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addenda and errata

Characterization, crystallization and preliminary X-ray analysis of bifunctional dihydrofolate reductase–thymidylate synthase from *Plasmodium falciparum*. Erratum

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In the paper by Chitnumsub *et al.* (2004) the details for one of the authors were given incorrectly. The name of the second author is Jirundon Yuvaniyama (instead of Yavaniyama) and his affiliation is Center for Protein Structure and Function and Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.

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Chitnumsub, P., Yavaniyama, J., Vanichtanankul, J., Kamchonwongpaisan, S., Walkinshaw, M. D. & Yuthavong, Y. (2004). *Acta Cryst.* D**60**, 780–783.

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Characterization, crystallization and preliminary X-ray analysis of bifunctional dihydrofolate reductase–thymidylate synthase from *Plasmodium falciparum*

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The full-length *pfdhfr-ts* genes of the wild-type TM4/8.2 and the double mutant K1CB1 (C59R+S108N) from the genomic DNA of the corresponding *Plasmodium falciparum* parasite have been cloned into a modified pET(17b) plasmid and expressed in *Escherichia coli* BL21 (DE3) pLysS. Conditions for the expression and purification of the *P. falciparum* dihydrofolate reductase–thymidylate synthase (PfDHFR-TS) have been established that yield ∼1 mg of the soluble active enzyme per litre of culture. The purified enzymes have been crystallized using a modified microbatch method with PEG 4000 as the primary precipitating agent. X-ray diffraction data were collected to 2.50 and 2.64 Å resolution under cryogenic conditions from single crystals of the two PfDHFR-TS proteins in complex with NADPH, dUMP and either Pyr30 or Pyr39. Preliminary X-ray analysis indicated that the crystals belong to the orthorhombic space group $P2_12_12_1$, with two molecules per asymmetric unit and ∼52% solvent content ($V_M \simeq 2.6$ Å^3 Da^{-1}). The use of a particular type of baby oil in the microbatch setup appeared to be beneficial to PfDHFR-TS crystallization and a preliminary comparison with another commonly used oil is described.

1. Introduction

Dihydrofolate reductase (DHFR), an important enzyme in the thymidylate cycle, catalyzes the reduction of dihydrofolate to tetrahydrofolate with concurrent oxidation of NADPH (Ferone, 1977). DHFR is a validated molecular target for antifolate drugs, such as pyrimethamine (Pyr) and cycloguanil (Cyc), against malarial parasites as these drugs block the DHFR activity, leading to depletion of thymidylate, incomplete DNA synthesis and eventually cell death (Ivanetich & Santi, 1990). Resistance to antifolates has occurred through point mutations at various amino-acid residues surrounding the DHFR active site (Cowman *et al.*, 1988; Foote *et al.*, 1990; Peterson *et al.*, 1988, 1990; Sirawaraporn *et al.*, 1997). Homology models of *Plasmodium falciparum* DHFR have been built based on sequence alignment of the DHFR sequences of *Escherichia coli*, *Lactobacillus casei*, human, chicken liver and *Pneumocystis carinii* to explain the interactions between the malarial DHFR and its inhibitors which give rise to the resistance and to guide the design of new inhibitors (Rastelli *et al.*, 2000; Warhurst, 2002). The models, however, could not provide adequate information on the structural assembly of the malarial DHFR, which exists as a homodimeric bifunctional enzyme dihydrofolate reductase–thymidylate synthase (PfDHFR-TS). Our recent success in determining crystal structures of this crucial drug target in complex with a potent inhibitor WR99210 and an available Pyr drug has allowed insights into interdomain interactions and also opened a new dimension in the design of new drugs to fight against malaria (Yuvaniyama *et al.*, 2003). However, more structures of wild-type and mutant PfDHFR-TS enzymes in complex with different classes of inhibitors are needed in order to extend our understanding and to develop new antifolate antimalarials. In this paper, we provide for the first time details of the production, purification and crystallization of PfDHFR-TS wild-type enzyme (TM4/8.2) and the C59R+S108N double-mutant enzyme (K1CB1) and also describe the preliminary crystallographic analysis of two new complexes of the bifunctional PfDHFR-TS enzymes with rationally designed 2,4-diaminopyrimidine derivatives Pyr30 and Pyr39 (Fig. 1). Pyr30 (also known as SO3) is the *m*-chloro analogue of pyrimethamine, while Pyr39 is a pyrimethamine derivative without the *p*-chloro atom and with a 6-*n*-hexyl group in place of the 6-ethyl group. They both retain binding affinity to the mutant enzymes and have good antimalarial activities against mutant parasites (Kamchonwongpaisan *et al.*, 2004). The crystal structures of the complexes in comparison with the complex with pyrimethamine (Yuvaniyama *et al.*, 2003) should give insights into development of new effective compounds against resistant parasites.

