Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria

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We have exploited the recently developed ability to transfect the malaria parasite *Plasmodium falciparum* to investigate the role of polymorphisms in the enzyme dihydropteroate synthase (DHPS), identified in sulfadoxine-resistant field isolates. By using a truncated form of the *dhps* gene, specific mutations were introduced into the endogenous gene by allelic replacement such that they were under the control of the endogenous promoter. Using this approach a series of mutant *dhps* alleles that mirror *P.falciparum* variants found in field isolates were found to confer different levels of sulfadoxine resistance. This analysis shows that alteration of Ala437 to Gly (A437G) confers on the parasite a 5-fold increase in sulfadoxine resistance and addition of further mutations increases the level of resistance to 24-fold above that seen for the transfectant expressing the wild-type *dhps* allele. This indicates that resistance to high levels of sulfadoxine in *P.falciparum* has arisen by an accumulation of mutations and that Gly437 is a key residue, consistent with its occurrence in most *dhps* alleles from resistant isolates. These studies provide proof that the mechanism of resistance to sulfadoxine in *P.falciparum* involves mutations in the *dhps* gene and determines the relative contribution of these mutations to this phenotype.

Keywords: drug resistance/Fansidar/malaria/sulfadoxine/transfection

Introduction

Malaria parasites are the cause of approximately two million deaths annually, and the most severe form of the disease arises from infection with *Plasmodium falciparum*. The sulfone/sulfonamide (sulfa) group of compounds are potent inhibitors of *P.falciparum* and are used for the control of malaria. However, the widespread appearance of resistant parasites, particularly in southeast Asia, has reduced their utility (Landgraf *et al.*, 1994). Importantly, this group of drugs is still effective in Africa where resistance is less prevalent. Sulfadoxine is the most commonly used sulfa drug and is usually used in a combination with pyrimethamine known as Fansidar, as the two drugs show a marked synergism in their anti-malarial action (Chulay *et al.*, 1984).

Sulfadoxine acts as a competitive inhibitor of the enzyme dihydropteroate synthase (DHPS) (Triglia *et al.*, 1997) which is in the folate biosynthetic pathway and couples *p*-aminobenzoic acid (pABA) with 6-hydroxymethyl-dihydropterin pyrophosphate to yield 7,8-dihydropteroate (Brown, 1971). In *P.falciparum*, DHPS is a bifunctional enzyme (PPPK-DHPS) with 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK), the enzyme preceding DHPS in the folate biosynthetic pathway (Brooks *et al.*, 1994; Triglia and Cowman, 1994). The *P.falciparum dhps* gene from sulfadoxine-resistant and -sensitive parasites has been cloned and differences between the amino acid sequences encoded by them have been identified, suggesting that they may be involved in the mechanism of resistance to this drug (Brooks *et al.*, 1994; Triglia and Cowman, 1994; Wang *et al.*, 1995, 1997a; Triglia *et al.*, 1997).

Expression of variant forms of *P.falciparum* PPPK-DHPS in *Escherichia coli* and subsequent kinetic analysis of the purified enzymes has shown that the mutations decrease the affinity of binding to sulfadoxine, which is consistent with their importance in the mechanism of resistance to this drug (Triglia *et al.*, 1997). Additional evidence for the role of DHPS in sulfadoxine resistance has been obtained by analysis of a genetic cross between sulfadoxine-resistant and -sensitive *P.falciparum* parents in which the mutations in the *dhps* gene were shown to segregate with the drug-resistant phenotype (Wang *et al.*, 1997b). Results from this cross have also suggested that an additional gene that affects folate utilization can affect the level of sulfadoxine resistance.

The recent development of transfection in different *Plasmodium* species, including *P.falciparum* (van Dijk *et al.*, 1995; Wu *et al.*, 1995, 1996; Crabb and Cowman, 1996), has now made it possible to determine directly the role of allelic variants in phenotypes such as sulfadoxine resistance. Integration of plasmid constructs into the *P.falciparum* genome appears always to occur by single recombination events as no double recombination events have yet been described (Wu *et al.*, 1996; Crabb *et al.*, 1997a,b).

The inability to select double recombination events in this parasite makes it difficult to engineer gene replacements to determine the specific contribution of mutations in a gene to phenotypes such as drug resistance. In this study we have therefore made constructs containing truncated mutant *dhps* alleles which, when integrated into the *P.falciparum* genome, result in allelic exchange and expression of a functional mutant *dhps* gene under the control of its endogenous promoter. Exploiting a recently developed, highly sensitive assay that allows good resolution of sulfadoxine susceptibilities (Wang *et al.*, 1997c), we show that six different *pppk-dhps* mutant alleles transfected into sulfadoxine-sensitive para-

sites confer different levels of sulfadoxine resistance. This demonstrates that mutations in the *dhps* gene are responsible for sulfadoxine resistance in *P.falciparum*.

