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Heterologous Expression of Active Thymidylate Synthase–Dihydrofolate Reductase from *Plasmodium falciparum*†

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ABSTRACT: The coding sequence of the bifunctional thymidylate synthase–dihydrofolate reductase (TS–DHFR) from a moderately pyrimethamine-resistant strain (HB3) of *Plasmodium falciparum* was assembled in a pUC expression vector. The coding sequence possesses unique *Nco*1 and *Xba*1 sites which flank 243 bp of the DHFR gene that include all point mutations thus far linked to pyrimethamine resistance. Wild-type (3D7) and highly pyrimethamine-resistant (7G8) TS–DHFRs were made from this vector by cassette mutagenesis using *Nco*1–*Xba*1 fragments from the corresponding cloned TS–DHFR genes. Catalytically active recombinant TS–DHFRs were expressed in *Escherichia coli*, albeit at low levels. Both TS and DHFR coeluted upon gel filtration and copurified upon affinity and anion exchange chromatography. Gel filtration and SDS–PAGE indicated that the enzyme was a dimer with identical 67-kDa subunits, characteristic of protozoan TS–DHFRs. Amino-terminal sequencing gave 10 amino acids which perfectly matched the sequence predicted from the nucleotide sequence. The recombinant TS–DHFR was purified to homogeneity by 10-formylfolate affinity chromatography followed by Mono Q FPLC. The inhibition properties of pyrimethamine toward the purified recombinant enzymes show that the point mutations are the molecular basis of pyrimethamine resistance in *P. falciparum*.

In most organisms, thymidylate synthase (TS) and dihydrofolate reductase (DHFR) are separate, monofunctional enzymes. DHFR is usually a monomer of about 20 kDa (Blakley, 1984), and TS is a dimer of identical subunits of about 35 kDa each (Santi, & Danenberg, 1984). However, in protozoa, the enzymes exist on the same 55–70-kDa polypeptide chain, with the DHFR domain at the amino terminus and TS at the carboxy terminus, separated by a junction peptide. The native bifunctional protein is comprised of two such subunits (Ivanetich & Santi, 1990).

The TS–DHFR[1] of *Plasmodium falciparum* is one of the

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[1] Abbreviations: TS–DHFR, thymidylate synthase–dihydrofolate reductase; H_2folate, 7,8-dihydrofolate; H_4folate, tetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; MTX, methotrexate; PVDF, poly(vinylidine difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Pyr, pyrimethamine; Amp, ampicillin; ON, oligonucleotide; nt, nucleotide; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonate.

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few well-defined drug targets for malaria. The DHFR inhibitors pyrimethamine (Pyr) and cycloguanil have been used as antimalarial agents for several decades, but the emergence of resistant parasites has reduced the utility of these drugs. Anti-folate resistance in *Plasmodium* appears to be linked to structurally altered DHFR (Cowman et al., 1988; Peterson et al., 1988), and DHFR preparations from several Pyr-resistant strains show decreased affinity to the drug (Chen et al., 1987; Zolg et al., 1989). Recently, TS-DHFR genes from wild-type and anti-folate-resistant strains of *P. falciparum* have been sequenced (Bzik et al., 1987; Cowman et al., 1988; Foote et al., 1990; Peterson et al., 1988, 1990). Interestingly, independent isolates of Pyr-resistant *P. falciparum* DHFRs have common point mutations. In all Pyr-resistant isolates examined, the Ser 108 of the sensitive strain is converted to Asn; strains with higher resistance to Pyr also have a Asn 51/Ile mutation or a Cys 59/Arg mutation. The latter mutations are not found alone, which suggests that highly resistant strains arise through sequential mutation of the Ser 108 mutant. Resistance to the anti-folate cycloguanil is linked to Ala 16/Val and/or Ser 108/Thr mutations, and cross resistance to both Pyr and cycloguanil has been associated with Ile 164/Leu, along with Ser 108/Asn and Cys 59/Arg mutations.

Studies of the TS-DHFR of *P. falciparum* have been hampered by the limited quantities of protein which can be obtained from the parasite, and formidable efforts have been required to obtain even small amounts of this enzyme (Chen & Zolg, 1987; Zolg et al., 1989). Clearly, recombinant proteins will be necessary to study details of the effect of TS-DHFR point mutations on anti-folate resistance and to use modern methods of drug design to discover new antifolate antimalarials.

Attempts at expression of *Plasmodium* TS-DHFR have thus far been unsuccessful, probably because of the high A+T content of the DNA. In this report we describe the construction of expression vectors containing the TS-DHFR coding sequence from wild-type and two Pyr-resistant strains of *P. falciparum*. The vectors are flexible in that they permit construction of all known Pyr-resistant sequences by simple cassette mutagenesis. We describe the expression of catalytically active TS-DHFR in *Escherichia coli*, as well as purification and initial characterization of the enzyme. Finally, we show that the mutants possess the expected resistance to inhibition by Pyr, which definitively demonstrates the relationship between point mutations in DHFR and drug resistance.