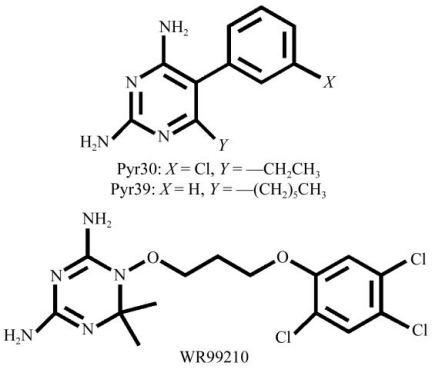

Figure 1
Structures of the potent *P. falciparum* dihydrofolate inhibitors Pyr30, Pyr39 and WR99210.

2. Methods and results

2.1. Cloning and expression of PfDHFR-TS

The TM4/8.2 (wild type) and K1CB1 (C59R+S108N double mutant) PfDHFR-TS genes were cloned using PCR amplification from genomic DNA of the corresponding *P. falciparum* strains with oligonucleotide primers 5′-GCC AGC **AAG CTT** ATG ATG GAA CAA GTC TGC GAC GTT-3′ and 5′-GCC AGC **GGT ACC** AAT ATT AAG CAG CCA TAT CCA TTG A-3′, which encompass *Hin*dIII and *Kpn*I restriction sites (in bold), respectively. The PCR was carried out using *pfu* DNA polymerase (Promega) at 367 K for 5 min, followed by 30 cycles of 367 K for 1 min, 323 K for 1 min and 345 K for 2 min and finally an additional 345 K for 10 min. The derived fragment of 1.8 kbp complete PfDHFR-TS gene was subsequently ligated into the *Hin*dIII and *Kpn*I sites of a modified pET17b (Novagen) vector, from which a total of 47 bases including the *Nde*I site before the *Hin*dIII cloning site had been excised, and then transformed into *E. coli* BL21(DE3)pLysS. The recombinant PfDHFR-TS protein was expressed as a soluble active form as determined by tritium FdUMP labelling. An overnight pre-culture of the transformant was grown from a single colony with shaking at 310 K in Luria–Bertani (LB) medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. Using a 1.5% inoculum from the overnight pre-culture, 12 l of the *E. coli* cells were grown in LB medium containing 100 µg ml^{-1} ampicillin to a mid-log phase ($OD_{600} \simeq 0.8$) at 310 K and then induced with 0.4 m*M* IPTG for 20 h at 293 K. Cells were collected by centrifugation at 10 500*g* for 8 min.

