Mutational analysis of Plasmodium falciparum dihydrofolate reductase: The role of aspartate 54 and phenylalanine 223 on catalytic activity and antifolate binding

ARTICLE *in* MOLECULAR AND BIOCHEMICAL PARASITOLOGY · JUNE 2002

Impact Factor: 1.79 · DOI: 10.1016/S0166-6851(02)00035-X · Source: PubMed

ELSEVIER

Molecular & Biochemical Parasitology 121 (2002) 185–193

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com

Mutational analysis of *Plasmodium falciparum* dihydrofolate reductase: the role of aspartate 54 and phenylalanine 223 on catalytic activity and antifolate binding

Worachart Sirawaraporn [a,*], Rachada Sirawaraporn [a], Suganya Yongkiettrakul [b], Amornpol Anuwatwora [b], Guilio Rastelli [c], Sumalee Kamchonwongpaisan [b], Yongyuth Yuthavong [b]

[a] *Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Rd., Bangkok 10400, Thailand*

[b] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Rama 6 Rd., Bangkok 10400, Thailand*

[c] *Dipartimento di Scienze Farmaceutiche, Universita' di Modena e Reggio Emilia, Via Campi, 183-41100 Modena, Italy*

Received 29 August 2001; received in revised form 26 January 2002; accepted 4 February 2002

Abstract

The catalytic activity and ability to confer resistance to antifolates of *Plasmodium falciparum* dihydrofolate reductase (pfDHFR) through single and double mutations at Asp-54 and Phe-223 were investigated. A single Asp54Glu (D54E) mutation in the pfDHFR domain greatly decreased the catalytic activity of the enzyme and affected both the K_m values for the substrate dihydrofolate and the K_i values for pyrimethamine, cycloguanil and WR99210. The Phe223Ser (F223S) single mutant had unperturbed kinetics but had very poor affinity with the first two antifolates. The ability to confer high resistance to the antifolates of F223S enzyme was, however, abolished in the D54E+F223S double mutant enzyme. When D54E mutation was present together with the A16V+S108T double mutation, the effects on the K_m values for the substrate dihydrofolate and the binding affinity of antifolates were much more pronounced. The severely impaired kinetics and poor activity observed in A16V+S108T+D54E enzyme could, however, be restored when F223S was introduced, while the binding affinities to the antifolates remained poor. The experimental findings can be explained with a model for substrate and inhibitor binding. Our data not only indicate the importance of Asp-54 of pfDHFR in catalysis and inhibitor binding, but also provide evidence that infer the potentially crucial function of the C-terminal portion of pfDHFR domain.

Keywords: *Plasmodium falciparum*; Dihydrofolate reductase; Mutation; Active site residues

1. Introduction

The dihydrofolate reductase (DHFR) domain of *Plasmodium falciparum* bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a validated target for antifolate antimalarials. Two potent inhibitors of *P. falciparum* DHFR (pfDHFR), pyrimethamine (Pyr) and cycloguanil (Cyc), have been widely employed for the treatment of malaria. Unfortunately, the widespread use of the drugs has resulted in rapid emergence of drug-resistant parasites and hence compromised the clinical utilities of the drugs. It has been well accepted that antifolate resistance in *P. falciparum* is associated with point mutations in the DHFR domain of the DHFR-TS bifunctional protein (see [1,2] for review). Recently, molecular modeling studies and in vitro inhibition of recombinant wild-type and mutant *P. falciparum* DHFRs have provided significant insight into the active site residues of the wild-type and resistant pfDHFRs responsible for binding to antifolate antimalarials such as Pyr, Cyc and WR99210. The latter is a promising antimalarial dihydrotriazine antifolate with potent antimalarial activity against both drug-sensitive

Abbreviations: Cyc, cycloguanil; MTX, methotrexate; pfDHFR, *Plasmodium falciparum* dihydrofolate reductase; *pfdhfr*, the gene encoding pfDHFR; Pyr; pyrimethamine.

* Corresponding author. Tel.: +66-2-201-5605; fax: +66-2-248-0375.

E-mail address: scwsr@mahidol.ac.th (W. Sirawaraporn).

0166-6851/02/$ - see front matter
PII: S0166-6851(02)00035-X

and drug-resistant malaria [3,4]. These and other studies have contributed greatly to the understanding of drug resistance and to the implications on rational inhibitor design with the goal to circumvent drug resistance in malaria [5–9].

Despite increasingly detailed studies on the molecular basis of antifolate resistance in *P. falciparum*, relatively little is known about the active site residues and the effects of their mutations on catalysis and inhibitor binding. Alignment of the primary amino acid sequence of pfDHFR with DHFRs from a number of other sources revealed substantial homology in many regions of the sequences [7–10]. Crystal structural studies of *Escherichia coli* and *Lactobacillus casei* DHFRs [11–13] revealed that an aspartate residue (Asp-27 in *E. coli* and Asp-26 in *L. casei*) located in the active site cavity of the enzyme is important and plays a crucial role in enzyme, presumably through formation of a hydrogen-bond network and proton donation to the substrate [13–15]. For the DHFRs of higher organisms, Glu or Asp is present at the equivalent position and its importance in enzyme catalysis has also been noted [16,17]. Previous studies on mutagenesis of *E. coli* DHFR have given considerable insight into the reaction mechanism and roles of amino acids involved in catalysis and inhibitor binding [14,18–20]. In *E. coli*, Asp-27 was shown to be a critical residue in the active site of the enzyme with respect to catalysis and inhibitor binding [14,21,22]. Data from structural studies revealed that Asp-27 forms hydrogen bonding with the 2-amino group and N-3 of pteridine ring of the folate molecule [13]. Mutations of Asp-27 to Ser (D27S) or Asp-27 to Asn (D27N) dramatically affected the activity of the enzyme [14]. Of particular interesting was the finding that mutations at the C-terminal portion of *E. coli* DHFR, i.e. Phe-137 to Ser (F137S), Phe-153 to Ser (F153S) or Ile-155 to Asn (I155N) can rescue the impaired activity of *E. coli* DHFR caused by D27S mutation [23].