Results

Transfection of a full-length *pppk-dhps* gene results in pseudo-polyploidy

In order to test if mutations identified in the *dhps* gene of *P.falciparum* are responsible for sulfadoxine resistance we initially made two constructs in the transfection plasmid pHC1 (Crabb *et al.*, 1997b), both of which contain the full-length *pppk-dhps* gene (Brooks *et al.*, 1994; Triglia and Cowman, 1994). The first encoded the wild-type *pppk-dhps* gene as a control, while the second contained the *pppk-dhps* allele from W2mef, a highly sulfadoxine-resistant isolate possessing three amino acid differences in the *dhps* domain compared with the wild-type gene (Triglia *et al.*, 1997). The constructs were transfected into the sulfadoxine-sensitive cloned line D10 and transfectants selected for integration. Southern hybridization analysis of different cloned transfectants showed that the plasmid had integrated between one and four copies by single recombination events producing parasites with multiple copies of the *pppk-dhps* gene under control of either the endogenous promoter or the introduced calmodulin promoter (data not shown). Analysis of the transfectants showed that those carrying the W2mef allele were resistant to considerably higher levels of sulfadoxine than those in which the wild-type gene had been introduced. However, the presence of different numbers of integrated and expressed copies of the *pppk-dhps* gene made it difficult to quantitate the contribution of the mutations to the drug resistance phenotype and to extend this type of analysis to the role of individual mutations.

Homologous recombination of a truncated *pppk-dhps* gene leads to allelic exchange

We thus sought to ensure that integration of the *pppk-dhps* transfection plasmids by a single recombination event would result in just a single active gene that would be driven from the endogenous promoter. To this end, we used a *pppk-dhps* gene from which the 5'-most 100 bp had been removed, corresponding to the codons for the initiation methionine and the subsequent 33 amino acids (Figure 1). Integration of this construct into the endogenous *pppk-dhps* gene by homologous recombination should insert the mutations in *dhps* that are 3' to the recombination event by allelic exchange. Importantly, multiple plasmid insertion events would not create additional full-length *pppk-dhps* genes as they would be truncated and unlikely to express functional enzyme.

Using this approach, truncated *pppk-dhps* genes from a sulfadoxine-sensitive isolate and five different resistant forms were inserted into the plasmid vector pHC1 (Crabb *et al.*, 1997b) such that they would integrate into the endogenous *pppk-dhps* gene as described above (Figure 1). Transfectants were selected on pyrimethamine and grown for 3-week periods without drug pressure to select for parasites that contain integrated copies of each plasmid. All transfectants were cloned and one clone of each, called D10-sakaa, D10-sgkaa, D10-sgkga, D10-agkaa, D10-ageaa and D10-fgkas (Table I), was analysed further.

Integration of truncated *dhps* plasmids occurs at the *pppk-dhps* gene on chromosome 8

To determine if integration of the transfection plasmids had occurred, chromosomes from each clone were separated by pulsed-field gel electrophoresis (PFGE) (Chu *et al.*, 1986), transferred to nylon filters and hybridized with both the *dhps* and *Toxoplasma gondii dhfr* genes (Figure 2). The ethidium bromide stained chromosome pattern was identical for D10 and the six transfected clones, indicating that all were derived from the parental, pyrimethamine-sensitive parasite line. As expected, the *P.falciparum dhps* probe hybridized to chromosome 8 in the parental D10 line (Brooks *et al.*, 1994; Triglia and Cowman, 1994) and in the transfected clones. The *T.gondii dhfr* probe did not hybridize to D10 but gave an otherwise identical hybridization pattern showing, in the six different transfected *P.falciparum* lines, that integration had occurred on chromosome 8 in each case.

To prove that integration of the plasmid on chromosome 8 had occurred at the *dhps* locus, Southern blot analysis was used to probe genomic DNA of both untransfected D10 and the six transfectants. The *dhps* probe was hybridized to *EcoRI*-*Clal*-digested DNA (Figure 3A). In D10 a band of 3.6 kb was observed, whereas the transfected clones gave bands of 2.9, 4.2 and 4.8 kb, indicating that the transfected plasmid construct had indeed integrated into the *pppk-dhps* gene. It was evident from band intensities in the D10-sgkaa and D10-fgkas samples that the 4.2 kb *EcoRI*-*Clal* fragment was present at a higher copy number with respect to the other transfected clones. The copy number of this band in the six transfectants was determined using phosphorimage analysis and found to be in a 3:1 ratio with respect to the 2.9 kb band in both D10-sgkaa and D10-fgkas, but in a 1:1 ratio in the other clones. This indicated that two copies of the transfection plasmid had integrated into D10-sakaa, D10-agkaa, D10-sgkga and D10-ageaa, whereas D10-sgkaa and D10-fgkas had each acquired four copies (Figure 3C).

