Heterologous Expression and Characterization of the Bifunctional Dihydrofolate Reductase−Thymidylate Synthase Enzyme of *Toxoplasma gondii*†

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ABSTRACT: We have expressed catalytically active *Toxoplasma gondii* dihydrofolate−thymidylate synthase (DHFR-TS) and the individual TS and DHFR domains in *Escherichia coli* using the T7 promoter of pET-15b. DHFR-TS constituted approximately 10% of the total soluble cell protein and was purified using methotrexate-Sepharose chromatography to yield 10 mg of homogeneous DHFR-TS per liter of culture. The DHFR domain was recovered as insoluble inclusion bodies which could be unfolded and refolded to recover soluble, active enzyme. The TS domain was overexpressed as a soluble protein by growing the cells at 24 °C; this is the first report of the expression of an active TS domain from a bifunctional enzyme. The k_{cat} and K_m values for DHFR-TS are similar to those of other previously characterized protozoan DHFRs and TSs. The antimicrobial antifolates, TMP and Pyr, inhibit DHFR activity of the bifunctional protein in accord with their effects in crude enzyme preparations and *in vivo* systems. Kinetic parameters and K_i values for TMP and Pyr with the isolated DHFR domain were identical to the values for DHFR in the bifunctional enzyme. Evidence of kinetic channeling of the dihydrofolate product of TS to the DHFR domain in the bifunctional enzyme was obtained by kinetic and inhibition studies. Properties such as yield, stability, and activities of the recombinant *T. gondii* DHFR-TS provide clear advantages over other bifunctional DHFR-TSs as a model for future studies.

Thymidylate synthase (TS)[1] and dihydrofolate reductase (DHFR) catalyze sequential reactions in the *de novo* synthesis of dTMP. Both TS and DHFR exist as distinct, monofunctional enzymes in sources as varied as bacteriophage, bacteria, fungi, viruses, and mammals (Ackermann & Potter, 1949; Blakley & Benkovic, 1984). In most genera, TS is a dimer of identical subunits of about 35 kDa, whereas monofunctional DHFRs are found as monomers of about 20 kDa. In contrast, TS and DHFR exist on the same polypeptide chain in protozoa (Ivanetich & Santi, 1990) and plants (Lazar et al., 1993). The bifunctional DHFR-TS is a dimer of identical subunits with an amino-terminal DHFR domain connected to a TS domain by a linker region that varies in length depending on the source of the enzyme.

The protozoan parasite *Toxoplasma gondii* is a ubiquitous organism capable of infecting a wide range of vertebrate hosts. It is estimated that approximately one-third of the US population is infected by *T. gondii* and 10−90% of the population elsewhere. Infection poses particular dangers for AIDS patients (Luft & Remington, 1992) and neonates. Although acute toxoplasmosis is effectively treated with a combination of an antifolate and a sulfonamide (Beaman et al., 1992), allergic reactions to sulfa drugs often preclude use of this combination. Hence, there is a need for new chemotherapeutic agents to treat toxoplasmosis.

One approach toward the development of antimicrobial agents involves the identification and exploitation of differences between the molecular structure of key parasitic enzymes and their mammalian counterparts. The susceptibility of *T. gondii* to antimicrobial antifolates and the qualitative differences between protozoan and human DHFRs suggests the bifunctional enzyme as a prime chemotherapeutic target. However, since only sparse amounts of protein are obtainable from the protozoan source (Kovacs et al., 1990), there is a severe limitation on the nature and scope of studies that can be carried out. We therefore sought to develop a heterologous expression system which would provide sufficient enzyme for such studies.

The genomic and cDNA sequences encoding DHFR-TS enzyme from *T. gondii* have recently been determined (Roos, 1993) and used to complement enzyme-deficient *Escherichia coli* and express drug-resistant alleles in transgenic parasites (Donald & Roos, 1993, Donald & Roos, 1994). In the present paper, we describe high-level expression of catalytically active *T. gondii* DHFR-TS and the individual DHFR and TS domains in *E. coli.* We also report the purification and characterization of these enzymes and demonstrate the phenomenon of substrate channeling between the TS and DHFR domains of the bifunctional protein.