Materials and Methods

Materials. pGem3(*Xba*1) containing a 4.9-kb *Xba*1 fragment and pUC13(5′R1) containing a 2-kb *Eco*RI fragment from strain HB3 TS-DHFR were described previously (Cowman et al., 1988). The 243-bp *Nco*1–*Xba*1 fragments of the coding sequences of wild-type strain 3D7 and Pyr-resistant strain 7G8 were PCR-amplified fragments obtained from the corresponding DNA (A. Cowman, unpublished results). *E. coli* K12 strain χ2913 (thy A572), which carries a deletion in the TS gene, was a gift from Dr. R. Thompson (University of Glasgow). The DHFR-deficient *E. coli* D3-157 (Singer et al., 1985) was from S. Singer (Burroughs Wellcome), and *E. coli* TB1 was from T. O. Baldwin (Texas A&M). Oligonucleotide synthesis and protein sequencing were performed by the UCSF Biomolecular Resource Center. 10-Formylfolate was prepared from folic acid (Banerjee et al., 1982) and coupled to aminohexyl-Sepharose CL-6B (Dann et al., 1976). Immobilon P membranes were from Millipore. All other reagents have been previously described (Grumont et al., 1988; Meek et al., 1985) or were of the highest purity commercially available.

DNA Manipulations. General methods for DNA manipulations were performed as described (Sambrook et al., 1989). DNA fragments were purified by agarose gel electrophoresis using low melting temperature agarose or electroelution. For fragment ligation of the six synthetic oligonucleotides forming nt 1–138 (ON3), 2.5 pmol of each was annealed and the mixture ligated; the fragment was ligated into the *Nco*1-digested pUC18 PL2 at molar ratios of 5:1 and 10:1. DNA sequences of constructs were confirmed by restriction analysis and dideoxy DNA sequencing.

Preparation of Cell Extracts. Bacterial clones were grown in LB containing 50 μg/mL Amp at 37 °C. To obtain maximum TS and DHFR, cells were grown for 12 h and frozen at −20 °C until use. Cell paste from 5 L of transformed *E. coli* χ2913 cells was resuspended in 50 mL of buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) containing a mixture of six protease inhibitors (Meek et al., 1985). Cells were disrupted by sonication, and extracts were clarified by centrifugation at 30000*g* for 1 h at 4 °C (Grumont et al., 1988).

Purification of TS-DHFR. Crude extract (~50 mL; 7-L culture) was circulated at 0.5 mL/min through a 10-formylfolate–Sepharose column (1.0 × 4.0 cm) equilibrated with buffer A for 2–3 h at 4 °C. The column was washed (1 mL/min) with buffer A containing 0.45 M NaCl until protein was undetectable in the effluent (~80 mL). H_2folate (4 mM) in the same buffer (20 mL) was applied onto the column at a flow rate of 0.5 mL/min, and 5-mL fractions were collected. Fractions (5–10 mL) containing activity were pooled and concentrated.

One-fourth of the concentrated sample (ca. 0.5 mL, 1 mg of protein) from affinity chromatography was desalted on a PD-10 (Sephadex G-25) column (Pharmacia) and applied to a Mono Q HR5/5 column preequilibrated with buffer A. The column was washed with 5 mL of buffer A (0.2 mL/min) before application of a gradient to 0.2 M NaCl in buffer A (30 mL) and then to 1 M NaCl in buffer A (10 mL). The TS-DHFR eluted at 60–80 mM NaCl. Active fractions (5 mL) were pooled and concentrated to ~1 mL against buffer A. In a control experiment, host *E. coli* DHFR eluted from Mono Q FPLC at 600–700 mM NaCl.

Protein Analysis. Protein determinations (Read & Northcote, 1981), electrophoresis on 12% SDS-PAGE, and staining with Coomassie Blue R250 (Laemmli, 1977) were as described. Proteins were concentrated at 4 °C by use of CentriPrep concentrators (Amicon). DHFR and TS activities were determined spectrophotometrically as described (Meek et al., 1985). One unit of enzyme activity is the amount of enzyme required to produce 1 nmol of product per minute at 25 °C. For determination of kinetic parameters, one substrate was kept constant at 50 μM and the other varied between 2 and 50 μM. For K_i determinations, pyrimethamine concentrations used were 1–3 nM for 3D7, 1–11 nM for HB3, and 200–1100 nM for 7G8; K_i values were determined by varying H_2folate with NADPH constant at 50 μM. Reactions were initiated by addition of about 1–4 units mL^{-1} TS-DHFR (0.1–0.5 nM, based on subunit size), and initial velocities were recorded over several minutes, during which time rates were linear. Kinetic parameters were evaluated with a nonlinear least-squares fit of the data to the Michaelis–Menten equation (Cleland, 1979). Covalent TS-[^{3}H]FdUMP-CH_2-H_4folate complexes were formed by incubating enzyme with 0.1 μM [^{3}H]FdUMP (20 Ci/mmol), 0.1 mM CH_2-H_4folate, and 6.5

Table I: Synthetic Oligonucleotide Duplexes Used in Construction of *P. falciparum* TS-DHFR Expression Vectors