Homologous recombination events occurred 5' to the *dhps* mutations

Although integration of the plasmid constructs had occurred in the endogenous *pppk-dhps* gene, it was possible that this had taken place 3' to the mutations in *dhps*, which would result in the absence of these mutations in the expressed allele. It was thus important to verify that the recombination event had occurred 5' of the mutations in *dhps* to give allelic exchange. The presence of an *AvaII* site, associated with the Ala-Gly substitution at amino acid 437, was used to determine if the D10-sgkaa, D10-sgkga, D10-agkaa, D10-ageaa and D10-fgkas cloned lines had undergone integration events 5' of this position. *AvaII*-digested genomic DNA of both untransfected D10 and the six transfected clones was hybridized with the *dhps* probe (Figure 3B). If recombination had occurred 5' to the *AvaII* site in the mutant *dhps* genes, a hybridizing DNA band of 3.1 kb would be expected, and this was observed for D10-sgkaa, D10-sgkga, D10-agkaa, D10-ageaa and D10-fgkas. As the *pppk-dhps* gene transfected into D10-sakaa did not have the Gly437 codon mutation there was no *AvaII* site and bands of 8.0, 6.7 and 16 kb were obtained. These results confirmed that in all cases the recombination events had occurred 5' of the

Fig. 1. Schematic diagram of a single-site crossover at the endogenous *pppk-dhps* gene with a transfection plasmid construct containing truncated *pppk-dhps*. The filled triangles represent introns. The arrows within the *pppk-dhps* gene represent positions where mutations have been introduced into the various allelic exchange constructs. Mutations have been found in codons 436, 437, 540, 581 and 613 (see Table I). The arrows labelled #1–#7 correspond to the position of the oligonucleotides used in this study. The diagram of the genomic DNA following integration assumes that crossing-over occurred upstream of the first mutation (position 436).

Table I. Sulfoxadine resistance levels in D10 parasites transfected with various *dhps* alleles

Parasite clone	<i>dhps</i> alleles transfected	Amino acid ^a					$K_i(\mu\text{M})^b$ <i>E.coli</i> -expressed	IC ₅₀ (ng/ml)	Fold-increase ^c
		436	437	540	581	613			
D10	None	–	–	–	–	–		32 ± 8	–
D10-sakaa ^d	D10	S	A	K	A	A	0.14 ± 0.01	129 ± 63	–
D10-sgkaa	3D7	S	G	K	A	A	1.39 ± 0.23	614 ± 229	4.8
D10-sgkga	K1	S	G	K	G	A	16.2 ± 2.7	679 ± 142	5.3
D10-agkaa	Tak9/96	A	G	K	A	A	19.9 ± 4.0	773 ± 103	6.0
D10-ageaa	PR145	A	G	E	A	A	98.3 ± 7.5	1258 ± 379	9.8
D10-fgkas	W2mef	F	G	K	A	S	112 ± 10	3087 ± 336	24

^aEncoded by the transfected *dhps* allele.

^bValues taken from Triglia *et al.* (1997).

^cIC₅₀ relative to D10-sakaa.

^dRepresents the genotype of the transfected *dhps* allele as shown in column three.

437 codon and that the endogenous allele had been replaced by the mutant *dhps* alleles.

The integrated *dhps* alleles are transcribed

To determine whether the integrated *dhps* alleles were transcribed, DNase-treated poly(A)⁺ RNA was made from each clone and cDNA synthesized for reverse transcriptase–polymerase chain reaction (RT–PCR), prior to amplification with *dhps* (primer 1; Figure 1) and *hsp86* 3' (primer 2; Figure 1) oligonucleotides. The expected product of 1323 bp was obtained for the six transfectants. When the PCR products were digested with *Ava*II, D10-sgkaa, D10-sgkga, D10-agkaa, D10-ageaa and D10-fgkas gave fragments of 959 and 364 bp, indicating that a *dhps* gene encoding the Gly437 mutation is transcribed in these transfected lines (Figure 4). The D10-sakaa RT–PCR product was resistant to digestion with *Ava*II as expected,

as the *pppk-dhps* allele transfected was the wild-type. Similar RT–PCR experiments were performed as controls using two oligonucleotides that were both from the *dhps* gene, to test if the disrupted endogenous *dhps* gene was still transcribed. Residual transcription from this gene was at an almost undetectable level (<1% of total) in all of the *P.falciparum* lines transfected with a *dhps* gene encoding Gly437 (data not shown). In addition, this gene is truncated and unlikely to express a functional enzyme. Sequencing of RT–PCR products amplified with oligonucleotides #1 and #2 (Figure 1) confirmed that the genotype of the exchanged *dhps* allele in each clone was as expected for each transfectant (Table I). The cDNA synthesized from RNA isolated from each of the different lines was tested for the absence of DNA contamination by using oligonucleotides across the intron of the *pabAB* gene (T.Triglia and A.F.Cowman, in preparation). None of the RNA

samples used for further analysis showed any such contamination. Therefore allelic exchange has replaced the endogenous *pppk-dhps* and the new alleles with the desired mutations are all transcribed.

