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The role of tryptophan-48 in catalysis and binding of inhibitors of *Plasmodium falciparum* dihydrofolate reductase

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Abstract

Dihydrofolate reductases (DHFRs) from *Plasmodium falciparum* (Pf) and various species of both prokaryotic and eukaryotic organisms have a conserved tryptophan (Trp) at position 48 in the active site. The role in catalysis and binding of inhibitors of the conserved Trp48 of PfDHFR has been analysed by site-specific mutagenesis, enzyme kinetics and use of a bacterial surrogate system. All 19 mutant enzymes showed undetectable or very low specific activities, with the highest value of k_{cat}/K_m from the Tyr48 (W48Y) mutant (0.12 versus 11.94 M^{-1} s^{-1}), of about 1% of the wild-type enzyme. The inhibition constants for pyrimethamine, cycloguanil and WR99210 of the W48Y mutants are 2.5–5.3 times those of the wild-type enzyme. All mutants, except W48Y, failed to support the growth of *Escherichia coli* transformed with the parasite gene in the presence of trimethoprim, indicating the loss of functional activity of the parasite enzyme. Hence, Trp48 plays a crucial role in catalysis and inhibitor binding of PfDHFR. Interestingly, W48Y with an additional mutation at Asn188Tyr (N188Y) was found to promote bacterial growth and yielded a higher amount of purified enzyme. However, the kinetic parameters of the purified W48Y+N188Y enzyme were comparable with W48Y and the binding affinities for DHFR inhibitors were also similar to the wild-type enzyme. Due to its conserved nature, Trp48 of PfDHFR is a potential site for interaction with antimalarial inhibitors which would not be compromised by its mutations.

Keywords: Dihydrofolate reductase; Mutagenesis; *Plasmodium falciparum*; Malaria

1. Introduction

Plasmodium falciparum dihydrofolate reductase (PfDHFR; EC 1.5.1.3) is a part of the bifunctional enzyme, DHFR-thymidylate synthase (DHFR-TS), responsible for sequential reactions in the synthesis of deoxythymidine 5′-monophosphate (dTMP). The enzyme is one of the few well-defined drug targets for malaria, with antifolates like pyrimethamine and cycloguanil, and combinations with sulfa drugs, playing important roles in treatment of *P. falciparum* infections over many decades. However, resistance to these antifolates is a major problem, necessitating the search for new, effective drugs. The mechanism of resistance has been shown to involve mutations at various residues of the DHFR, causing reduced binding of the drugs (Hyde, 2002; Yuthavong, 2002; Yuthavong et al., 2005, 2006). These residues include Ala16, Ser108, Cys59, Asn51 and Ile164. These mutations also resulted in reduced binding of the substrates and reduced catalytic activity of the enzyme, but not to the extent of making the enzyme inactive and ineffective in carrying out its biological function. Hence, although they may have some role in substrate binding and catalysis, these residues are not essential residues, required for effective functioning of malarial DHFR. Apart from investigation of the role of Asp54 (Sirawaraporn et al., 2002), there is as yet scant information on the

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essential residues for malarial DHFR. Knowledge of the essential residues is potentially important for design of new inhibitors which work through interaction with these non-mutating residues.