Oligonucleotide	Sequence
Polylinker duplex (ON1)	
	5'-CGGATCCATGGTCTAGATCGTCGACA 3'
	3'-CATGGCCTAGGTACCAGATCTAGCAGCTGTTCGA 5'
3' end (ON2)	
	5'-TGGATATGGCTGCTTAAGATATCGTCGACA 3'
	3'-ACCTATACCGACGAATTCTATAGCAGCTGTTCGA 5'
5' end (ON3)[a]	
ON3A	5'-CATGATGGAACAAGTCTGCGACGTTTTCGATATCTATGCCATATGTGCAT 3'
ON3B	3'-TACCTTGTTCAGACGCTGCAAAAGCTATAGATACGGTATACACGTACAACATTCC 5'
ON3C	5'-GTTGTAAGGTTGAAAGCAAAAATGAGGGGAAAAAAAAATGAGGTTTTTAAT 3'
ON3D	3'-AACTTTCGTTTTTACTCCCCTTTTTTTTACTCCAAAAATTATTGATGT 5'
ON3E	5'-AACTACACATTTAGAGGTCTAGGAAATAAAGGAGTATTAC 3'
ON3F	3'-GTAAATCTCCAGATCCTTTATTTCCTCATAATGGTAC 5'
Ribosome binding site (ON4)	
	5'-CGTAAGAGGAGTTAAA 3'
	3'-TCGAGCATTCTCCTCAATTTCTAG 5'

[a] ON3A–ON3F were ligated to form ON3, as described in the text.

mM HCHO in 20 mM TES, 25 mM $MgCl_2$, 1 mM EDTA, and 75 mM 2-mercaptoethanol for 15 min at 25 °C. Analysis of the covalent complex was performed by SDS–12% PAGE. The Coomassie-stained gel was soaked with an autoradiography enhancer (Enlightning, Du Pont) for 15 min at ambient temperature, dried, and exposed on KODAK X-OMAT film at −80 °C for 48 h before development. Amino-terminal sequencing was performed on protein electroblotted to Immobilon P (Matsudaira, 1987) by use of an ABI 470A protein sequencer/120A PTH analyzer.

Gel filtration of *Plasmodium* TS–DHFR was performed using Superose 12 HR10/30 FPLC (Pharmacia). The crude extract (1 mL, ~7 mg/mL) in 50 mM potassium phosphate, pH 7.2, and 0.15 M NaCl was eluted with the same buffer at 0.2 mL/min. The column was calibrated with 13.7–232-kDa standards.

Complementation of TS-Deficient E. coli. Plasmids were transformed into the TS-deficient *E. coli* K12 strain χ2913 and plated on minimal agar plates containing 50 μg/mL Amp, ± 50 μg/mL Thd. Colonies which express active TS could be visualized on plates lacking Thd after overnight incubation at 37 °C.

Results

Cloning the Coding Sequence. The two overlapping genomic clones containing the *P. falciparum* TS–DHFR gene used here were contained in plasmids pUC13(5′R1) and pGem3(*Xba*1). As previously described (Cowman et al., 1988), pUC13(5′R1) has a 2.0-kb *Eco*RI gene fragment composed of 0.6 kb of 5′ untranslated sequence followed by 1.4 kb of coding sequence; pGem3(*Xba*1) has a 4.8-kb *Xba*1 fragment composed of 1.5 kb of 3′ coding sequence followed by 3.3 kb of 3′ untranslated sequence. The 1824-bp coding sequence of *P. falciparum* TS–DHFR was assembled with a 138-bp synthetic oligonucleotide at the 5′ end (nt 1–138), a 243-bp genomic *Nco*1–*Xba*1 fragment from pUC13(5′R1) (nt 139–382), a 1427-bp genomic *Xba*1–*Bbv*1 fragment from pGem3(*Xba*1) (nt 383–1810), and a synthetic oligonucleotide at the 3′ end (nt 1811–1824) (Figure 1).

Figure 1 illustrates how the parent plasmic pTDSD(HB3) was constructed. First, the *Kpn*1–*Hin*dIII fragment of the polylinker of pUC18 was replaced by an oligonucleotide linker (ON1, Table I) to give pUC18PL2 which had restriction sites *Sst*1–*Kpn*1–*Bam*H1–*Nco*1–*Xba*1–*Sal*1–*Hin*dIII designed for introduction of fragments of the coding sequence. Second, a 1427-kb 5′ *Xba*1–3′ blunt end fragment containing nucleotides 383–1810 was generated by digestion of pGem3(*Xba*1) with *Bbv*1, filling in with T_4 polymerase to form a blunt end followed by cleaving with *Xba*1. In a triple ligation, this fragment and a synthetic oligonucleotide (ON2) containing the last 14 bases of the coding sequence, a stop codon, and a *Hin*dIII overhang were cloned into the *Xba*1/*Hin*dIII site of pUC18 PL2 to give pWF1. Third, the 243-bp *Nco*1–*Xba*1 fragment from the HB3 gene in pUC13(5′R1) was introduced into the *Nco*1–*Xba*1 site of pWF1 to give pWF2, which contains all but the first 140 bases of the coding sequence. Fourth, a synthetic oligonucleotide (ON3) with a 5′ *Nco*1-compatible cohesive end and a 3′ *Nco*1 site, and which encodes the first 47 amino acids of the coding sequence, was prepared by ligation of six oligonucleotides (ON3A–ON3F) and cloned into the *Nco*1 site of pUC18 PL2. This produced plasmids with two orientations of the synthetic fragment. In the desired orientation, there was a single *Nco*1 site at nt 138; the 5′ *Nco*1 cloning site is destroyed to accommodate Met 2; the 140 bp of coding sequence was identical with the natural fragment except for a T to C change at nt 33 which introduced a silent *Eco*RV site to facilitate identification. The plasmid with the appropriate orientation was designated pUC18 PL2/143. Next, the *Nco*1–*Sal*1 fragment of pWF2 was transferred to pUC18 PL2/143 to give the parent plasmid pTDPF(HB3),