Fig. 2. Integration of truncated *dhps* alleles occurs at chromosome 8. Chromosomes of D10 and cloned parasites following transfection of D10 with the six *dhps* alleles were separated by PFGE and stained with ethidium bromide. Chromosomal DNA was then blotted to a nylon membrane and first probed with a 391 bp PCR-amplified *dhps* fragment then, after removal of the signal, reprobed with a 210 bp PCR-amplified *T.gondii dhfr-ts* probe.

The new *pppk-dhps* alleles are expressed

To determine whether the integrated *pppk-dhps* alleles in the different transfectants were expressed, proteins from synchronized trophozoites of each parasite line were separated by SDS-PAGE and transferred to nitrocellulose. Antibodies to *dhps* detected a protein band of 83 kDa in the D10 parent and the six transfectants (Figure 5), a size which corresponds to the predicted molecular weight of the 706 amino acid bifunctional enzyme (Brooks *et al.*, 1994; Triglia and Cowman, 1994). There was no evidence for translation of truncated proteins that may have initiated from internal methionine codons, although we did observe consistent reactivity with an unidentified 92 kDa band in all the transfected clones. Probing of an identical filter using anti-hsp70 antibodies (Bianco *et al.*, 1986) showed that there was an approximately equal amount of protein loaded in each lane, suggesting that the level of expression of the full-length PPPK-DHPS enzyme in the transfected parasite lines is similar to that in the untransfected D10 parent.

The mutant *dhps* alleles confer sulfadoxine resistance

To determine whether the different mutant alleles of *pppk-dhps* render D10 parasites resistant to sulfadoxine, parasites were firstly adapted to growth in a low folate/pABA medium to permit accurate measurement of the

Fig. 3. Restriction analysis of genomic DNA showing allelic replacement of the endogenous D10 *dhps* gene. DNA from D10 and the six integrated clones following allelic replacement were digested with *EcoRI*-*ClaI* (A) and *AvaII* (B) and the fragments separated electrophoretically before blotting to nylon membranes. Both blots were probed with the 391 bp *dhps* fragment represented by the solid bar in (C). (C) The structure of the integration events into the D10 *dhps* gene is shown, together with the position of the restriction enzyme sites *EcoRI* (R)-*ClaI* (C) and *AvaII* (A) that were used for the Southern hybridization experiments shown in (A) and (B). The scale is shown at the bottom of the panel. $\pm$ Av indicates the *AvaII* site which is generated when D10 is transfected with all constructs encoding G437 (see Table I).

Fig. 4. *Ava*II digestion of RT-PCR products derived from poly(A)⁺ RNA from the parasite clones containing the six integrated mutant *dhps* alleles. The left panel shows RT-PCR products from each clone digested with *Ava*II. The oligonucleotides used for amplification were located 364 bp 5' of the *Ava*II site in *dhps* [5'-TCCATTCCTCAT-GTGTATACAAC-3' (#7)] and within the hsp86 3' sequence (#2; see Materials and methods). The right panel is included as a control and shows that cDNA derived from the D10-ageaa integrated clone is free of contaminating genomic DNA (all samples were tested but only D10-ageaa is shown). D10 genomic DNA and D10-ageaa cDNA were amplified with oligonucleotides derived from a different gene involved in folate biosynthesis (*pabAB*). The oligonucleotides amplify a 545 bp fragment from genomic DNA and a fragment of 259 bp from cDNA because of the presence of two introns. The oligonucleotides used were 5'-AATTGAAATAGTCCTAGGG-3' and 5'-AAAATATATATT-CCACGTCAGTGT-3'.

inhibitory concentration of sulfadoxine that kills 50% of the parasites (IC₅₀) (Wang *et al.*, 1997b,c). The D10 parental line had an IC₅₀ of 32 ± 8 ng/ml whereas the D10-sakaa transgenic (transfected with the wild-type allele of *pppk-dhps*) was consistently more resistant to the drug (129 ± 63 ng/ml) (Table I). Both of these parasite lines express the wild-type form of the PPPK-DHPS enzyme and the increase in the level of resistance may reflect a small increase in the level of expression of the protein, or the addition of the modified *T.gondii dhfr* gene resistant to pyrimethamine may somehow affect the level of sulfadoxine resistance.