With respect to pfDHFR, alignment of the sequences has allowed identification of Asp-54 and Phe-223 to be equivalent to *E. coli* Asp-27 and Phe-153, respectively [7,8,10]. As found in *E. coli* DHFR, pfDHFR carrying mutations at Ala-16, Asp-54, Ser-108 and Phe-223 (A16V+D54N+S108T+F223S) have previously been reported [24]. These and other lines of evidence support the hypothesis that the underlying mechanism of enzyme catalysis and inhibitor binding of pfDHFR might be similar to that of *E. coli* enzyme. To test the hypothesis, we constructed a number of DHFR mutants carrying both single mutations (D54E, D54N and F223S) and double mutations (D54E+F223S and D54N+F223S), using the previously described synthetic gene encoding for both wild-type and A16V+S108T DHFRs [25] as templates. We report the expression and purification of these mutant enzymes, their kinetics and inhibition by Pyr, Cyc and WR99210 of the mutant enzymes. We show that Asp-54 of *P. falciparum* DHFR is crucial for catalytic activity. The effects of mutation at Phe-223 (F223S) on the binding of antifolates and the ability of F223S mutation to rescue D54E mutant DHFR are described.

2. Materials and methods

2.1. Materials

The restriction endonucleases and DNA ligases were from New England Biolabs and Promega. The plasmid extraction and purification kits were the products of QIAGEN. Custom oligonucleotide synthesis and fluorescent automated DNA sequencing were performed at the Bioservice Unit, BIOTEC Center, National Science and Technology Development Agency (NSTDA), Thailand. The recombinant pUC-based plasmid harboring the wild-type synthetic gene for pfDHFR [26] was used as the template for the construction of the pfDHFR mutants. The *P. falciparum dhfr* (*pfdhfr*) gene fragments were subsequently subcloned into the *Nde*I–*Hind*III sites of the pET-17b expression vector (Novagen). *E. coli* DH5α was employed as a general host strain for plasmid propagation, while *E. coli* BL21(DE3)pLysS was used as a host to express the pfDHFRs in all expression experiments. Pyrimethamine and NADPH were purchased from Sigma. Cycloguanil and WR99210 were the generous gifts from Burroughs Wellcome and Jacobus Pharmaceutical, NJ, respectively. H_2folate [27] and Methotrexate Sepharose CL-6B affinity resin ($\sim$1 μmol methotrexate ml^{-1} wet resin) [28] were prepared as described. All other reagents used were the purest grades available commercially.

2.2. Methods

pUC-pfDHFR (D54E) was constructed by cassette mutagenesis between the *Sac*II and *BstE*II sites of the pUC-pfDHFR (wild-type) [26] using a 66/73-bp oligonucleotide duplex, *ONd54mix-T* (5′-GGTCTGGGCAACAAAGGGGTCCTGCC GTGGAAATGCAACTCTCTA**NNN**ATGAAATACTTCTGCGCG3′) and *ONd54mix-B* (5′-GTAACCGCGCAGAAGTATTTCAT**NNN**TAGAGAGTTGCATTTCCACGGC AGGACCCCTTTGTTGCCCAGACCGC-3′). Annealing of the two oligonucleotides formed duplex with *Sac*II and *BstE*II compatible overhangs (underlined), with the degenerate codon **NNN** coding for all possible amino acids at residue 54. Mutation at Phe 223 to Ser (F223S) of the pUC-pfDHFR was achieved by replacement of the *Spe*I–*Hind*III gene fragment with a 64/64-bp oligonucleotide duplex, *ONf223s-T* (5′-CTAGTAACAACACTACTCTGGAC**TCG**ATCATCTACAAGAAGA CCAACAACTAATAGCGGCCGCA-3′)

and *ONf223s-B* (5′-AGCTTGCGGCCGCTA TTAG-TTGTTGGTCTTCTTGTAGATGAT**CGA**GTCCAG-AGTAGTGTTGTTA-3′). Annealing of *ONf223s-top* and *ONf223s-bot* generated an oligonucleotide duplex with overhangs of *Spe*I and *Hind*III at the 5′ and 3′ end, respectively, with codon **TCG** for Ser as indicated in bold. Mutants containing single mutation (D54E and D54N) and double mutations (D54E+F223S and D54N+F223S) were first constructed in pUC vector using the wild-type and A16V+S108T mutant *pfdhfrs* [26] as templates. Upon verification of the mutations by DNA sequencing, the gene fragments ($\sim$0.7 kb) encoding the corresponding mutant *pfdhfrs* in the pUC vector were excised by *Nde*I and *Hind*III double digestion. The excised DNA fragments were extracted from the gel, ligated into the corresponding sites of pET-17b vector, and transformed into *E. coli* BL21(DE3)pLysS for further expression of the mutant enzymes.