Based on the crystal structure of PfDHFR-TS (Yuvaniyama et al., 2003) and analogy with the enzyme from other species, Trp48 of pfDHFR, equivalent to Trp22 in the *Escherichia coli* enzyme and Trp24 of the human enzyme, is expected to be an essential residue involved in substrate binding through hydrogen bonding with a water molecule which in turn hydrogen-bonds to Asp54 and C4 oxygen of the substrate (Beard et al., 1991; Warren et al., 1991; Yuvaniyama et al., 2003; Yuthavong et al., 2005). This paper reports the results of mutation analysis of the role of Trp48, which shows that among the 20 possible residues at position 48, tryptophan is indeed by far the most effective one for the activity of the enzyme. Mutation to any of the 19 other amino acids resulted in drastic reduction of enzyme activity, and the Tyr (W48Y) mutant is the only one with measurable activity and can support bacterial growth, albeit poorly, in the presence of trimethoprim which inhibits the host enzyme. The ability to support bacterial growth is greatly improved by another mutation at N188Y, demonstrating the possibility of compensating mechanisms. Further investigation showed that in this case the compensating mutation is linked with an increased expression of the catalytically active mutant enzyme, which still has a specific activity similar to the single W48Y mutant.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other DNA-modifying enzymes were obtained from New England Biolabs and Promega Corporation. The recombination pUC18-based plasmids harboring the synthetic gene for wild-type pfDHFR (Sirawaraporn et al., 1993) was used as a template for the construction of all mutants in this study. *E. coli* DH5α (Life Technologies, Inc.) was used as a host strain for plasmid-mediated transformation and general manipulation of the recombinant constructs. The pET-17b vector (Novagen) was used for the construction of recombinant expression plasmids harboring a mutant PfDHFR synthetic gene, while *E. coli* BL21(DE3)pLysS (Novagen) was used as a host strain for the expression experiments. The plasmids purification kit and gel extraction kit were obtained from Qiagen. Methotrexate (MTX), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and folic acid were purchased from Sigma. MTX-Sepharose CL-6B (Meek et al., 1985) and DHF (Friedkin, M., Crawford, E.J., Misra, D., 1962. Reduction of folate derivatives with dithionite in mercaptoethanol. Fed. Proc. Fed. Am. Soc. Exp. Biol. 21, 176) were prepared as described. Oligonucleotide synthesis and DNA sequencing were performed at the Bioservice Unit, BIOTEC Center, National Science and Technology Development Agency (NSTDA), Thailand.

2.2. Cassette mutagenesis of residue 48 of PfDHFR

Saturated mutation of PfDHFR at residue 48 was constructed by cassette mutagenesis between *Sac*II and *Xba*I sites of the pUC-PfDHFR (wild-type) (Sirawaraporn et al., 1997) using a 42/48-bp oligonucleotide duplex with degenerate codons (NNG/T) at residue 48. The duplex of 5′-GGT CTG GGC AAC AAA GGG GTC CTG CCG (NNG/T) AAA TGC AAC AGC-3′ and 5′-C TAG GCT GTT GCA TTT (A/CNN) CGG CAG GAC CCC TTT GTT GCC CAG ACC GC-3′ contained *Sac*II compatible overhang (underlined) but without *Xba*I overhang. The ligation products were back cut with *Xba*I to remove the wild-type template and transformed into *E. coli* DH5α. The recombinant clones grown on Luria Bertani (LB) agar containing 100 μg ml^{-1} ampicillin were randomly selected for sequencing. In some cases, specific codons were used in place of NNK to obtain the missing mutants. The identified mutants were digested with *Nde*I and *Hind*III to excise the 0.7 kb fragment of DHFR. The resulting fragments were gel purified and then ligated into *Nde*I/*Hind*III sites of pET-17b expression vector. The recombinant pET-PfDHFR mutants were transformed into *E. coli* BL21(DE3) pLysS and plated on to LB agar supplemented with 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol.

2.3. Expression of mutant PfDHFRs

Fresh overnight culture from a single colony of the transformants was used to inoculate 0.5–1 L of LB agar supplemented with 100 μg ml^{-1} ampicillin. The cultures were grown at 37 °C until OD_{600} reached 0.5–0.6 at which time isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.4 mM. After growing for an additional 24 h at 20 °C, the cultures were harvested by centrifugation at 4 °C, 7000*g* for 10 min. The cell pellets were resuspended with lysis buffer (20 mM potassium phosphate buffer, pH 7, 10 mM dithiothreitol, 50 mM KCl, 0.1 mM EDTA, 20% Glycerol) and ruptured by French Press with the pressure of 18,000 psi, three times. The lysed cell was centrifuged and the supernatant (soluble crude) was collected for enzyme assay and protein determination. The DHFR mutants with active and/or detectable DHFR activities were further purified by affinity chromatography using Methotrexate–Sepharose resin (Sirawaraporn et al., 1993) followed by anion exchanger as described previously (Chitnumsub et al., 2004).