Importantly, a single mutation in the *dhps* gene converting Ala437 to Gly437 (A437G) increased the level of resistance of the parasite 5-fold to 614 ± 229 ng/ml compared with that seen for D10-sakaa (Table I). Addition of the further mutation A581G in D10-sgkga or S436A in D10-agkaa further increased the level of resistance to the drug, but by a smaller margin, suggesting that these amino acid changes have a lesser effect on the ability of sulfadoxine to inhibit the enzyme. The mutation at residue 540 (K540E) increased the level of resistance ~2-fold for the transfectant D10-ageaa compared with D10-sgkaa but was 10-fold more resistant to the drug compared with the D10 allele (D10-sakaa). The highest level of resistance was obtained with the W2mef allele of *pppk-dhps* (D10-fgkas), which originates from a highly sulfadoxine-resistant line of *P.falciparum*. The insertion of the three amino acids corresponding to this allele

Fig. 5. The PPPK-dhps enzyme is expressed in clones containing the integrated mutant *dhps* alleles. Proteins from synchronized trophozoites were separated by SDS-PAGE and blotted to nitrocellulose. The upper filter was probed with anti-dhps antibodies and the lower filter with antibodies to the *P.falciparum* hsp70 protein.

yielded a sulfadoxine-resistance phenotype that was 24-fold higher compared with that obtained with the wild-type allele (D10-sakaa). The pattern of increasing resistance to sulfadoxine as the number of mutations rises suggests that resistance to this drug arose by an accumulation of mutations due to increasing levels of drug pressure. Importantly, these results prove conclusively that mutations in the *dhps* gene of *P.falciparum* confer resistance to this drug on the parasite.

Discussion

The recently developed ability to transfect *P.falciparum* (Crabb and Cowman, 1996; Wu *et al.*, 1996) provides a powerful means to investigate the role of *dhps* mutations in the mechanism of resistance to sulfadoxine. We have devised a novel allelic replacement strategy to introduce mutant *pppk-dhps* genes at the endogenous locus, retaining transcriptional control from the autologous promoter. Although the 3' non-coding region of the *pppk-dhps* gene is replaced with the 3' end of hsp86 in these constructs (Figure 3C), this appears not to affect RNA or protein expression levels (Figures 4 and 5). This approach permits us for the first time to analyse specific alterations in the DHPS molecule against a constant genetic background outside of the *dhps* locus, and also represents the first systematic study of a phenotype in *Plasmodium* using transfection. Our analysis of the different transfectants that express mutant *dhps* enzymes proves that these alterations are responsible for the sulfadoxine-resistance phenotype and allows a direct determination of the contribution of the different amino acid substitutions to the final level of drug resistance.

The most common mutation found in the sulfadoxine-resistant isolates so far examined is A437G (Brooks *et al.*, 1994; Triglia and Cowman, 1994; Plowe *et al.*, 1997; Triglia *et al.*, 1997; Wang *et al.*, 1997a) and it has been observed in some samples to be the sole alteration in DHPS. This suggests that it is normally the first mutation selected by sulfadoxine drug pressure, although some exceptions have been seen (Wang *et al.*, 1997a). This is supported by the observations that this single alteration

leads to a 10-fold increase in the K_i of the enzyme for sulfadoxine and can confer on transfected parasites a 5-fold higher level of drug resistance. Thus A437G may be the closest equivalent in DHPS to the S108N alteration in *P.falciparum* DHFR that is almost always present in pyrimethamine-resistant parasites (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Wang *et al.*, 1997a).

An additional DHPS mutation of S436A or A581G caused only a small increase in the level of sulfadoxine resistance, suggesting that these mutations play a lesser role in the phenotype. This is also in agreement with measurements for sulfadoxine using purified PPPK-DHPS derived from the 3D7, K1 and Tak9/96 alleles, where the K_i increased by a relatively small amount in the case of each mutation (Triglia *et al.*, 1997). This is in contrast to the addition of the K540E mutation to those at positions 436 and 437, which increased the level of sulfadoxine resistance a further 2-fold, and ~10-fold with respect to the D10-sakaa (wild-type) transfected allele. The allele that gave the highest level of sulfadoxine resistance after transfection was W2mef, which occurs in the most highly resistant parasites found so far. The addition of S436F plus A613S to the A437G mutation increased sulfadoxine resistance in this transfectant a further 5-fold (24-fold relative to wild-type) to an IC_{50} level of ~3 µg/ml, very similar to that seen previously in isolates that naturally contain this allele (Wang *et al.*, 1997b). The sulfadoxine resistance phenotypes measured in the *in vitro* cultures of the transfectants thus parallel closely the results seen both for the kinetic parameters of the corresponding purified enzymes (Triglia *et al.*, 1997) and for natural isolates in culture (Wang *et al.*, 1997b). We conclude that in these conditions the mutations observed in *P.falciparum dhps* are solely responsible for the sulfadoxine-resistance phenotype.