2.3. Expression and purification of mutant pfDHFRs

A fresh overnight culture from a single colony of *E. coli* BL21(DE3)pLysS cells harboring the gene for mutant pfDHFR was used to inoculate 1–4 l of LB media supplemented with 100 μg ml^{-1} ampicillin. The culture was grown at 37 °C until A_{600} reached 0.5–0.6, at which time the expression of pfDHFR was initiated by addition of IPTG at a final concentration of 0.4 mM. Initial experiments to assess expression of mutant pfDHFRs were performed by growing the cells with vigorous shaking at 37 °C for 3 h, a condition previously exploited for expression of pfDHFR which requires unfolding and refolding to recover the enzyme activity [26]. For subsequent experiments, expression of pfDHFR was performed by growing the cells at 20 °C for 18–20 h after IPTG induction, a condition that yielded highly active soluble pfDHFR. The cells were harvested by centrifugation at 10 000 × *g* for 10 min at 4 °C, washed once with $\sim$200–250 ml of cold phosphate-buffered saline, and resuspended in $\sim$20–50 ml of buffer A (20 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 10 mM DTT). The cells were then disrupted by two cycles of French press at 18 000 psi. The clear extract obtained after centrifugation at 30 000 × *g* for 1 h at 4 °C was applied onto an MTX-Sepharose CL-6B affinity column (1.5 × 5.0 cm), and the enzymes were affinity purified according to the procedure previously described [26].

2.4. Kinetics and inhibition studies of mutant pfDHFRs

The activity of pfDHFR was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm at 25 °C. The standard pfDHFR assay of 1 ml reaction was composed of 100 μM H_2folate, 100 μM NADPH, 50 mM TES, pH 7.0, 75 mM β-mercaptoethanol, 1 mg ml^{-1} bovine serum albumin and $\sim$0.005–0.01 U of purified enzyme. Unless otherwise specified, the final concentration of H_2folate was kept constant at 100 μM, and the reaction was initiated with H_2folate. One unit of DHFR activity is defined as the amount of enzyme which catalyzes the generation of 1 μmol of product min^{-1} at 25 °C. Steady-state kinetics and inhibition studies of the mutant enzymes by Pyr, Cyc, and WR99210 were performed as described previously [26]. Analysis of the kinetic and inhibition data was done by nonlinear least squares fitting to the equation $IC_{50} = K_i\ (1+[S]/K_m)$ [29].

2.5. Protein analysis

Protein concentrations were determined by Coomassie Blue G dye binding method using bovine serum albumin as a standard [30]. SDS-PAGE (12.5%) was performed according to the method of Laemmli [31].

2.6. Molecular modeling of the mutant pfDHFR-inhibitor complexes

The homology models of the wild-type and A16V+S108T pfDHFRs [8] were used as templates to generate mutant models by replacement of the amino acids Asp54 to Glu (D54E), and/or Phe223 to Ser (F223S). Modeling and energy minimization of the mutant models were performed using several modules including homology, builder, and discover modules of *Insight II* program installed in Silicon Graphic Station (Model Indy). The reduced form of the cofactor (NADPH), H_2folate and Cyc were also adopted from the previous models [8]. Following the assembly of the enzyme models with the cofactor and substrate H_2folate or Cyc, energy minimization was performed, with 5000 steps of conjugated gradient minimization, distance-dependent dielectric constant, fixing the cofactor and substrate/inhibitor in space, constraints on the nitrogens of Cyc (N-1 and 6-amino group) and H_2folate (2-amino group and N-3) to the carboxylic oxygens of Asp54/Glu54 with a generic distance of 1.0–2.0 Å. The minimization was performed without restraint on the backbone atoms to allow dynamic movement of the atoms.

3. Results

Nineteen mutants were constructed by replacing the codon for Asp-54 of the synthetic gene encoding the pfDHFR domain with all other amino acids. The mutants were characterized with respect to their ability to express active enzyme, kinetic properties, and inhibition by antifolate antimalarials. Of the nineteen mutants

investigated, Glu-54 DHFR was the only second most active enzyme, with DHFR activity representing about 2% of the wild-type enzyme. Eleven mutants (Ala, Val, Pro, Ser, Thr, Met, Asn, Gln, Trp, Lys and His) had undetectable DHFR activity in the crude lysate, and seven (Ile, Phe, Gly, Cys, Leu, Tyr and Arg) showed remarkably low DHFR activity, ranging from 0.03 to 0.2% of the wild-type enzyme (Table 1). The results clearly indicated the importance of Asp-54 for enzyme catalysis. We then proceeded to construct pfDHFR mutants with single and double mutations at residues 54 and 223 of the pfDHFRs and characterized their ability to express the mutant DHFRs in *E. coli*. Under the conditions used to obtain catalytically active *P. falciparum* wild-type DHFR (20 °C for 18–20 h), all mutants yielded a major expressed protein band of molecular mass ~27 kDa in the crude extracts upon SDS-PAGE (data not shown). Except for D54N and D54N+F223S mutants which poorly expressed the enzyme, all other mutants gave substantial quantities of active soluble DHFR at variable levels. The successful expression of pfDHFR was monitored by the appearance of an intense protein band of ~27 kDa on SDS-PAGE and the detectable DHFR activity as determined spectrophotometrically.

Despite considerably low DHFR activity detected in the crude extracts of the mutants as compared to the wild-type, the mutant enzymes were purified to homogeneity using a single-step affinity chromatography on MTX-sepharose column according to the procedure previously exploited for purification of the wild-type pfDHFR [26]. Attempts have not been made, however, in the present study to recover the DHFR activity from the D54N and D54N+F223S mutants, from which the expression yielded inactive inclusion bodies in *E. coli*.