2.4. Enzyme assays and salt effects

The activity of PfDHFR was determined spectrophotometrically at 25 °C according to the method previously described (Sirawaraporn et al., 1993, 1997) using a Hewlett

Packard UV–vis spectrophotometer (HP 8453). The reaction (1 mL) contained 100 μM DHF, 100 μM NADPH in 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.0, 75 mM β-mercaptoethanol, 1 mg ml^{-1} BSA, and appropriate amount of purified enzyme (0.001–0.005 U) to initiate the reaction. Where specified, enzyme activity was determined in the presence of NaCl or KCl at final concentration of the salts ranging from 50 to 1500 mM, or urea at 150–6000 mM. To study the effect of pH, a series of assay buffer was prepared to have a pH range of 4–9 and used in place of the TES buffer (Warren et al., 1991; Sirawaraporn et al., 1993).

2.5. Inhibition by antifolates

The inhibition of the enzymes with pyrimethamine and cycloguanil was investigated in the above mixture and in the presence of antifolate. For the determination of kinetic parameters, the kinetic reactions at 340 nm were followed. The K_i values of the inhibitors for the wild-type and mutant enzymes were determined using the non-linear least square fit of the data to the Michaelis–Menten equation, assuming the inhibitors bind competitively to the enzyme active site.

2.6. Random mutagenesis of PfDHFR gene using error-prone PCR approach

The pET-PfDHFR mutants were mixed equally and subjected to error-prone PCR (Giver et al., 1998; Chusacultanachai et al., 2002). The reaction mixture contained 0.1 ng of the mutant genes, 40 pmol of the sense primer (5′-GAAG GAGATATACATATGATGGAACAG-3′), 40 pmol of the antisense primer (5′-GATCCGAGCTCGGTACCAAGCTTG-3′), 1 mM 2′-deoxycytidine 5′-triphosphate (dCTP), 1 mM 2′-deoxythymidine 5′-triphosphate (dTTP), 0.2 mM 2′-deoxyadenosine 5′-triphosphate (dATP), 0.2 mM 2′-deoxyguanosine 5′-triphosphate (dGTP), 1× PCR buffer, and 5 U of *Taq* DNA polymerase. The conditions for the PCR cycle were 40 cycles of 95 °C 1 min denaturation, 68 °C 1 min annealing and 72 °C 1 min extension, with a final extension cycle of 72 °C for 10 min. The amplified PCR product of approximately 700 bp was digested with *Nde*I/*Hind*III at 37 °C for 3 h, gel purified, and ligated into the corresponding sites of pET-17b expression plasmid at 16 °C for 16 h. The ligated product was transformed into *E. coli* DH5α and grew on LB agar plates containing 100 μg ml^{-1} ampicillin. A library of pET-PfDHFR mutants was then extracted from approximately 20,000 colonies of the transformants, and retransformed into *E. coli* BL21(DE3)pLysS.

2.7. Screening of compensating mutations using bacterial complementation assay in the presence of trimethoprim

Escherichia coli BL21(DE3)pLysS was transformed with pET-PfDHFR mutant libraries. The transformed cells were plated on LB agar plates containing 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol. Colonies were pooled and reinoculated into minimal media (MM) containing 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol. The bacterial culture was allowed to grow at 37 °C until OD_{600} reached ~0.6, then aliquots of 100 μl of the 1:10 dilution of the cultured cells were spread on MM agar plates containing 100 μg ml^{-1} ampicillin, 35 μg ml^{-1} chloramphenicol and 4 μM trimethoprim (Chusacultanachai et al., 2002). All of the colonies grown over 1–3 nights at 37 °C were picked up. Plasmids were extracted for DNA sequencing.

3. Results

3.1. Obtaining 19 Try48 PfDHFR mutants

The cassette mutagenesis method was employed to derive all possible mutant enzymes of DHFR at residue 48 where the *Sac*II/*Xba*I fragment of pUC-PfDHFR was replaced by the mixed oligonucleotide cassette carrying the degenerate codon coding for all of the possible 19 common amino acids. Fifteen mutants of PfDHFR at residue 48 were obtained from 94 clones selected randomly. The replacement of Trp48 by His, Phe, Tyr and Cys was achieved by the same method using the specific codons, CAT, TTT, TAT and TGT, respectively. The sequences of the mutant genes were checked to ensure correct assignment of the mutants.

3.2. Low to no catalytic activities of all Try48 mutants, except W48Y

The mutant DHFRs were expressed as soluble enzymes following IPTG induction at 20 °C, at approximately the same levels as the wild-type enzyme (data not shown). The specific activities of these DHFRs in the crude extracts, which should reflect relative activities of the pure enzymes, are given in Table 1. All of the mutant enzymes have very low or no activity. The most active mutant, Tyr48 (W48Y), has only a small fraction of the activity of the wild-type (Trp48) enzyme, with k_{cat}/K_m of only 1% of the wild-type enzyme.