Table II: TS and DHFR Activities in Crude Extracts of pDTSD-Transformed *E. coli* Hosts[a]

host	plasmid	condition	DHFR (nmol min^{-1} mg^{-1})	TS (nmol min^{-1} mg^{-1})
TB1		LB	2.4	0.13
TB1	pTDSD(HB3)	LB + Amp	10.1	0.55
HB101		LB	1.2	0.50
HB101	pTDSD(HB3)	LB + Amp	10.5	0.60
χ2913		LB	2.5	ND[b]
χ2913	pTDSD(HB3)	LB + Amp	16.2	0.18
χ2913		minimum + Thd	3.7	ND[b]
χ2913	pTDSD(HB3)	minimum + Amp	13.0	0.15
χ2913	pTDSD(HB3)	minimum + Thd + Amp	18.2	0.18
χ2913	pTDSD(3D7)	minimum + Amp	6.6	0.21
χ2913	pTDSD(7G8)	minimum + Amp	6.0	0.05
D3-157		LB	ND[b]	0.37
D3-157	pTDSD(HB3)	LB + Amp	20.4	0.86

[a] All cultures were grown for about 12 h to stationary phase. [b] Not detectable.

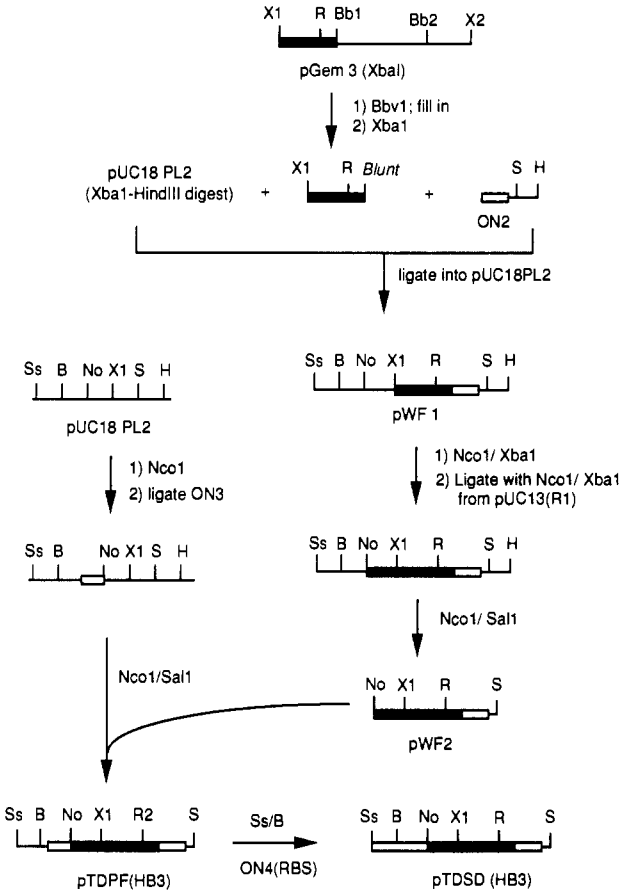

FIGURE 1: Construction of the expression vector pTDSD(HB3) containing the *P. falciparum* TS–DHFR gene. The solid bars represent sequences derived from gDNA, and open bars are sequences from synthetic oligonucleotides (ON2 and ON3). Restriction endonuclease sites are Ss for *Sst*1, K for *Kpn*1, B for *Bam*H1, No for *Nco*1, X for *Xba*1, S for *Sal*1, H for *Hin*dIII, R for *Eco*RI, and Bb for *Bbv*1.

which contains the entire TS–DHFR coding sequence of the moderately Pyr-resistant HB3 line. Finally, a 16/24-bp synthetic oligonucleotide containing a ribosomal binding sequence (ON4) was introduced 10 bases upstream from the start codon at the *Sst*1/*Bam*H1 sites of parent plasmid pTDPF(HB3) to give pTDSD(HB3).

With pTDSD(HB3), modifications at the target amino acid positions 51, 59, and/or 108 can simply be made by cassette mutagenesis of the appropriate 243-bp *Nco*1–*Xba*1 fragments from cloned TS–DHFR genes into the *Nco*1–*Xba*1 large fragment. Thus, the *Nco*1–*Xba*1 fragment from the 3D7 TS–DHFR gene amplified by PCR was introduced into the corresponding sites of pTDSD(HB3) to give pTDSD(3D7) which contains the wild-type Ser 108 instead of the Asn in HB3. Likewise, the *Nco*1–*Xba*1 fragment from 7G8 was cloned into pTDSD(HB3) to give pTDSD(7G8) which contains Asn 51/Ile and Ser 108/Asn.