The kinetic parameters of the purified mutant pfDHFRs and their inhibition by Pyr, Cyc and WR99210 were investigated and are summarized in Table 2. A single mutation at residue 54 (D54E) of the wild-type pfDHFR caused a remarkable decrease (~87 fold) in the catalytic efficiency (k_{cat}/K_m) of the enzyme compared with the wild-type pfDHFR. Unlike the effect seen for D54E mutation, a single F223S mutation on the wild-type pfDHFR background did not perturb the kinetic properties of the enzyme. Instead, when introduced together with the D54E mutation, the resulting D54E+F223S double mutant produced enzyme that had k_{cat} and k_{cat}/K_m values that were ~2.5- and 1.6-fold, respectively, higher than those from the single D54E mutant enzyme. When the D54E mutation was introduced into A16V+S108T double mutant, the resulting A16V+S108T+D54E triple mutant produced enzyme that was ~10-fold poorer in substrate binding, and ~300-fold less efficient than the A16V+S108T mutant pfDHFR (Table 2). The F223S mutation with the A16V+S108T background also did not affect the kinetics of the A16V+S108T mutant enzyme. Instead, the resulting triple mutant (A16V+S108T+F223S) yielded an enzyme that had k_{cat} and k_{cat}/K_m values that were ~2.7- and 5-fold, respectively, higher than the A16V+S108T pfDHFR. Interestingly, when F223S was introduced into the A16V+S108T+D54E triple mutant to yield A16V+S108T+D54E+F223S quadruple mutant, the kinetic properties and catalytic efficiency of the enzyme were restored to a value comparable to that observed for the A16V+S108T double mutant enzyme.

Table 1
Activities of DHFR in the crude extracts of wild-type (D54) and single mutants containing all possible mutations at amino acid residue 54

Recombinant enzyme	Expression*	Specific activity¶ (nmol min^{-1} mg^{-1})		Kinetic parameters		Inhibition constant (K_i, nM)	
		Refold§ enzyme	Crude‡ enzyme	K_m H_2folate (μM)	K_m NADPH (μM)	Pyr	Cyc
Asp (D)	+	17000±1520	2500±83	13±5#	5±1#	1.5±0.2#	2.6±0.3#
Glu (E)	+	345±66	17±0.2	23±2‡‡	11±0.4‡‡	10±0.3‡‡	54±4‡‡
Ile (I)	+	nd	5.3±1.1	–	–	–	–
Phe (F)	+	nd	3.4±0.4	–	–	–	–
Gly (G)	+	nd	2.5±0.1	–	–	–	–
Cys (C)	+	nd	2.2±0.1	–	–	–	–
Leu (L)	+	nd	2.1±0.6	–	–	–	–
Tyr (Y)	+	nd	1.2±0.2	–	–	–	–
Arg (R)	+	nd	0.7±0.3	–	–	–	–
Other mutants†	+	nd	nd	–	–	–	–

* Expressed protein (~27 kDa) seen on SDS-PAGE.
¶ Average values (mean±S.D.) from three independent determinations.
§ From inclusion bodies of 0.5–1.0 l of cell culture.
‡ From 100 ml of IPTG-induced *E. coli* culture.
† pfDHFRs with Ala, Val, Pro, Ser, Thr, Met, Asn, Gln, Trp, Lys and His at residue 54 were expressed but the enzyme activities were undetectable.
# Data from [25].
‡‡ Data obtained from refolded enzyme.
nd, DHFR activity not detected.

Table 2
Kinetics and inhibition by Pyr, Cyc and WR99210 of D54E, F223S and D54E + F223S mutant pfDHFRs in the wild-type and A16V + S108T mutant background

Recombinant enzyme	Expression‡	Kinetic parameters				K_i (nM)		
		K_m H_2folate (μM)	K_m NADPH (μM)	k_{cat} (s^{-1})	K_{cat}/K_m*	Pyr	Cyc	WR99210
Wild-type§	+	13 ± 5	5 ± 1	68	5.2	0.72 ± 0.1	1.1 ± 0.13	0.9 ± 0.1
D54E	+	44 ± 2.7	4.6 ± 0.3	2.7	0.06	11.3 ± 0.7	77.0 ± 5.8	13.4 ± 1.6
F223S	+	9.4 ± 1.6	10 ± 1.5	67	7.0	667 ± 38	4206 ± 359	31.3 ± 2.3
D54E + F223S	+	64 ± 12	13 ± 1.2	6.7	0.1	26 ± 4	100 ± 12	4.3 ± 0.6
A16V + S108T	+	11 ± 1.3	24 ± 1	13	1.2	2.4 ± 0.2	676 ± 5	5 ± 0.54
A16V + S108T + D54E‡‡	+	138 ± 9.8	15.5 ± 2	0.6	0.004	50 ± 4.4	26 120 ± 2600	26 ± 2.2
A16V + S108T + F223S	+	5.8 ± 0.5	7.8 ± 1.2	35	6.0	300 ± 27	4000 ± 170	14.4 ± 1.3
A16V + S108T + D54E + F223S	+	16.5 ± 2.5	11 ± 1	27	1.6	790 ± 80	10 770 ± 890	35 ± 2.8

Purified from the soluble fractions of the recombinant clones.
‡ Indicated by the presence of ~27-kDa protein band on SDS-PAGE.
* Calculated from the K_m values for H_2folate.
§ Data from [25].