The catalytic activity of these mutant enzymes was further investigated through its ability to complement host DHFR in the presence of trimethoprim (Chusacultanachai et al., 2002). As shown in Fig. 1, in the presence of 4 μM trimethoprim which completely inhibits the host DHFR, only the wild-type and to a lesser extent W48Y PfDHFR can support bacterial growth, reflecting the fact that the other mutant enzymes have very low activity.

3.3. Poor kinetic properties and binding affinity to antifolate inhibitors of W48Y PfDHFR

In order to characterise the W48Y PfDHFR further, the enzyme was purified and its kinetic properties and characteristics of inhibition pyrimethamine (Pyr), cycloguanil (Cyc) and WR99210 were determined (Table 1). The

Table 1
Activities, kinetic properties and inhibitor-binding affinity of *Plasmodium falciparum* dihydrofolate reductases (PfDHFR) in wild-type and mutants of residue 48

Enzyme	Wild-type (W48)	W48Y	W48Y+N188Y
Specific activity (μmol/min/mg)			
Crude protein extract	0.82 (100%)	0.025 (3.0%)[a]	0.24 (28.9%)
Purified protein	151	6.3	6.5
Enzyme yield			
mg/l	6.25 (100%)	0.39 (6.2%)	2.02 (31.7%)
U/l	949	2.44	13.1
Kinetic parameters			
K_m (μM)			
DHF	5.1 ± 0.9[b]	23.1 ± 5.8	32.4 ± 0.52
NADPH	2.7 ± 0.8	4.5 ± 0.7	3.45 ± 0.33
k_{cat} (s^{-1})	60.9	2.8	3.3
k_{cat}/K_m (M^{-1} s^{-1})[c]	11.9	0.12	0.10
K_i (nM)			
Pyr	0.72 ± 0.08	1.74 ± 0.08	1.01 ± 0.16
Cyc	1.03 ± 0.09	5.48 ± 0.49	1.99 ± 0.13
WR99210	0.44 ± 0.05	1.54 ± 0.22	1.21 ± 0.08

[a] Eighteen other single mutant DHFRs showed the same expressed product on SDS–PAGE, but their DHFR activities were less than 2% of the wild-type enzyme, i.e., Val 1.4%, Lys 0.8%, Thr 0.8%, Gly 0.8%, etc.
[b] Mean and SEM were calculated from three determinations.
[c] Calculated from the K_m values for DHF.

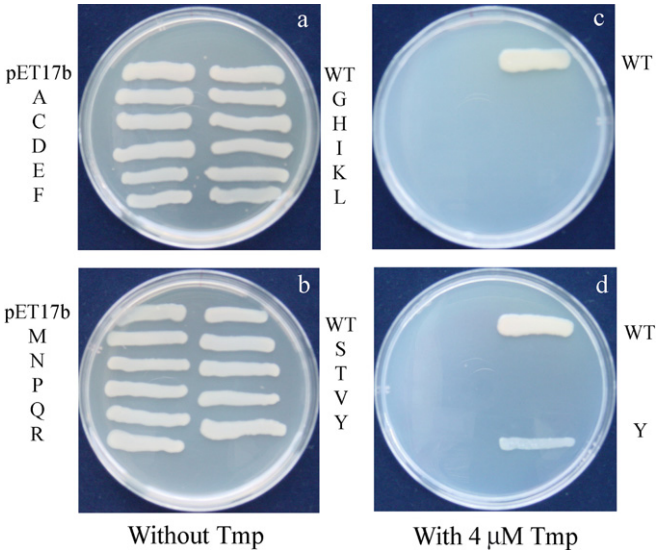

Fig. 1. Complementation effect of *Plasmodium falciparum* dihydrofolate reductases (PfDHFR) on bacterial growth transformed with the PfDHFR gene with all possible amino acids at residue 48. (a and b) Control with no trimethoprim (Tmp); (c and d) in the presence of 4 μM trimethoprim.