2.2. Purification of PfDHFR-TS

The cell pellets were resuspended in buffer *A* [0.1 m*M* EDTA, 10 m*M* DTT, 50 m*M* KCl, 20 m*M* potassium phosphate buffer pH 7.2 containing 20%(*v*/*v*) glycerol] and lysed at 69 MPa using a French Press (Thermo Dynamics Ltd). The lysate was clarified by centrifugation at 27 000*g* for 1 h. The supernatant (crude extract) was loaded onto a methotrexate (MTX) affinity column (Meek *et al.*, 1985), extensively washed with buffer *A* containing 0.5 *M* KCl until no protein was detected in the effluent and then eluted with 4 m*M* 7,8-dihydrofolate (H_2folate) in buffer *B* [0.1 m*M* EDTA, 10 m*M* DTT, 50 m*M* KCl, 50 m*M* *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) pH 8.2 containing 20%(*v*/*v*) glycerol]. The DHFR-active fractions were pooled, concentrated and H_2folate was partially removed by exchanging with buffer *B* using a Vivaspin-20 ultrafiltration membrane with 30 kDa cutoff (Sartorious). The DHFR-TS enzyme sample was further purified on a Q-Sepharose column equilibrated with buffer *B* that contained no KCl. A linear gradient of 0–0.5 *M* KCl in buffer *A* was applied over 3 h at a flow rate of 1 ml min^{-1}. The PfDHFR-TS protein was eluted in the range 100–150 m*M* KCl. The DHFR-active fractions were pooled and concentrated and the solution was exchanged with buffer *A* three times in a Vivaspin-20 ultrafiltration membrane with 30 kDa cutoff by 3500*g* centrifugation at 277 K. The protein concentration was determined with Bradford protein assay using BSA as a standard (Bradford, 1976). The purity of the protein sample is shown in Fig. 2.

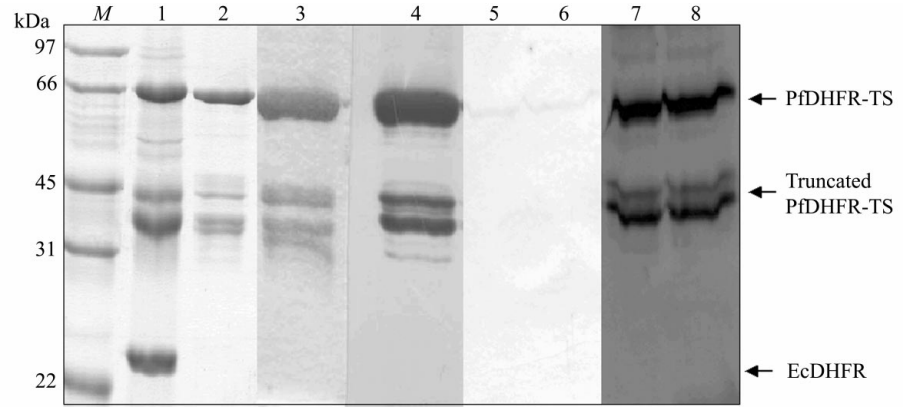

Figure 2
Coomassie-blue stained SDS–PAGE (12%) and tritium FdUMP-labelling film showing expression and purification of the PfDHFR-TS. Lane *M*, low-molecular-weight protein markers as indicated; lanes 1 and 2, purified PfDHFR-TS samples from MTX and Q-Sepharose columns, respectively; lanes 3 and 4, Coomassie blue staining and autoradiograph of Q-purified PfDHFR-TS with tritium FdUMP labelling and tritium signal enhancer embedding, respectively; lanes 5 and 6, dissolved crystals of PfDHFR-TS–WR99210–NADPH–dUMP complex; lanes 7 and 8, tritium FdUMP-labelling films of lanes 5 and 6, respectively.

2.3. Dihydrofolate reductase activity assay

The activity of DHFR was determined spectroscopically at 340 nm by monitoring the declining absorption of NADPH at 298 K using a reaction molar absorptivity of 12 300 M^{-1}cm^{-1} (Hillcoat *et al.*, 1967; Meek *et al.*, 1985). The activity was assayed with 100 µ*M* of both NADPH and DHF in a 1 ml cocktail (50 m*M* TES buffer pH 7.0 containing 75 m*M* β-mercaptoethanol, 1 mg ml^{-1} BSA) on a Hewlett–Packard UV–Vis spectrophotometer (HP8453) using a single acquisition. The reaction was initiated with the enzyme (of activity 0.01 µmol min^{-1}).