The sensitivity of the mutant pfDHFRs toward Pyr, Cyc and WR99210 was investigated and are summarized in Table 2. The single D54E mutant pfDHFR had slightly less affinity with both Pyr and WR99210, with K_i values of 11.3 ± 0.7 and 13.4 ± 1.6 nM, respectively. However, the K_i value for Cyc of the D54E pfDHFR was ~70-fold higher than the wild-type enzyme, suggesting the mutant enzyme conferred more resistance to Cyc than Pyr and WR99210. The F223S single mutant enzyme, however, had far lower affinity with Pyr (K_i = 667 ± 38 nM) and Cyc (K_i = 4206 ± 359 nM), but only moderately affected the binding affinity of WR99210 (K_i = 31.3 ± 2.3 nM). When F223S mutation was introduced into the D54E mutant, the K_i values for Pyr, Cyc and WR99210 of the resulting D54E + F223S double mutant enzyme were greatly reduced to the range observed for the D54E single mutant enzyme (Table 2).

As previously reported, the A16V + S108T mutant pfDHFR conferred high resistance to Cyc but remained susceptible to Pyr [26,32,33]. When tested against WR99210, the A16V + S108T mutant enzyme was relatively sensitive to compound, with the K_i value of 5 ± 0.54 nM (Table 2). Additional mutations of D54E and F223S to the A16V + S108T mutant pfDHFR yielded triple A16V + S108T + D54E and A16V + S108T + F223S mutant enzymes which conferred high resistance to Pyr and Cyc, with the K_i values that were 20- and 39-fold, respectively, higher than that of the A16V + S108T enzyme. Likewise, introducing both D54E and F223S mutations into the A16V + S108T enzyme gave quadruple A16V + S108T + D54E + F223S mutant pfDHFR that also conferred high resistance to Pyr and Cyc. It is interesting, however, to note that resistance to WR99210 was only very modest for all the mutants tested, with the increased K_i values that ranged between 15- and 35-fold of the values for the wild-type enzyme (Table 2).

The drastic effect of D54 mutation and the ability to rescue the activity of D54E mutant enzyme by additional mutation at F223 (F223S) of the triple mutant (A16V + S108T + D54E) can be explained by a model of binding between the enzyme and substrate H_2folate (A, B and C) and inhibitor such as Cyc (D, E and F) (Fig. 1). As previously reported from the model of pfDHFR [6–8], one of the carboxylate oxygen of D54 (or E54) can form hydrogen bond to the 2-amino group of the substrate H_2folate (bonding a in Fig. 1A–C) or 6-amino group of Cyc (bonding a in Fig. 1D–F) which in turn forms a hydrogen bond with T185 (bonding b in Fig. 1A–E). The other carboxylate oxygen of D54 (or E54) hydrogen-bonds to N-3 of substrate H_2folate (bonding c in Fig. 1A–C) or N-1 of Cyc (bonding c in Fig. 1D–F). In the binding model, the D54E mutation was found to be slightly displaced from these interactions due to a bulkier side chain of E54 (Fig. 1B and E). As a consequence, the steric constraint in Cyc binding imposed by the A16V mutation would be much more pronounced in the mutant harboring both A16V + D54E mutations. The F223S mutation was found to displace T185, probably through formation of hydrogen bonding between the side chains. T185 normally serves to hold both substrate and inhibitors through hydrogen bonding with residue 54. With new hydrogen bonding with S223, T185 could only maintain hydrogen bonding with E54, in turn hydrogen bonded with the substrate, but was too distant to interact with E54, hydrogen bonded to Cyc (Fig. 1C and F). Such displacement of T185 and the lack of hydrogen bonding with the inhibitor might be an explanation for the inability of Cyc to bind the