Expression of Recombinant TS–DHFR. In initial studies of the expression of TS–DHFR, we examined several different *E. coli* strains as hosts for pTDSD expression vectors (Table II). With *E. coli* TB1, DHFR and TS levels in extracts of pTDSD(HB3) transformants were significantly higher (4-fold) than in untransformed cells; in transformed HB101 cells, DHFR was high, but TS levels were similar to those of the controls. To facilitate analysis, we examined *E. coli* hosts which were deficient in TS or DHFR. All three of the expression plasmids complemented growth of TS-deficient *E. coli* χ2913, showing that catalytically active TS was expressed. In crude extracts of the host *E. coli* χ2913, TS activity was undetectable, and no TS–[^{3}H]FdUMP–CH_2-H_4folate complex was detectable on SDS–PAGE; in extracts from cells transformed with each of the three expression vectors, there was significant TS activity. The DHFR activities in transformed *E. coli* χ2913 cells were also significantly higher than those found in the host cell (4-fold for HB3; 2-fold for 3D7 and 7G8). The DHFR activity in crude extracts of pTDSD-(HB3)-transformed DHFR-deficient *E. coli* D3-157 was high and again demonstrated that expression of the enzyme emanated from the vector. The DHFR/TS activity ratios were erratic, presumably because of uncontrolled degradation of TS (see below). On the basis of these results, we chose *E. coli* χ2913 for further studies.

The TS–DHFR activities and growth of *E. coli* χ2913 harboring pTDSD (HB3) in minimum media with 50 μg/mL Amp were investigated over a period of 22 h (Figure 2). The activity of DHFR followed cell growth and leveled after about 12 h, whereas TS activity sharply declined after about 7 h. At 6.5 h the DHFR/TS ratio was ~12 and increased dramatically with time due to diminished TS activity. By 22 h, the DHFR activity was approaching its maximum, but the TS activity was only ~25% of that at 6.5 h, and the DHFR/TS ratio was ~77. Loss of TS activity was previously observed with *Leishmania* TS–DHFR and shown to be a result of proteolysis (Garvey & Santi, 1985). With this possibility in mind, we transformed the protease-deficient cell lines *E. coli* CAG 456 and FB 974 (Baker et al., 1984) with pTDSD (HB3), but enzyme activity was not detected in crude extracts of the transformants (data not shown).

Superose 12 gel filtration of extracts of χ2913 harboring pTDSD(HB3) showed that the TS and DHFR activities coeluted as a single peak of about 110 ± 15 kDa, compared

Table III: Purification of TS–DHFR Expressed in *E. coli* Transformed with pTDSD(HB3)

purification step	vol (mL)	protein (mg)	DHFR units (nmol/min)	DHFR sp atc. (units/mg)	x-fold purification	yield (%)
crude extract	58	4042	55 379	13.7		100
affinity chromatography	1.7	3.7	25 731	6 936	506	46
Mono Q FPLC	1.6	0.34	22 634	66 571	4859	41

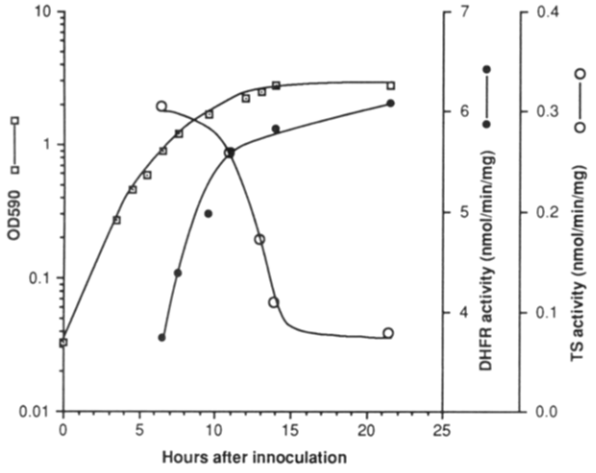

FIGURE 2: Expression of *Plasmodium* TS–DHFR in *E. coli* χ2913 harboring pTDSD(HB3). 10 mL of an overnight culture of *E. coli* χ2913/pTDSD(HB3) was inoculated into 1 L of minimum media supplemented with 50 μg/mL Amp. Growth was monitored by A_{590}, and 50-mL aliquots of cells were harvested and disrupted; DHFR and TS specific activities were determined: (□) OD_{590}; (●) DHFR (nmol min^{-1} mg^{-1}); (○) TS (nmol min^{-1} mg^{-1}).