2.4. Thymidylate synthase activity assay

TS activity was determined spectroscopically at 340 nm by monitoring the increase in absorbance arising from conversion of 5,10-methylenetetrahydrofolate (6R-CH_2H_4folate) to H_2folate at 298 K using a reaction molar absorptivity of 6400 M^{-1} cm^{-1} (Meek *et al.*, 1985). The reaction was assayed in a 1 ml cocktail of 150 µ*M* 6R-CH_2H_4folate and 125 µ*M* dUMP in 50 m*M* TES buffer pH 7.4 containing 25 m*M* $MgCl_2$, 6.5 m*M* formaldehyde, 1 m*M* EDTA and 75 m*M* β-mercaptoethanol on a Hewlett–Packard UV–Vis spectrophotometer (HP8453) using multiple acquisitions. The reaction was initiated with either the enzyme (~0.005–0.007 µmol min^{-1}) or dUMP. The use of tritium FdUMP for TS labelling was performed according to a previously described procedure (Roberts, 1966; Sirawaraporn *et al.*, 1990). Briefly, the enzyme was incubated at 298 K for 30 min with 10 µl of 3 m*M* 6R-CH_2H_4folate, 0.1 µl of 0.66 µ*M* (^{3}H)-FdUMP (15 Ci mmol^{-1}; Moravek Biochemicals, Brea, CA, USA) in 50 m*M* TES buffer pH 7.4 containing 25 m*M* $MgCl_2$, 1 m*M* EDTA, 6.5 m*M* formaldehyde

and 75 m*M* β-mercaptoethanol. The (^{3}H)-FdUMP–protein complexes were analysed on 12% SDS–PAGE running at a constant voltage of 150 V. After electrophoresis, the Coomassie-blue stained gel was soaked with an autoradiography enhancer (NEN, Dupont) with gentle shaking for 60 min at room temperature, washed with cold water, dried and exposed on Hyperfilm (Hyperfilm MP, Amersham Pharmacia Biotech) at 193 K for 3 d before film development.

2.5. Crystallization and data collection

PfDHFR-TS was crystallized at room temperature (298 K) using the microbatch method (Chayen *et al.*, 1992; D'Arcy *et al.*, 1996) in a 60-well plate (1 mm diameter at the bottom of each well) covered with either 6 ml of Al's oil (Hampton Research) or baby oil (Cussons; a mixture of mineral oil, olive oil and vitamin E; PZ Cussons, Thailand). The inhibitor–protein mixture was prepared by mixing 60 µl of the purified PfDHFR-TS protein (15 mg ml^{-1}) in 20 m*M* potassium phosphate buffer pH 7.0 containing 0.1 m*M* EDTA, 10 m*M* DTT, 50 m*M* KCl and 20%(*v*/*v*) glycerol with 6 µl of each the following solutions: NADPH, dUMP and either Pyr39 or Pyr30 at a concentration of 10 m*M* each. The mixture was equilibrated on ice for 30 min for complete complex formation. Crystallization was set up on a microplate by first pipetting 1 µl of the protein mixture into the well soaked with oil followed by 1 µl of crystallization solution, without mixing. In the initial screen at room temperature, small crystals of needle, plate and prismatic morphology were observed together with precipitated protein in several different conditions. Crystals of prismatic form grew in a drop containing 0.1 m*M* sodium acetate pH 4.6, 25%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium acetate (solution 1) within a week, whereas needle and plate forms were found in 0.1 m*M* sodium citrate pH 5.5, 25%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium acetate (solution 2) and in 0.1 m*M* sodium acetate pH 4.6, 25%(*w*/*v*) PEG 4000 and 0.2 *M* calcium acetate (solution 3). After optimization of solution 1 by varying the concentrations of both PEG 4000 and salts, large single crystals (>0.2 mm in length) grew within 2 d in 0.1 m*M* sodium acetate pH 4.6, 20%(*w*/*v*) PEG 4000 and 0.22 *M* ammonium acetate (Fig. 3*c*). In addition, well formed rod-shaped crystals with strong birefringence grew in a drop of optimized solution 3 by lowering the PEG 4000 concentration to 18%. We found that crystal growth was very sensitive to temperature during crystallization setup; many small crystals formed immediately at temperatures below 298 K. Single crystals of the TM4/8.2–Pyr39–NADPH–dUMP and K1CB1–Pyr30–NADPH–dUMP complexes were flash-frozen in liquid nitrogen after a 10 s dip in a corresponding crystallization solution containing 20%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data were collected under a cold nitrogen stream (100 K) at Cu $K\alpha$ wavelength (1.5418 Å) on an FR591 rotating-anode X-ray generator equipped with a κCCD detector. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997); statistics are shown in Table 1.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Wild type–Pyr39	K1CB1–Pyr30
Unit-cell parameters (Å)	a = 57.985, b = 156.673, c = 164.668	a = 56.628, b = 156.014, c = 165.131
Space group	$P2_12_12_1$	$P2_12_12_1$
Resolution (Å)	99.0–2.64 (2.75–2.64)	50.0–2.50 (2.59–2.50)
No. observed reflections	177237	238105
No. unique reflections	41802	50688
Completeness (%)	96.3 (82.4)	98.6 (95.1)
$I/\sigma(I)$	9.2 (1.79)	13.7 (1.79)
$R_{\rm merge}$ (%)	8.9 (41.6)	8.4 (44.2)
Molecules per asymmetric unit	2	2