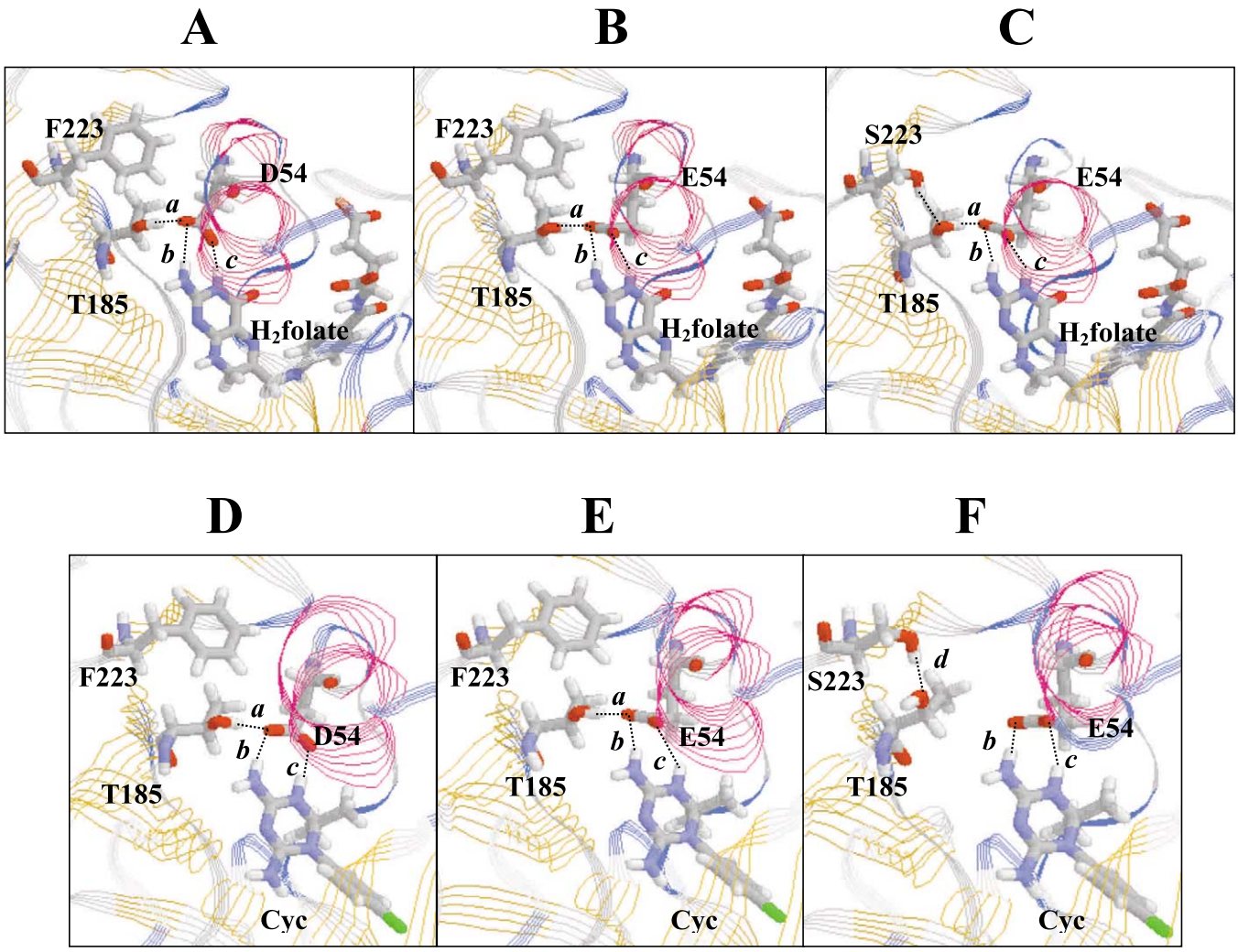

Fig. 1. Modeling studies showing binding of H2folate (A–C) and Cyc (D–F) to active site residues of wild-type pfDHFR (A and D); A16V+S108T+D54E mutant pfDHFR (B and E); and A16V+S108T+D54E+F223S mutant pfDHFR (C and F). The residues shown are the side chain residues of D54 (or E54) located on αB strand (pink), T185 and F223 (or S223) on βF and βH strands, respectively (brown). Possible hydrogen bondings are: a, side-chain hydroxyl group of T185 with the carboxylate oxygen of D54 (or E54); b, carboxylate oxygen of D54 (or E54) with 2-amino group of ligand (H_2folate or Cyc); c, carboxylate oxygen of D54 (or E54) with N-3 of substrate H_2folate or N-1 of Cyc; d, side-chain hydroxyl group of T185 with side-chain hydroxyl group of S223. Colors shown for the ligand (H_2folate and Cyc) are: carbon, grey; hydrogen, white; nitrogen, blue; oxygen, red; chloride, green.

mutant pfDHFR and hence the molecular basis of Cyc resistance in these mutants (Fig. 1F).

4. Discussion

We have described the study which addresses the importance of residue D54 of pfDHFR and have identified the mutation at residue F223 which can partially rescue the impaired pfDHFR activity causing by D54E mutation. Such compensating effects on enzyme activity occurred only with the background of A16V+S108T mutation, i.e. with the quadruple mutant A16V+S108T+D54E+F223S. Our working hypotheses were based on evidence from sequence alignment and extensive structural analyses of *E. coli* and *L. casei* DHFR-substrate-cofactor complexes [14,18–20], which suggested the likelihood that residue D54 of *P. falciparum* could be crucial for DHFR activity, and mutation of the D54 of pfDHFR would affect the binding affinity of substrate and inhibitors. The importance of D54 of pfDHFR is clearly demonstrated by our observation that only the wild-type pfDHFR with D54, but not mutants harboring other amino acids, is catalytically active (Table 1).

Results obtained from previously described pfDHFR models [6–9] suggest that the overall topology and folding of pfDHFR domain of the bifunctional pfDHFR-TS is very similar to that of DHFRs from other organisms [34]. Such topological arrangement

would place F223 on the βH strand of the DHFR domain and therefore raises the possibility, as observed in *E. coli* DHFR [23], that F223 mutation in pfDHFR might be responsible for a second-site suppressor in rescuing the impaired DHFR activity resulting from D54E mutation. Indeed, *P. falciparum* harboring F223S and D54N+F223S with the A16V+S108T background have previously been identified from FCR3 isolate under Pyr pressure in vitro [24], but neither of the mutation sites have been reported from malaria parasites in the field. Our observation that mutant pfDHFRs containing D54N and D54N+F223S are catalytically inactive may partly explain the observation in nature. However, further investigations are needed to understand the nature of mutations and to explain drug resistance in malaria parasite.