W48Y mutation showed 4.5- and 2-fold increases in K_m for DHF and NADPH, respectively; and a decrease in k_{cat} of 20–50-fold based on the 104-fold decrease in the catalytic efficiency (k_{cat}/K_m) of the enzyme compared with the wild-type PfDHFR. The inhibition constants of Pyr, Cyc and WR99210 for this mutant enzyme were also affected with K_{si}-values of 1.7, 5.5 and 1.5 nM, respectively, a 2.5–5.3-fold increase over the values for the wild-type enzyme. These indicate that the mutation of Trp48 to Tyr48 interferes with the binding of substrate and inhibitors to the enzyme.

3.4. Reduced stable of W48Y PfDHFR at neutral pH or high concentrations of urea or salts conditions

The effects of pH, salt, and urea on catalytic activity of the W48Y mutant were determined in comparison with the wild-type enzyme. As shown in Fig. 2a, the optimum pH of W48Y mutant was shifted towards acidic values, with activity of more than 60% at pH 4.0–6.5 while wild-type enzyme retains its activity above 60% at pH 4.0–7.5. In the presence of urea, the W48Y mutant lost its catalytic activity by more than 20% at concentrations of urea as low as 1 M, while the wild-type enzyme retains its catalytic activity above 80% at concentrations of urea up to 4 M (Fig. 2b).

The effects of KCl and NaCl salt on catalytic activity of the W48Y PfDHFR are shown in Fig. 2c and d. Both KCl and NaCl salts have drastic effects in decreasing catalytic activity of the mutant PfDHFR. The catalytic activity of the mutant enzyme declined rapidly with increasing salt concentration, while the wild-type enzyme was activated at low concentration of the salts, and its activity remained significant even at concentrations approaching 1 M.

3.5. Compensating mutation with N188Y increased expression of catalytically active W48Y+N188Y PfDHFR enzyme

In order to find out whether the activity of the W48X mutants can be improved by additional mutation, a library