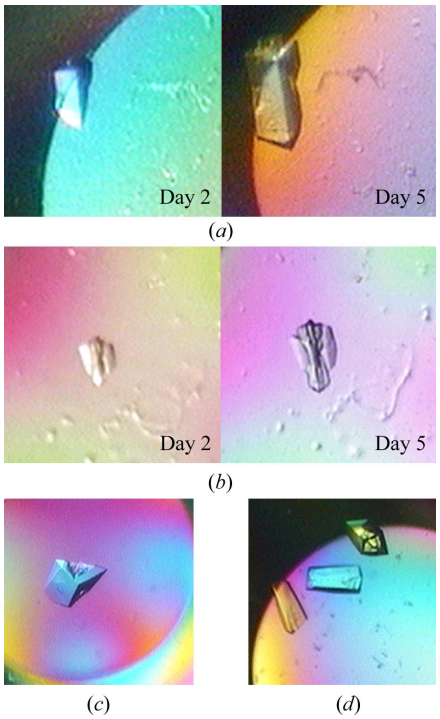

Figure 3
Crystals of (*a* and *b*) PfDHFR-TS K1CB1–Pyr30–NADPH–dUMP and (*c* and *d*) TM4/8.2–Pyr39–NADPH–dUMP complexes grown under the same crystallization conditions using baby oil (*a* and *c*) and Al's oil (*b* and *d*). The crystallization drop under Al's oil (*b*) nucleated faster than a similar drop under baby oil (*a*). Typical crystal dimensions of 0.1 × 0.2 × 0.1 mm were obtained from different optimized conditions under baby oil (*c*) and Al's oil (*d*).

3. Discussion

Purification of *P. falciparum* DHFR-TS using MTX resin *via* affinity binding with the DHFR domain was supposed to readily remove all other proteins in the crude extract, leaving only PfDHFR-TS bound to the resin. However, in addition to the 24 kDa *E. coli* DHFR, the MTX-purified PfDHFR-TS also contained major protein impurities with molecular weights of about 35 and 42 kDa as seen on SDS–PAGE. The results of tritium FdUMP labelling (Fig. 2) and N-terminal amino-acid sequencing of these 35 and 42 kDa proteins indicated that they were PfDHFR-TS proteins with truncated C-termini which could have resulted from protein degradation or incomplete translation. To prevent possible proteolysis of the PfDHFR-TS, a cocktail of protease inhibitors comprising 20 µg ml^{-1} aprotinin, 20 µg ml^{-1} leupeptin and 0.5 m*M* phenylmethylsulfonyl fluoride (Meek *et al.*, 1985) was added to the lysis buffer before cell disruption. The purified PfDHFR-TS protein obtained still exhibited the truncated enzyme bands, suggesting that these were likely to arise from incomplete protein translation or that proteolysis might have taken place while the protein was in the cell. Although the truncated proteins have much lower molecular weight than PfDHFR-TS, they could not be separated by gel-filtration chromatography and were co-eluted from a column as a single broad peak, suggesting that they are associated quite tightly. This finding is in agreement with a previous report (Sirawaraporn *et al.*, 1990). Further attempts using Q-Sepharose anion-exchange chromatography successfully removed the *E. coli* DHFR and some of the truncated proteins. Anion-exchange chromatography was thus used as the second column to improve the enzyme purity. The protocol yielded ~1 mg of PfDHFR-TS enzyme

