihydrofolate (DHF) and the cofactor, NADPH and the catalytic activity, k_{cat} and the k_{cat}/K_m for DHF for each complex. We tested complexes that carried the DHFR domains of the wild type, the triple mutant (51I/59R/108N), the quadruple mutant (51I/59R/108N/164L) including a novel quadruple mutant, (51I/59R/108N/213A). The kinetic data of the wild-type and mutant enzymes are summarized in Table 1.

The kinetics data from the present study showed that the K_m values for DHF of the mutant enzymes were 2-4 fold lower than that of the wild type ($p < 0.001$, 2 tailed t-test). The k_{cat} value for the wild-type was comparable to that of the mutant enzymes. As a consequence of the 2-fold higher of calculated enzyme efficiency, the calculated k_{cat}/K_m values for the mutant enzymes were 2-fold higher than that of the wild-type enzyme. In contrast, the K_m values for NADPH cofactor of both mutant enzymes were slightly higher than that of the wild type enzyme ($p = 0.0261$ for triple and $p = 0.0535$ for quadruple).

The DHFR-TS that carried the novel 51I/59R/108N/213A quadruple DHFR domain was analyzed. The K_m value for DHF was found to be about 2-fold lower than that for the triple and quadruple mutant enzymes and about 4-fold lower than that for the wild-type enzyme ($p < 0.01$ for all comparisons). However, the k_{cat} values for all the three proteins were compromised. As a result the k_{cat}/K_m values for the mutant enzymes were similar and were significantly better than that of the wild type ($p = 0.017$).

Discussion

The demonstration that the triple and quadruple mutant DHFR-TS complex enzymes are more active *in vitro* than the wild type is important for two reasons. First, based on the assay of the isolated DHFR domains, it has been widely assumed that both mutant enzymes were far less efficient than the wild type. This assumption and a some field studies [30,31] supported the hope that in the absence of drug selection, parasites that carry these alleles would have compromised fitness relative to the wild type. Our data show that both enzymes are more efficient *in vitro* than the wild type. Thus, it seems more likely that these alleles may have been rapidly selected and widely disseminated because they are both resistant to antifolates, and encode enzymes with adequate activity. Second, their adequate enzyme activity may explain the persistence of the highly mutant alleles in *P. falciparum* populations in Southeast Asia, even under conditions of low pyrimethamine usage [16,17,32,33].

The K_m values of the mutant enzymes for both substrates determined in the present study are within the same range compared to those previously reported for the DHFR domain alone and the partially purified DHFR-TS enzyme [29]. The discrepancy between the K_m values of the wild-type DHFR domain reported earlier [15] and the DHFR-TS complex in the present study could be attributed to the different structural topology of the two proteins particularly in different experimental conditions. However, the k_{cat} values differ from those that we calculated. The relative efficiency of the enzyme, k_{cat}/K_m , was almost 13-fold and 6-fold lower for the triple and quadruple mutants, respectively when the isolated DHFR domain was assayed [15]. In contrast, our data suggest that the mutant enzymes are a bit more efficient than the wild type. There are two principal differences between the two studies. First in this work, we have assayed the DHFR activity in the context of the DHFR-TS complex. We have noticed that the DHFR-TS complex is stable at 4°C for at least 3 weeks, supporting the idea that the enzyme is in its native conformation. In contrast, the DHFR domain alone is relatively unstable *in vitro*, and repeated attempts to purify and concentrate the domain for crystallography studies produced disordered aggregates, suggesting that it may not be as stable as its native form (JMW, data not shown). Second, in the prior studies, the DHFR domain was isolated from inclusion bodies, denatured and renatured prior to assay, whereas we have assayed only the soluble fraction isolated from the bacterial lysates. Both of these differences make it difficult to compare the two results, but they are likely to affect the concentration of active enzyme, and would both affect the calculation of the k_{cat} . In any case, it is possible that the activity of the DHFR-TS enzyme may reflect more accurately the behavior of the endogenous enzyme.