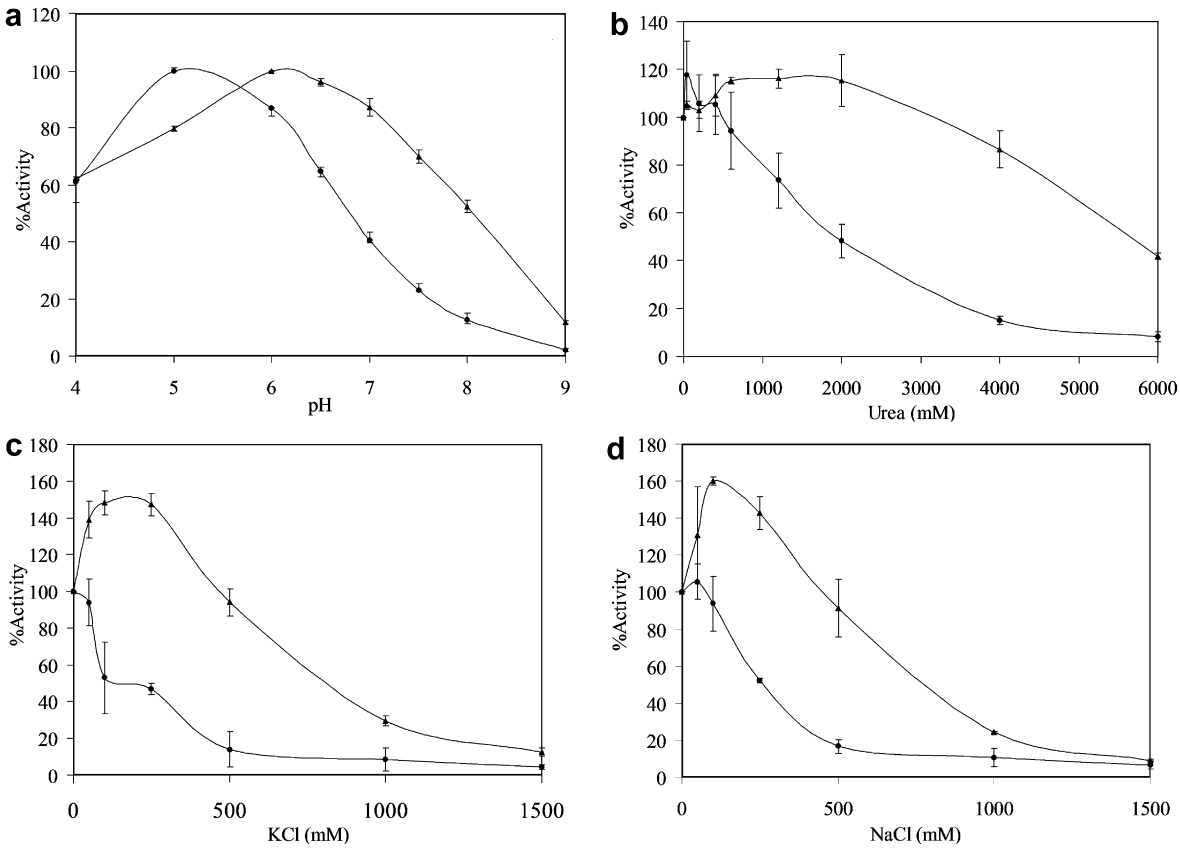

Fig. 2. Low structural stability of *Plasmodium falciparum* dihydrofolate reductases (PfDHFR) Tyr48 mutant. Effect of pH (a), Urea (b), KCl (c) and NaCl (d) on catalytic activity of Tyr48 PfDHFR (●) in comparison with the wild-type Trp48 enzyme (▲).

of mutants was randomly created by error-prone PCR and expressed under the T7 promoter of the pET-17b expression vector in *E. coli* BL21(DE3)pLysS. The transformants were plated on a LB plate with and without 4 μM trimethoprim. Over 20 colonies on both plates were randomly picked up for sequencing. We found that with about 0.1% mutation rate, only W48Y and W48Y+N188Y mutants were recovered from the trimethoprim plate. We also found that the W48Y+N188Y mutant supported bacterial growth to a greater extent than W48Y. Following a 24 h IPTG induction of 0.6 OD cultures at 20 °C, the crude activity of W48Y+N188Y was found to be 9.5-fold higher than that of the W48Y mutant while SDS–PAGE showed a similar expression level (data not shown). Following purification, the yield of W48Y+N188Y (2.02 mg/l culture) was about five times higher than the W48Y mutant (0.39 mg/l culture), compared with wild-type with a 6.25 mg/l yield. However, the specific activities of both mutants were not different (6.5 versus 6.3 μmol/min/mg). The kinetic properties of the two mutants are similar; only slightly higher K_m for DHF but lower K_m for NADPH of the double mutant was observed in comparison with those of the W48Y mutant (Table 1). The turnover number, k_{cat}, of W48Y+N188Y and W48Y were also similar. In the presence of 1 M urea, the catalytic activity of the wild-type enzyme (99.9 ± 10.1%) was not affected while the catalytic activity of the double (75.0 ± 6.2%) and single (66.6 ± 9.6%) mutants was affected by about 25% and 35%, respectively. These indicate that the additional mutation at N188Y does not affect the kinetic properties of the enzyme, but affects the stability of the mutant and thus the proportion of the catalytically active enzyme in the total expression.

4. Discussion

We have shown that Trp48 of pfDHFR is an essential residue both for catalysis and binding of inhibitors currently used as antimalarial drugs. Only Tyr, among 19 other amino acids in this position, can replace Trp with retention of residual enzyme activity, sufficient to be measured kinetically and to give viability to *E. coli* transformed with the mutant gene, when the host enzyme is suppressed. Although some other mutants, such as Val, Lys, Thr and Gly, showed enzyme activity of 0.8–1.4% of the wild-type enzyme, these were not at a sufficient level to complement *E. coli*. These results are consistent with our earlier observation that an enzyme activity of more than 1.8% of the wild-type enzyme is needed to complement the *E. coli* in our system (Chusacultanachai et al., 2002).

Kinetics studies of the W48Y mutant enzyme reveal that mutation from Trp48 to Tyr48 affects both DHF and

NADPH binding and, consequently, a drastic decrease in the catalytic efficiency (k_{cat}/K_m) of the mutant enzyme. The affinities for inhibitors (Pyr, Cyc and WR99210) of the Tyr mutant enzyme are also far less than those for the wild-type enzyme (Table 1). Trp48 is therefore important for both catalysis and inhibitor binding.