Interestingly, transfection of the *dhps* allele found in sulfadoxine-sensitive parasites consistently gave a somewhat higher level of resistance (by ~100 ng/ml) when compared in parallel assays with the D10 parent. Analysis by immunoblots using anti-DHPS antibodies indicated that there were approximately equal levels of expression of the PPPK-DHPS enzyme; however, it is possible that a small elevation in protein expression, not easily apparent from the immunoblots, contributed to the increased level of resistance. Any increased expression could not be due to a more active promoter as the endogenous *pppk-dhps* promoter controls expression of the inserted gene. However, the 3' untranslated end of the expressed *pppk-dhps* gene consists of *hsp86* sequence, which may alter the stability of the mRNA and increase protein expression. A second possibility is that co-transfection of a heterologous, mutant *dhfr* gene might have a small but significant effect on the absolute level of sulfadoxine resistance. The unnatural situation where two *dhfr* genes are expressed (the *T.gondii* gene on chromosome 8 and the *P.falciparum* wild-type on chromosome 4) may affect folate pools such that sulfadoxine becomes somewhat less inhibitory. A similar effect is seen in *T.gondii* where transfection of the wild-type *DHFR* gene increases the level of resistance to pyrimethamine (Reynolds and Roos, 1998). Whatever the cause of the baseline shift in sulfadoxine resistance, subtraction of its value from those measured for the transfectants carrying mutant alleles brings the latter into

even closer agreement with previously derived figures for naturally occurring mutant strains (Wang *et al.*, 1997b). It also suggests that the fold-increases of resistance above the wild-type level we report here are minimum estimates.

Although the highest values of both K_i and IC_{50} for the different alleles occur when the three mutations S436F, A437G and A613S are present, this combination has only rarely been seen to date in the many field samples analysed from diverse regions of the world. Most frequently, we have observed the double mutant A437G,A581G form in samples collected from areas of high-level Fansidar resistance or from patients for whom antifolate treatment had failed (Wang *et al.*, 1997a), indicating that this level of mutation may be sufficient in many cases to influence the outcome of Fansidar challenge. Two other triple mutant forms, the S436A,A437G,K540E type tested here and the A437G,K540E,A581G type, appear to be common in Thailand (Triglia *et al.*, 1997) and parts of South America (Plowe *et al.*, 1997), respectively. It is possible that the S436F,A437G,A613S form is not widely disseminated in the field because it suffers in some way from reduced fitness, despite its very high resistance to sulfadoxine.

The three-dimensional crystal structure of the *P.falciparum* DHPS enzyme has not yet been obtained. However, insights can be gained into the effect of the various mutations on inhibitor and substrate binding by comparison with the recently solved structures of the *Staphylococcus aureus* (Hampele *et al.*, 1997) and *E.coli* (Achari *et al.*, 1997) homologues, particularly the latter, where crystals of the enzyme-inhibitor complex were analysed. Thus, an oxygen from the sulfonamide was shown to accept a hydrogen bond from the guanidinium group of Arg63 in *E.coli* DHPS and additionally, the aniline amine group of the drug and the carbonyl group of Thr62 are only 3.5 Å from the hydroxymethyl group of the dihydropterin substrate (Table II). Arg63 in *E.coli* DHPS corresponds to Gly437 in *P.falciparum*, which is the important mutant residue found in most sulfadoxine-resistant parasites (Table II). Thr62 corresponds to Ser436 which, when changed to Ala or Phe, also increases sulfadoxine resistance levels in *P.falciparum*. This suggests that both residues 436 and 437 in *P.falciparum* DHPS are very close to, or involved in, both substrate- and inhibitor-binding. Although the *P.falciparum* residues 581 and 613 do not correspond to residues in *E.coli* DHPS directly involved in substrate or inhibitor binding, they are next to, or very close to, residues whose bacterial counterparts are important in this binding (Achari *et al.*, 1997). Interestingly, residue 540, where the Lys to Glu transition has a large effect on both K_i for sulfadoxine (Triglia *et al.*, 1997) and the level of resistance in live parasites (Table I), is not close to any amino acid residues implicated in substrate or inhibitor binding. In fact, most mutations in the DHPS enzymes of *S.aureus* (Hampele *et al.*, 1997) and *E.coli*, (Achari *et al.*, 1997) that confer resistance to sulfa drugs are also not located in the substrate- or inhibitor-binding sites, which defies a simple interpretation of their roles in the resistance mechanism.

The ability to transfect *P.falciparum* was developed only recently and integration occurs by a single recombination event (Crabb and Cowman, 1996; Wu *et al.*, 1996; Crabb *et al.*, 1997a). Previously, alleles of the *dhfr-ts* gene

Table II. Mutations in *P.falciparum* DHPS in relation to residues involved in substrate and inhibitor binding in *E.coli* and *S.aureus*

dhppp binding ^a			pABA/Su binding ^b			Mutations in <i>Pf</i> DHPS
<i>E.coli</i>	<i>S.aureus</i>	<i>P.falciparum</i>	<i>E.coli</i>	<i>S.aureus</i>	<i>P.falciparum</i> ^c	
L21	L10	L394				
N22	N11	N395				
V23	V12	V396				
D56	D45	D430				
S61	S50	S435	S61	S50	S435	
T62	T51	S436	T62	T51	S436	A or F436
R63	R52	A437	R63	R52	A437	G437
P64	P53	P438				
V95	V83	I481				
D96	D84	D482				
T97	T85	T483				
I114	I102	L501				
N115	N103	N502				
D116	D104	D503				
M139	M128	M529				
						E540
L184	L166	F574				
D185	D167	D575				
P186	P168	I576				
F190	F172	F580	F190	F172	F580	
						G581
L215	L197	F603				
			S219	S201	S607	
R220	R202	R608	R220	R202	R608	
K221	K203	K609	K221	K203	K609	
S222	R204	R610				
						S or T613
I254	V238	I685				
R255	R239	R686				
V256	V240	V687				
H257	H241	H688	H257	H241	H688	
D258	N242	D689				