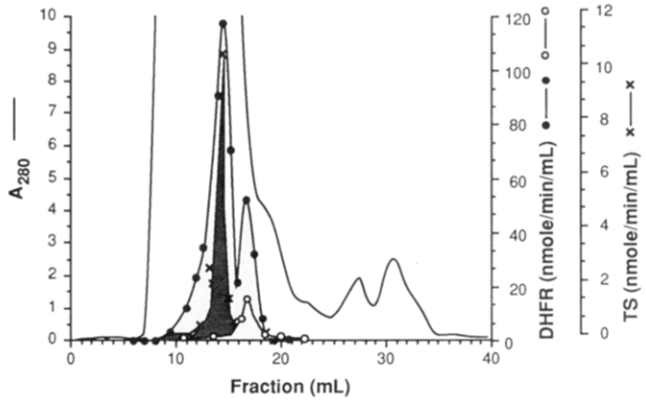

FIGURE 3: Superose 12 HR chromatography of a crude extract of *E. coli* χ2913/pTDSD(HB3). (—) A_{280}; DHFR activity (light shading) from (○) *E. coli* χ2913 and (●) *E. coli* χ2913/pTDSD-(HB3); (×) TS activity (dark shading) coelutes with the DHFR peak of pTDSD(HB3). The markers used (mass; V_e/V_0) were catalase (232 kDa; 1.30), BSA (67 kDa; 1.47), ovalbumin (43 kDa; 1.54), chymotrypsinogen (25 kDa; 1.69), and ribonuclease (13.7 kDa; 1.77).

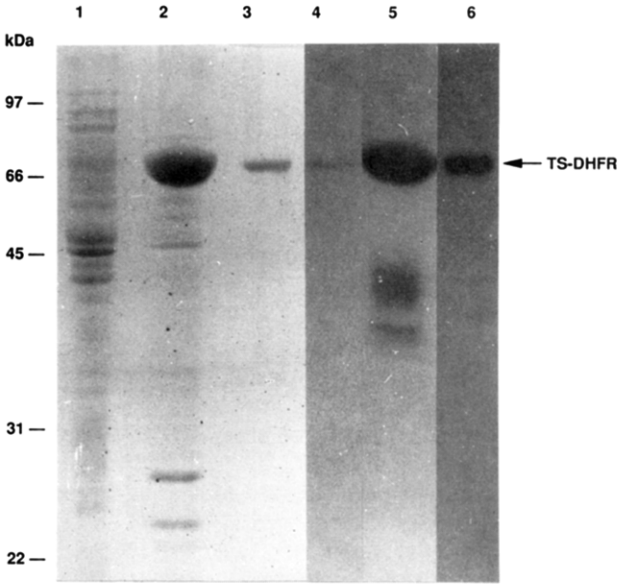

FIGURE 4: SDS–PAGE of TS–DHFR treated with [^{3}H]FdUMP and CH_2-H_4folate. Lanes 1–3 were stained with Coomassie Blue R250; lanes 4–6 are autoradiograms of lanes 1–3, respectively. Lanes 1 and 4, crude extract from *E. coli* χ2913/pTDSD(HB3); lanes 2 and 5, after 10-formylfolate–Sepharose column; lanes 3 and 6, after Mono Q FPLC. Molecular mass markers were as indicated: phosphorylase B, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa. The arrow shows the 67-kDa TS–DHFR.

with the calculated size of 143 kDa (Figure 3); the recovery of DHFR was about 40%, and the DHFR/TS ratio was ~12. A small trailing peak of DHFR activity with M_r of ~20 000 was probably host *E. coli* DHFR which is 18.1 kDa; indeed, a control extract from the host χ2913 showed a DHFR peak eluting with M_r of 20 000. Crude extracts of χ2913 harboring pTDSD (HB3) were treated with [6-^{3}H]FdUMP and CH_2-H_4folate to form the covalent ternary complex with TS (Santi & Danenberg, 1984) and analyzed by SDS–PAGE. Upon autoradiography, there was a band with subunit M_r of 67 000 (Figure 4, lane 4) and no indication of host TS (M_r = 291 000). When treated with [6-^{3}H]FdUMP and CH_2-H_4folate, the control extract from the host χ2913 showed no radioactive complex upon SDS–PAGE (data not shown). After affinity chromatography, there still were several faint radioactive bands with M_r of ~35 000–40 000 (Figure 4, lane 5) indicating proteolysis. Since the putative proteolysis products retained their ability to form the FdUMP complex, we concluded that they possess the TS domain.

Purification and Characterization. Initial attempts at purification of TS–DHFR using MTX–Sepharose and hydroxyapatite (HTP, Bio-Rad) chromatography were not promising. In crude extracts, the enzyme bound tightly to the MTX–Sepharose affinity matrix but could not be eluted with high concentrations of H_2folate, a result confirming earlier reports (Chen & Zolg, 1987; Garrett et al., 1984). The enzyme in crude extract bound to hydroxyapatite and was eluted at 250 mM potassium phosphate, pH 7.2; although this served to remove contaminating host DHFR, which eluted at 50 mM potassium phosphate, the purification was only 6-fold.