Mutation of D54 of *P. falciparum* DHFR exerted drastic effects on both catalytic activity and inhibitor binding (Table 1). The increase in K_m for H_2folate and K_i for Pyr, Cyc and WR99210 is not unexpected as similar observations were reported for *E. coli* DHFR [14,23]. Indeed, the decrease in affinity for binding observed in D54E mutant pfDHFR compared to its wild-type counterpart is understandable by modeling, which shows distortion in the hydrogen bonding system as a result of an extra CH_2 of E54 side chain at the active site of the enzyme. The observation that all other mutants, except for D54E, did not show detectable DHFR activity (Table 1) together with the kinetic data reported in Table 2 supports the hypothesis that the carboxylate group of the side chain at position 54 of pfDHFR is crucial for catalytic activity both in the wild-type and in the A16V+S108T mutant pfDHFR. Indeed, the D54E mutational effects on the latter were much more pronounced, markedly affecting both k_{cat} and K_m values for the substrate. Results from modeling studies are in good agreement with the kinetic data and provide insight into explanation of why the D54E mutant enzyme binds poorly to H_2folate. Although the effects of F223S mutation on the interactions between substrate and active site residues remain to be further elucidated, our preliminary results from Cyc-binding suggested that the hydrogen bonding originally formed between D54 and T185 was disrupted in the F223S mutant, rendering the already-weak hydrogen bonding system even weaker. The interaction between S223 and T185 served to hold the latter in place at the position that the mutated E54 side chain could form stable hydrogen-bondings with the 2-NH_2 group and N-3 of the pteridine ring of H_2folate (Fig. 1C). Nevertheless, the fact that Ser is relatively small compared with Phe and that T185 was held back as a result of interaction with S223 (Fig. 1F) would make inhibitors like Cyc interact with E54, which in turn cannot interact with T185, leading only to weak binding and inhibition (Table 2). As for the large reduction in binding of Cyc, and to a lesser extent Pyr and WR99210, our model shows that E54 carboxylate is displaced from the optimal position assumed by D54. Introduction of S223 leads to the ability of its hydroxyl to hydrogen-bond with T185, which normally forms hydrogen bonding with the carboxylate of residue 54, leading to less favorable binding with the inhibitor. The A16V+S108T mutations introduce steric restrictions to Cyc binding [8], exacerbating the reduction in binding. The relatively small effect on WR99210 may be due to its greater flexibility compared with the other two inhibitors [8].

The increase of enzyme activity of the D54E+F223S mutant with the presence of A16V+S108T mutations can be explained through modeling studies from the effect on the orientation of the carboxylate group of residue 54 and T185 which is hydrogen-bonded to it in the wild-type enzyme. The residue S223 of the mutant enzymes can form hydrogen bonding with T185, affecting the orientation of E54 which is already suboptimal compared to D54. The A16V+S108T mutations which reduce the free space available in the active site may have an overall effect of putting the carboxylate of E54 in a more favorable position for binding and catalysis.

Alternatively, the suppressing mutations may work through conformational changes of the enzyme, as concluded for F153 and I155 of *E. coli* DHFR [23] and for F137 of this enzyme [35,36]. The hypothesis can be tested but a more definitive conclusion has to await further detailed determination of the structures of the wild-type and mutant enzymes and their modes of binding to the substrates, as the results from modeling studies can only be considered as guidance to the correct structure, and that the data obtained must be exploited with caution, particularly when studies were performed using the DHFR domain of the bifunctional DHFR-TS enzyme which could affect the validity of the model.

It should be noted that F223S mutation either alone or in combination with A16V+S108T led to a large decrease in binding affinity with the inhibitors with little impairment of enzyme activity. It might have been expected, therefore, that the mutation would occur in nature during evolution of drug resistance. The fact that this was not found in nature needs further explanation.

In conclusion, we have described the importance of D54 of pfDHFR by demonstrating that mutation at this residue resulted in remarkable loss of enzyme activity and substrate/inhibitor binding affinities. We also reported that pfDHFR with F223S mutation did not exhibit impaired kinetics but weakened binding with Pyr, Cyc and, to a lesser extent WR99210. The severely impaired kinetics and poor activity observed in A16V+S108T mutants with D54E additional mutation could be restored by F223S mutation. The above experimental observations can be understood from the model of binding of substrates and inhibitors and could provide better understanding of the interactions among active

site residues in pfDHFR and aid in structure-based design of new families of antifolate inhibitors.

Acknowledgements

We are grateful to Mr Kanueng Chitcholtan for technical assistance. This work was supported in part by grants to W.S. (TDR, project ID 990568), to S.K. (DDR), and to Y.Y. (MMV, project 99009; EU, contract ICA4-CT-2001-10077).