The triple and quadruple mutant alleles are routinely found at high levels where ever SP has been intensively used [18]. However, various groups have used molecular methods to identify rare novel alleles of *dhfr* in field isolates from several locations [12,16,25,34-37]. The effect of these alleles on pyrimethamine-resistance in parasites is not known, although several alleles have been shown to confer pyrimethamine-resistance in a heterologous system. The failure of these rare alleles to come to prominence in the *P. falciparum* population is likely to depend mostly on stochastic processes; they simply fail to run the gauntlet of transmission [38]. However, the 51I/59R/108N/213V allele was observed in 4 widely separated African sites. The overall efficiency of the enzyme k_{cat}/K_m , is comparable to the common resistant variants because the far lower K_m for DHF compensates for the lower k_{cat} . However, these differences

may impair the performance of the enzyme *in vivo*, and provide a possible explanation for the allele remaining at low levels in the *P. falciparum* population.

The relationship between the *in vitro* activity of the purified protein and the overall fitness of the parasites that depend upon that enzyme is unknown, and probably unknowable. However, the very widespread distribution of parasites that carry the triple mutant allele, certainly suggests that this genotype may not severely compromise the fitness of the parasites that carry it. It has been suggested that the success of the parasites that carry the triple mutant allele could reflect contributions of other loci that compensate for a less than optimal DHFR activity [8-11,14]. However, if the DHFR activity of the drug resistant enzymes is equivalent to wild type, then there is no need to invoke compensatory loci.

When chloroquine was successfully withdrawn in Malawi in 1993, the prevalence of the K76T allele of *Pfcr* declined precipitously and there was a concomitant recovery of the clinical effectiveness of chloroquine treatment [13]. There have been suggestions of similar declines in chloroquine resistance in other areas (reviewed in [14]). These observations raised the hope that similar decreases in resistance might accompany withdrawal of other drugs. However, the cross resistance between the widely used antibiotic, trimethoprim and pyrimethamine already suggests that a true reduction in antifolate drug pressure might be difficult to achieve [39]. Moreover, if the parasites that carry pyrimethamine-resistant enzymes do not show lower fitness than their wild type counterparts, simply reducing drug pressure by withdrawing DHFR inhibitors would not be a successful strategy for “resurrecting” drugs of this class as effective antimalarials.

Acknowledgements

This work was supported by NIH grant AI 55604 awarded to CHS.

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Abbreviations

DHFR	dihydrofolate reductase
DHF	dihydrofolate
NADP	Nicotinamide adenine dinucleotide
SP	sulfadoxine-pyrimethamine

TS

thymidylate synthase

kD

Kilodalton

Figure 1. Molecular size of the DHFR-TS complex

The DHFR-TS complex was isolated as described in the methods, and analyzed by SDS-polyacrylamide gel electrophoresis. **A.** Non reducing conditions, **B.** Reducing conditions. Two micrograms of the first and second fractions eluted from the nickel column were added to loading buffer and separated on polyacrylamide gels as described in the methods.

Table 1

Kinetic parameters of the wild type and three pyrimethamine-resistant DHFR enzymes. Values are the average of at least 7 experiments $\pm$ the standard deviation.

Genotype	k_{cat} (sec^{-1})	K_m DHF (μM)	K_m NADPH (μM)	k_{cat}/K_m
WILD TYPE	1.24 ± 0.11	44.4 ± 12.1	23.2 ± 9.5	0.028
51I/59R/108N	1.23 ± 0.35	20.9 ± 5.5	37.9 ± 11.9	0.059
51I/59R/108N/164L	1.42 ± 0.57	20.7 ± 6.8	37.3 ± 13.2	0.069
51I/59R/108N/213A	0.68 ± 0.17	10.2 ± 5.1	30.9 ± 12.2	0.066