per litre of culture with TS and DHFR specific activities of 670 and 30 000 nmol min^{-1} mg^{-1}, respectively. The purified PfDHFR-TS showed a major band at 72 kDa and minor bands at 35 and 42 kDa on SDS–PAGE and tritium FdUMP-labelling gels (Fig. 2).

Despite the presence of truncated fragments in the purified PfDHFR-TS samples, measurement of its dynamic light scattering (at 1 mg ml^{-1}) using a DynaPro MSX (Protein Solutions) displayed a narrow unimodal distribution of hydrodynamic radius, suggesting suitable sample homogeneity for crystallization (Ferré-D'Amaré & Burley, 1997). The crystals obtained diffracted X-rays well and the identity of the bifunctional PfDHFR-TS protein in the crystals was verified with tritium FdUMP-labelling SDS–PAGE (Fig. 2). The protein from dissolved crystals, despite being below the detection limit of Coomassie staining (Fig. 2; lanes 5–6), showed a strong tritium signal from FdUMP–protein complexes, indicating the presence of active PfDHFR-TS protein in the crystal (Fig. 2; lanes 7–8). X-ray data sets of the two PfDHFR-TS proteins, TM4/8.2 and K1CB1, complexed with NADPH, dUMP and the inhibitor Pyr39 or Pyr30 were collected and processed to 2.64 and 2.50 Å resolution, respectively. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 57.98, b = 156.67, c = 164.67 Å for the TM4/8.2 complex and a = 56.63, b = 156.01, c = 165.13 Å for the K1CB1 complex. They were estimated to contain two molecules per asymmetric unit with ∼52% solvent content and a V_M of ∼2.6 $Å^3$ Da^{-1}.

Crystallization of the two PfDHFR-TS complexes appeared sensitive to air oxidation; we found that dithiothreitol or β-mercaptoethanol helped to promote crystal growth and stability. Moreover, attempts to reproduce the crystallization with the hanging-drop vapour-diffusion technique, in which the protein drops are exposed to air, were unsuccessful. The success of the microbatch method under oil could possibly be attributed to reduction of oxygen contact with the protein drop.

We found that Al's oil (Hampton Research) was very effective for crystallization trials in which broad ranges and combinations of precipitants were screened since it allows rapid evaporation of water and subsequent concentration of the crystallization drop (D'Arcy *et al.*, 1996). However, prolonged experiments using Al's oil over two weeks were undesirable owing to its volatility; the crystals consequently became 'rubbery', suggesting cross-linking of protein molecules, likely to have arisen from air oxidation. We have searched for a substitute for Al's oil for crystal optimization and finally chose baby oil (Cussons) owing to its lower volatility, purity and easy availability. In addition, the presence of vitamin E in this baby oil might also help as oxygen scavenger, although this aspect was not investigated further.

Preliminary investigation of crystal growth at 296 K using baby oil and Al's oil showed that crystal nucleation and growth were faster under Al's oil than baby oil (Fig. 3). As a result, crystals of PfDHFR-TS complexes tended to be multi-crystalline with Al's oil while corresponding crystals under baby oil took a longer time to reach their optimum sizes but grew in single crystalline form (Figs. 3*a* and 3*b*). However, under some conditions crystals of TM4/8.2–Pyr39–NADPH–dUMP grew well using both baby oil and Al's oil (Figs. 3*c* and 3*d*). Successful crystallizations of PfDHFR-TS and other proteins in our laboratories suggest the use of baby oil as a cheap effective alternative source of mineral oil for microbatch crystallization. This may be of importance for laboratories in countries with tropical climates such as Thailand where temperatures are more difficult to control and evaporation in microbatch is accelerated.

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