The expressed TS–DHFR from *E. coli* χ2913 harboring pTDSD(HB3) was purified about 5000-fold to homogeneity in about 40% yield with affinity chromatography on 10-formylfolate Sepharose and anion exchange on Mono Q FPLC (Table III; Figure 5); the same protocol was used to provide the purified enzymes from pTDSD(3D7) and pTDSD(7G8). After affinity chromatography, the preparation showed one major band on SDS–PAGE migrating with mass of 67 kDa and several minor bands of 40–50 kDa and 25 kDa (Figure 4, lane 2); the 67-kDa and 40–50-kDa proteins were labeled by preincubation with [^{3}H]FdUMP and CH_2-H_4folate, showing that each possessed an intact TS binding site. Further purification of the enzyme by Mono Q resulted in the homogeneous TS–DHFR eluting at 70–90 mM NaCl (Figure 4, lane 3); using the same system, host *E. coli* DHFR eluted late in the gradient at 600–700 mM NaCl. The pooled pu-

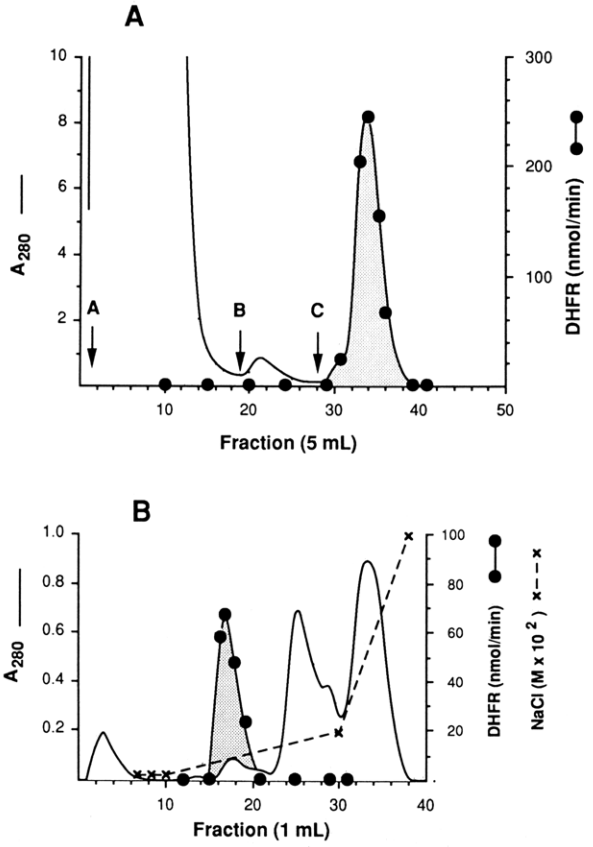

FIGURE 5: Purification of recombinant TS–DHFR. (Panel A) 10-Formylfolate–Sepharose CL-6B FPLC. Buffer A is 50 mM Tris, 1 mM EDTA, and 5 mM 2-mercaptoethanol, pH 7.5, buffer B is buffer A containing 0.45 M NaCl, and buffer C is buffer B containing 4 mM H_2folate. After buffer C, protein at A_{280} was not monitored because of interfering absorbance by H_2folate. (Panel B) Mono Q FPLC. (—) A_{280}; (●) DHFR activity (nmol min^{-1} mL^{-1}); (×) NaCl.

rified fractions from Mono Q FPLC showed a single 67-kDa protein band on SDS–PAGE which formed a radioactive covalent complex with [^{3}H]FdUMP and CH_2-H_4folate (Figure 4, lane 6); we think that we would have detected an impurity constituting 5–10% of the protein applied in the stained gel.

To further verify that the 67-kDa band on SDS–PAGE was *Plasmodium* TS–DHFR, the protein was electrotransferred onto an Immobilon P membrane (Matsudaira, 1987), and the excised band was subjected to microsequencing analysis. Ten cycles of analysis gave a major sequence of MMEQVCDVFD which perfectly matched the amino acid sequence predicted from the nucleotide sequence for the *Plasmodium* TS–DHFR gene (Bzik et al., 1987; Cowman et al., 1988). There was a minor preview sequence of about 15% which indicated some cleavage after the first Met at the amino terminus.

Kinetic Parameters. The kinetic parameters and inhibition constants for pyrimethamine (apparent K_m and K_i values) of the DHFR in purified recombinant wild-type and Pyr-resistant TS–DHFRs are given in Table IV. For the DHFR of HB3, we calculate a k_{cat} value of 80 s^{-1}, and k_{cat}/K_m values of 8×10^7 M^{-1} s^{-1} for H_2folate and 1.9×10^6 M^{-1} s^{-1} for NADPH. By double-reciprocal plot analysis, the inhibition by pyrimethamine was competitive with respect to H_2folate with the DHFR from HB3 and 7GB but appeared noncompetitive with the wild-type enzyme from strain 3D7. However, the low K_i value with 3D7 (lower than enzyme concentration) suggests a "tight-binding" inhibitor, so either type of inhibition would provide the apparent noncompetitive pattern (Williams & Morrison, 1979); further experimentation is required in the case of 3D7 before assignment of the inhibition type. The

Table IV: Kinetic Parameters of Purified Recombinant *P. falciparum* TS–DHFR[a]

parameter	3D7 Asn 51, Ser 108	HB3 Asn 51, Asn 108	7G8 Ile 51, Asn 108
K_m for H_2 folate (μM)	1.0 ± 0.1	11.0 ± 1	5.8 ± 0.5
K_m for NADPH (μM)	4.2 ± 0.2	1.1 ± 0.2	9.7 ± 3.6
K_i for pyrimethamine (nM)	0.10 ± 0.03	2.0 ± 0.4	230 ± 50

[a] Conditions used are described under Materials and Methods.

inhibition data clearly indicate that amino acid changes at positions 51 and 108 are responsible for conferring Pyr resistance. These kinetic parameters for recombinant TS–DHFRs are in good agreement with values reported or calculated from data for purified TS–DHFR from *P. falciparum* (Zolg et al., 1989).