Evidence from three-dimensional structures of DHFR enzymes in bacteria, vertebrate and parasite, such as *E. coli*, human and *P. falciparum* clearly shows that the equivalent Trp residue makes hydrophobic and van der Waals interactions with the bound NADPH, and the side chain indole interacts with the C4 oxygen of bound DHF through a bridge water molecule (Beard et al., 1991; Warren et al., 1991; Yuvaniyama et al., 2003). The conserved Trp24 of human DHFR has been mutated to phenylalanine (Huang et al., 1989; Beard et al., 1991) with consequent decrease in structural stability and enzyme efficiency (K_m increase for both DHF and NADPH, and k_{cat} decrease). The equivalent Trp22 in *E. coli* has also been mutated to phenylalanine and histidine with a decrease in enzyme activities (Warren et al., 1991). It was concluded that the role of Trp22 may be to position Met20 with respect to N5 of the DHF. However, it was reported that Trp22 can be replaced by Phe without substantial loss in activity and by Leu with 13% of the original activity (Ohmae et al., 2001).

The structure of pfDHFR-TS (Yuvaniyama et al., 2003) shows that Trp48, being near the NADPH binding site as in the other DHFRs, is involved in hydrogen bonding with a water molecule, which in turn hydrogen-bonds to D54 and C4 oxygen of the substrate. In the W48Y mutant, the water molecule is probably replaced by the hydroxyl group of the Tyr side chain, resulting in less binding affinity with the substrate. Trp48 also forms a part of the hydrophobic cluster, interacting with Leu46, Pro47, Ile187, Tyr191 and Thr219, and stabilising the compact structure. It is also in van der Waals contact with both substrates and inhibitors. From this structural arrangement, Trp48 has dual central roles, firstly as an anchor for catalysis and second as a mediator to lock up the Cys50-Leu46 loop for structural stability. Consequently, the substitution of Trp by 19 other amino acids must have resulted in abolition or drastic decrease of these interactions. Nonetheless, Tyr with a hydroxyphenyl side chain may still retain some interactions, sufficient to result in some residual enzyme activity, while substitution with other amino acids resulted in loss of activity.

Trp24 of human DHFR (Huang et al., 1989; Beard et al., 1991) and Trp22 in bacterial DHFR (Warren et al., 1991; Ohmae et al., 2001), equivalent to PfDHFR Trp48, have both been postulated to confer stability to the enzyme structure. The PfDHFR Tyr48 mutant was found to have lower structural stability than the wild-type enzyme with regard to denaturation by urea and salts (Fig. 2b, c and d), indicating that the mutant has lower structural stability than the wild-type enzyme, similar to human and bacterial counterparts.

An additional mutation of N188Y (W48Y+N188Y) was found to have little impact on the kinetic properties and inhibitor-binding affinities of the enzyme compared with the W48Y single mutant. These suggest that the active sites of these mutant enzymes are quite similar. However, the double mutant (W48Y+N188Y) was found to promote better growth of *E. coli* than the single mutant (W48Y). While the level of expression of the double mutant enzyme was similar to the single mutant, the enzyme activity of the former in the crude extract was higher, as was also the yield of the purified enzyme, indicating a higher proportion of the active enzyme in the expressed protein, with higher stability, as supported by the improved stability of the double mutant in the presence of 1 M urea, possibly combined with higher solubility. Since N188Y is located on the surface of the molecule, the Tyr residue might improve expression of the active enzyme through improving folding/solubility/stability of the enzyme W48Y. As Fig. 2 shows, the structural stability of the W48Y mutant has been lowered, probably through disturbance of the hydrophobic cluster of which Trp48 forms a part, and destruction of a hydrogen bonding network though molecular water in the active site. Examples of PfDHFR enzymes with improved solubility by mutating surface residues have been reported recently by our group (Japrung et al., 2005).

It is clear from our results that Trp48 is an important residue for catalytic activity, as well as for binding of inhibitors like Pyr, Cyc and WR99210. It is worthnoting, however, that Trp is not an absolutely essential residue, since Tyr can substitute for Trp, albeit poorly. The fact that an additional mutation N188Y of W48Y yielded a higher amount of active enzyme and thus supported bacterial growth better than that with only the W48Y mutation significantly implies that the function of Trp48 can be replaced by other residues if the overall activity of the enzyme can be maintained by additional, compensating mutations.

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