^aResidues in or near the binding site of the substrate 7,8-dihydropterin pyrophosphate dhppp in *E.coli* (Achari *et al.*, 1997) and *S.aureus* (Hampele *et al.*, 1997). Residues in *E.coli* and *S.aureus* that directly bind to dhppp are shown in bold. Equivalent residues in the *P.falciparum* DHPS are shown (Brooks *et al.*, 1994; Triglia *et al.*, 1994).

^b*Escherichia coli* residues in or near the pABA and sulfonamide (Su) binding site (Achari *et al.*, 1997). Residues in *E.coli* that directly bind to Su are shown in bold. Equivalent residues are shown for *P.falciparum* and *S.aureus* DHPS.

^cThese residues are from the *Pf*DHPS of sulfadoxine-sensitive isolates (Brooks *et al.*, 1994; Triglia *et al.*, 1994).

had been transfected into *P.falciparum* to show that mutations in this gene (Cowman *et al.*, 1988; Peterson *et al.*, 1988) can confer resistance to pyrimethamine (Wu *et al.*, 1996). However, these genes were used as the selectable marker and homologous recombination events were selected that created pseudo-diploid and pseudo-polyploid parasites with two or more functional copies of *dhfr-ts*, one or more of which were under the control of the histidine-rich protein 3 gene (*hrp3*) promoter (Wu *et al.*, 1996). This makes it difficult to determine the contribution of specific amino acids to the particular phenotype, owing to the expression of an endogenous wild-type gene as well as the multiple mutant *dhfr-ts* genes that have integrated by homologous recombination. To overcome this problem, we transfected plasmid constructs that contained only a partial *dhps* gene so that integration by a single recombination event would insert the required mutations and reconstitute the gene by allelic exchange. Although insertion of multiple copies of the transfection plasmid increased the copy number of the *dhps* gene, the extra copies all have the 5' end of the gene truncated and were shown not to be expressed. We were thus able to test directly the role of specific mutations in the DHPS enzyme on the sulfadoxine-resistance phenotype.

The *dhps* transfectants obtained were found to have between two and four copies of the plasmid inserted into the endogenous *pppk-dhps* gene. Insertion of these multiple copies was not due to challenge with higher concentrations of pyrimethamine, which would select for increased expression of DHFR, as lower levels of the drug also gave parasites with multiple inserts. It has been shown that plasmids transfected into *P.falciparum* replicate as episomes (Wu *et al.*, 1995) and are present in forms larger than a single copy, probably as concatamers (Crabb *et al.*, 1997a). Homologous recombination of such concatamers would result in insertion of multiple plasmids in a single crossover irrespective of the level of pyrimethamine used for selection. This is consistent with the observation that between one and four copies of the plasmid were inserted in independent clones obtained from the same transfection experiment.

In summary, we have used transfection to prove conclusively that the amino acid mutations identified in the DHPS enzyme of *P.falciparum* are responsible for the sulfadoxine-resistance phenotype in live parasites. The demonstration that we can replace the wild-type *dhps* allele to examine the effects on a drug resistance phenotype provides a powerful precedent for the analysis of other

phenotypes where amino acid substitutions are implicated. This is of particular importance in *P.falciparum* where integration occurs via a single recombination event, precluding gene replacement by double crossover. Our studies have provided new insights into the evolution of sulfa drug resistance in *P.falciparum* and indicate that high level resistance results from a stepwise accumulation of mutations in the *dhps* gene due to selection with increasing drug pressure. This has important implications for the analysis of the development and spread of drug resistance alleles in malaria endemic areas.

Materials and methods

Parasites and transfection

Parasites were maintained (Trager and Jensen, 1978) and synchronized by standard procedures. D10 parasites were transfected as described for transient expression (Wu *et al.*, 1995) with 150 µg plasmid DNA purified using CsCl density gradients. Parasites were cultured for 48 h post-transfection prior to selection with 0.1 µM pyrimethamine (Crabb and Cowman, 1996). Cultures were maintained for a further 18–21 days with selection before parasites were seen. Parasites containing integrated forms of the construct were obtained by removing the drug for 3 weeks followed by further selection on 0.1 µM pyrimethamine until parasites re-appeared. Parasites were cloned by the single-cell method without drug selection.