Discussion

The coding sequence of TS–DHFR from *P. falciparum* was assembled in an expression vector by a combination of synthetic oligonucleotides and portions of the cloned gene. Unique *Nco*1 and *Xba*1 sites separate a 243-bp *Nco*1–*Xba*1 fragment (nt 139–382) which encodes for all of the mutations thus far associated with Pyr resistance. Thus, expression vectors containing any of these mutations can readily be constructed by cassette mutagenesis of the appropriate fragments from the TS–DHFR gene of resistant *P. falciparum* isolates. Using this approach, we first constructed a vector expressing TS–DHFR from moderately Pyr-resistant strain HB3 which has a Ser 108/Asn mutation; we then converted it to contain sequences corresponding to TS–DHFR from *P. falciparum* wild-type strain 3D7 and highly Pyr-resistant strain 7G8 which has a double mutation of Asn 51/Ile + Ser 108/Asn.

The expression of authentic *P. falciparum* TS–DHFR in *E. coli* harboring these vectors was demonstrated as follows. First, transformation of a TS-deficient *E. coli* host resulted in genetic complementation of the deficiency, and TS and DHFR activities in extracts from transformed cells were significantly higher than those in control host extracts. Second, both activities copurified on three different chromatography systems used in this study. Third, gel filtration of the native protein showed that both enzyme activities migrated with an apparent mass of about 110 kDa, and SDS–PAGE showed a 67-kDa subunit which formed a covalent TS–[^{3}H]-FdUMP–CH_2-H_4folate complex. These are in accord with the reported masses for TS–DHFR of *P. falciparum* and substantially larger than the monofunctional counterparts found in host *E. coli*. Finally, amino-terminal sequencing of the first 10 amino acids from the purified protein perfectly matched the sequence predicted from the reported *P. falciparum* TS–DHFR nucleotide sequence.

The expressed TS–DHFR was not stable; particularly, the activity of TS decreased dramatically after 7-h culture. Despite precautions, we observed degradation products of 38–45 kDa which contained the TS binding site. Since the TS domain begins about 35 kDa from the carboxy terminus, we conclude that the site of proteolysis occurs within the 10-kDa junction peptide separating the DHFR and TS domains.

P. falciparum TS–DHFRs were purified by affinity chromatography on 10-formylfolate–Sepharose followed by anion exchange chromatography on Mono Q FPLC. The aforementioned degradation products were removed by the anion exchange step. Also, host *E. coli* DHFR binds tightly to Mono Q, so this step ensured removal of any contaminating host enzyme which could interfere with kinetic analysis. The pure protein gave a homogeneous 67-kDa band on SDS–PAGE

which formed a covalent complex with [^{3}H]FdUMP and CH_2-H_4folate.

From the specific activity of the homogeneous preparation, we estimate that the recombinant *P. falciparum* TS–DHFR is expressed to a level of about 0.02% in soluble crude extracts or about 0.1 mg/L of culture. Since similar expression systems express very high levels of the related *Leishmania* TS–DHFR (Grumont et al., 1988), we think that the high A+T content of the *Plasmodium* gene may be associated with the poor expression levels. Although the expression system clearly needs optimization, it is a significant improvement over isolating the enzyme from infected red blood cells. The system provided sufficient enzyme from wild type 3D7 and drug-resistant strains HB3 and 7G8 to answer pertinent questions about the relationship between drug resistance and point mutations in DHFR.

The purified recombinant enzymes showed kinetic properties similar to those reported for TS–DHFRs directly isolated from *P. falciparum* (Zolg et al., 1989). Pyr was a potent inhibitor of DHFR of wild-type 3D7 DHFR ($K_i \sim 0.1$ nM), about 20-fold poorer toward the moderately resistant HB3 Ser 108/Asn mutant, and 2300-fold poorer toward the highly resistant 7GB Asn 51/Ile + Ser 108/Asn double mutant. The results confirm that point mutations confer resistance of DHFR toward Pyr and support the proposals that such mutations are responsible for resistance of *P. falciparum* toward the drug.

In summary, the systems described here permit expression of catalytically active *P. falciparum* TS–DHFR in *E. coli*. They are particularly amenable to preparation of point mutations in the DHFR gene at the positions putatively linked to Pyr resistance. Although the expression needs to be optimized, sufficient amounts of wild-type and mutant proteins can now be obtained to undertake studies on enzyme structure, function, and inhibition. Drugs inhibitory to Pyr-resistant DHFR would be useful in treatment of malaria in regions where a resistant strain is established. Further, if, as speculated, Pyr-resistant DHFRs arise first through the Ser 108/Asn mutant, then a drug inhibitory to this mutant used in combination with Pyr could decrease the emergence of Pyr-resistant malaria.

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Registry No. NADPH, 53-57-6; H_2folate, 4033-27-6; pyrimethamine, 58-14-0.

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