References

[1] Cowman AF. The molecular basis of resistance to the sulfones, sulfonamides, and dihydrofolate reductase inhibitors. In: Sherman IW, editor. Malaria: Parasite Biology, Pathogenesis, and Protection. Washington DC: ASM Press, 1998.
[2] Sirawaraporn W. Dihydrofolate reductase and antifolate resistance in malaria. Drug Resist Updates 1998;1:397–406.
[3] Childs GE, Lambros C. Analogues of *N*-benzyloxydihydrotriazines: in vitro antimalarial activity against *Plasmodium falciparum*. Ann Trop Med Parasitol 1986;80:177–81.
[4] Canfield CJ, Milhous WK, Ager AL, et al. PS-15: a potent, orally active antimalarial from a new class of folic acid antagonists. Am J Trop Med Hyg 1993;49:121–6.
[5] McKie JH, Douglas KT, Chan C, et al. Rational drug design approach for overcoming drug resistance: application to pyrimethamine resistance in Malaria. J Med Chem 1998;41:1367–70.
[6] Warhurst DC. Antimalarial drug discovery: development of inhibitors of dihydrofolate reductase active in drug resistance. Drug Discov Today 1998;3:538–46.
[7] Lemcke T, Christensen IT, Jorgensen FS. Towards an understanding of drug resistance in malaria: three-dimensional structure of *Plasmodium falciparum* dihydrofolate reductase by homology building. Bioorg Med Chem 1999;7:1003–11.
[8] Rastelli G, Sirawaraporn W, Sompornpisut P, et al. Interactions of pyrimethamine, cycloguanil, WR99210 and their analogues with *Plasmodium falciparum* dihydrofolate reductase: structural basis of antifolate resistance. Biorg Med Chem 2000;8:1117–28.
[9] Santos-Filho OA, Hopfinger AJ. A search for sources of drug resistance by the 4D-QSAR analysis of a set of antimalarial dihydrofolate reductase inhibitors. J Comput Aided Mol Des 2001;15:1–12.
[10] Bzik DJ, Li WB, Horii T, Inselburg J. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc Natl Acad Sci USA 1987;84:8360–4.
[11] Bolin JT, Filman DJ, Matthews DA, Hamlin RC, Kraut J. Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 A resolution: I. General features and binding of methotrexate. J Biol Chem 1982;257:13650–62.
[12] Filman DJ, Bolin JT, Matthews DA, Kraut J. Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 A resolution: II. Environment of bound NADPH and implications for catalysis. J Biol Chem 1982;257:13663–72.
[13] Bystroff C, Oatley SJ, Kraut J. Crystal structures of *Escherichia coli* dihydrofolate reductase: the $NADP^+$ holoenzyme and the folate.$NADP^+$ ternary complex. Substrate binding and a model for the transition state. Biochemistry 1990;29:3263–77.
[14] Howell EE, Villafranca JE, Warren MS, Oatley SJ, Kraut J. Functional role of aspartic acid-27 in dihydrofolate reductase revealed by mutagenesis. Science 1986;231:1123–8.
[15] Blakley RL, Appleman JR, Freisheim JH, Jablonsky MJ. ^{13}C and ^{15}N nuclear magnetic resonance evidence that the active site carboxyl group of dihydrofolate reductase is not involved in the relay of a proton to substrate. Arch Biochem Biophys 1993;306:501–9.
[16] Oefner C, D' Arcy A, Winkler FK. Crystal structure of human dihydrofolate reductase complexed with folate. Eur J Biochem 1988;174:377–85.
[17] Davies JF, Delcamp TJ, Prendergast NJ, Ashford VA, Freisheim JH, Kraut J. Crystal structures of recombinant human dihydrofolate reductase complexed with folate and 5-deazafolate. Biochemistry 1990;29:9467–79.
[18] Adams J, Johnson K, Matthews R, Benkovic SJ. Effects of distal point-site mutations on the binding and catalysis of dihydrofolate reductase from *Escherichia coli*. Biochemistry 1989;28:6611–8.
[19] Murphy DJ, Benkovic SJ. Hydrophobic interactions via mutants of *Escherichia coli* dihydrofolate reductase: separation of binding and catalysis. Biochemistry 1989;28:3025–31.
[20] Warren MS, Brown KA, Farnum MF, Howell EE, Kraut J. Investigation of the functional role of tryptophan-22 in *Escherichia coli* dihydrofolate reductase by site-directed mutagenesis. Biochemistry 1991;30:11092–103.
[21] Villafranca JE, Howell EE, Voet DH, et al. Directed mutagenesis of dihydrofolate reductase. Science 1983;222:782–8.
[22] Appleman JR, Howell EE, Kraut J, Blakley RL. Role of aspartate 27 of dihydrofolate reductase from *Escherichia coli* in interconversion of active and inactive enzyme conformers and binding of NADPH. J Biol Chem 1990;265:5579–84.
[23] Dion A, Linn CE, Bradrick TD, Georghiou S, Howell EE. How do mutations at phenylalanine-153 and Isoleucine-155 partially suppress the effects of the aspartate-27 to serine mutation in *Escherichia coli* dihydrofolate reductase? Biochemistry 1993;32:3487–97.
[24] Tanaka M, Gu HM, Bzik DJ, Li WB, Inselburg JW. Dihydrofolate reductase mutations and chromosomal changes associated with pyrimethamine resistance of *Plasmodium falciparum*. Mol Biochem Parasitol 1990;39:127–34.
[25] Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, Santi DV. Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc Natl Acad Sci USA 1997;94:1124–9.
[26] Sirawaraporn W, Prapunwattana P, Sirawaraporn R, Yuthavong Y, Santi DV. The dihydrofolate reductase domain of *Plasmodium falciparum* thymidylate synthase-dihydrofolate reductase: gene synthesis, expression, and anti-folate resistant mutants. J Biol Chem 1993;268:21637–44.
[27] Friedkin M, Crawford EJ, Misra D. Reduction of folate derivatives with dithionite in mercaptoethanol. Fed Proc Fed Am Soc Exp Biol 1962;21:176.
[28] Meek TD, Garvey EP, Santi DV. Purification and characterization of the bifunctional thymidylate synthetase-dihydrofolate reductase from methotrexate resistant *Leishmania tropica*. Biochemistry 1985;24:678–86.
[29] Segal IH. Enzyme Kinetics: Behavior and Analysis of Steady-State and Rapid Equilibrium Enzyme Systems. New York: Wiley-Interscience, 1975.
[30] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[31] Laemmli UK. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature (London) 1970;227:680–5.
[32] Foote SJ, Galatis D, Cowman AF. Amino acids in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those

involved in pyrimethamine resistance. Proc Natl Acad Sci USA 1990;87:3014–7.
[33] Peterson DS, Milhous WK, Wellems TE. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA 1990;87:3018–22.
[34] Blakley RL. Dihydrofolate reductase. In: Blakley RL, Benkovics SJ, editors. Folates and Pteridines. New York: Wiley, 1984:191–253.
[35] Dunn SM, Lanigan TM, Howell EE. Dihydrofolate reductase from *Escherichia coli*: probing the role of aspartate-27 and phenylalanine-137 in enzyme conformation and the binding of NADPH. Biochemistry 1990;29:8569–76.
[36] Howell EE, Booth C, Farnum MF, Kraut J, Warren MS. A second-site mutation at phenylalanine-137 that increases catalytic efficiency in the mutant aspartate-27 serine *Escherichia coli* dihydrofolate reductase. Biochemistry 1990;29:8561–9.