Nucleic acids, pulsed-field gel electrophoresis and DNA sequencing

Genomic DNA and intact chromosomes were extracted from trophozoites as previously described (Triglia and Cowman, 1994). Southern blotting was carried out using standard procedures. *P.falciparum* chromosomes were separated by PFGE using a contour-clamped homogeneous electric field apparatus (Chu *et al.*, 1986) at 4.2 V/cm and a pulse time of 225 s for 72 h.

Poly(A)⁺ RNA was purified from each cloned transfectant using the MicroFast RNA extraction kit (Invitrogen). Any remaining genomic DNA was removed by treatment with RNase-free DNase I (Gibco-BRL) before conversion to cDNA using the MicroFast cDNA synthesis kit (Invitrogen). For sequencing of cDNA from clones containing integrated *dhps* alleles, cDNA was amplified using an oligonucleotide to *dhps*, 5'-GTATTTTGTGAACCTAACG-3' (#1) and an oligonucleotide to the *hsp86* 3' sequence, 5'-TATATATGTATATGGGGTGATG-3' (#2). This PCR product was sequenced with the #1 oligonucleotide and with the oligonucleotide 5'-TCCAATTGTGTGATTGTCCAC-3' (#3) (Figure 1).

Two DNA probes were used for Southern blots. The first was a 391 bp *P.falciparum dhps* fragment amplified using the oligonucleotides 5'-AAGAAGAAGAACAATCTATAGTG-3' (#4) and #3. The second probe was a 210 bp *T.gondii dhfr-ts* fragment amplified using the oligonucleotides 5'-ATGCAGAAACCGTGTGTCTGGTC-3' (#5) and 5'-CGACTGATGGAGAGGGAAGTCC-3' (#6) as shown in Figure 1. Relative band intensities were quantitated by exposure of blots to a PhosphorImager using Image-Quant software (Molecular Dynamics).

DNA constructs

The full-length *pppk-dhps* genes from D10 and W2mef were obtained from the previously made pTrc constructs (Triglia *et al.*, 1997) by digesting with *Bam*HI–*Hind*III. These fragments were subcloned into pGEM11Z (Promega) which resulted in *pppk-dhps* being flanked by *Xho*I sites at both ends. The *Xho*I fragments were finally cloned into the *Xho*I site of pHCl resulting in D10-FL and W2mef-FL which were used for stable transfection (Crabb *et al.*, 1997b).

Truncated *pppk-dhps* constructs were made by a two-step procedure. First, the *Avr*II site in pLITMUS 28 (New England Biolabs) was mutated to a *Xho*I site using PCR mutagenesis resulting in a vector with two *Xho*I sites flanking both a *Xba*I and an *Eco*RI site. Secondly, pTrc constructs for D10, 3D7, K1, Tak9/96, PR145 and W2mef (Triglia *et al.*, 1997) were digested with *Xba*I–*Eco*RI, which resulted in an almost complete deletion (98 bp) of exon 1 within the *pppk* domain. The six truncated *pppk-dhps* fragments were cloned into the *Xho*I-modified pLITMUS vector and *Xho*I used to obtain each truncated fragment. These fragments were finally cloned into pHCl as above (Crabb *et al.*,

1997b) to give six constructs which, when transfected into the D10 parasite, integrated and cloned, resulted in the following parasites lines: D10-sakaa, D10-sgkaa, D10-sgkaa, D10-agkaa, D10-ageaa and D10-fgkas (Table I).

Western blotting

Protein samples were analysed by SDS–PAGE and electrophoretically transferred to nitrocellulose as previously described (Triglia and Cowman, 1994). The anti-*P.falciparum* DHPS antibodies were raised in rabbits to *E.coli*-expressed DHPS enzyme. The antiserum was subsequently affinity-purified (Triglia and Cowman, 1994). The antibodies to *P.falciparum* HSP70 protein have been previously described (Bianco *et al.*, 1986). Bound antibody was detected with horse-radish peroxidase coupled sheep anti-rabbit IgG (Silenus Laboratories, Australia) and developed by ECL, a luminescence-based method (Amersham International).

In vitro sulfadoxine assay

For determination of the sulfadoxine resistance of a cloned parasite, cultures were first transferred to a folate pABA-free RPMI–HEPES medium containing 0.5% Albumax (Gibco-BRL), 0.2% glucose, 1 µg/ml hypoxanthine, 50 µg/ml gentamycin and re-supplemented with 100 ng/ml folate. After adaptation to this medium, parasites were transferred to the same medium devoid of folate for 48 h prior to drug challenge as described (Wang *et al.*, 1997b,c). Serial dilutions of sulfadoxine were then added and cultures incubated for a further 48 h before addition of [³H]-hypoxanthine to monitor growth. IC₅₀ values were defined as the concentration of sulfadoxine where parasite growth was half that of untreated controls. Each IC₅₀ value in Table I was derived from between six and eight separate experiments.

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