MATERIALS AND METHODS

Materials. *E. coli* strains lacking DHFR [D3−157 (*fol* 200)] (Singer et al., 1985), TS [MM295 (TS::Tn5)], or both

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[1] Abbreviations: TS, thymidylate synthase; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine 5′-monophosphate; DHFR, dihydrofolate reductase; H_2folate, 7,8-dihydrofolate; nt, nucleotides; DTT, dithiothreitol; MTX, methotrexate; BSA, bovine serum albumin; Pyr, pyrimethamine; TMP, trimethoprim; CnDHFR, *Cryptococcus neoformans* DHFR; LcTS, *Lactobacillus casei* TS.

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enzyme activities (NM522 *thyA*, Δfol::kan) (Howell et al., 1988) and PA414 (AB1157 *thyA* Δfol::kan) (Ahrweiler & Frieden, 1988) were used for TS and DHFR complementation analysis. The expression vector, pET-15b, and the host strains, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS, were from Novagen. pSL1190, the superlinker phagemid, was from Pharmacia. Partial cDNA clones of *T. gondii* DHFR-TS, pPCR31-13 which contains nt −41 to 970 and λgt11-c6 which contains nt 970 to 2164, have been described previously (Donald & Roos, 1993; Roos, 1993) χ2913recA-(DE3) was lysogenized using the Novagen kit. Bluescript (pKS+) was from Stratagene. The Wizard kit for plasmid isolation and purification and the pGEM vector were purchased from Promega. Restriction endonucleases, T4 ligase, and other DNA-modifying enzymes were products of New England Biolabs, Life Technologies Inc., or Boehringer Mannheim. *E. coli* DH5α-competent cells were used as a host for plasmid-mediated transformations and general manipulation of recombinant plasmids. GeneClean was obtained from BIO101, and Prep-A-Gen was from Bio-Rad. Ampicillin, MTX, and NADPH were purchased from Sigma. MTX-Sepharose CL-6B (∼1 μmol of MTX/mL of resin) (Meek et al., 1985) and H_2folate (Friedkin et al., 1962) were prepared as described. Oligonucleotides were synthesized by cyanoethyl phosphoramidite chemistry at the Biomolecular Resource Center at the University of California San Francisco.

DNA Manipulations. General methods for ligation and transformation of *E. coli* were performed as previously described (Sambrook et al., 1989). Plasmid DNA was prepared for restriction analysis and automated sequencing using the Wizard miniprep kit. DNA fragments were isolated by agarose gel electrophoresis and purified using Prep-A-Gen or GeneClean III kits. Automated DNA sequencing was performed at the Biomolecular Resource Center, University of California San Francisco, by the Taq dye terminator cycle sequencing chemistry.

Construction of pTgDHFR-TS. The 1.2 kb *Eco*RI fragment from λgt11-c6 was excised and cloned into *Eco*RI digested pPCR31-13 to yield plasmid, pTgDHFR-TS. DNA sequence analysis confirmed the coding sequence. pTgDHFR-TS contained *T. gondii* DNA sequences including the complete DHFR-TS gene (nt 1−1834) plus 41 nt upstream of the ATG initiation codon and 331 nt downstream of the TAG stop codon. This plasmid complements *E. coli* strains D3-157 and PA414.

T. gondii DHFR-TS Coding Cassette. A synthetic DNA duplex, ON1a (5′- CAGG**ACCTGC**TTAAC**ATG**CAGAAA-3′) and ON1b (5′- CCGGTTTCTG**CAT**GTTAA**GCAG-GT**CCTGGGCC-3′) was cloned into *Age*I/ *Apa*I digested pTgDHFR-TS to yield pMT1. The ON1 duplex provides a 32 bp DNA fragment containing the coding sequence for amino acids 1−3, *Apa*I and *Age*I compatible overhangs (underlined nt), and a *Bsp*MI site (in bold). The *Bsp*MI site can be cleaved to generate an *Nco*I overhang which allows direct cloning into expression vectors that possess an *Nco*I site at the ATG codon (bold and underlined).

T. gondii DHFR Coding Cassette. The gel purified 4358 nt *Apa*I−*Xho*I fragment from pMT1 was cloned into *Apa*I−*Xho*I digested pSL1190. ON2a (5′ TCGAAAAGAGACG**T-TA**A**CTCGAG**A-3′) and ON2b (5′-AGCTT**CTCGAG**T-**TAA**CGTCTCTTT-3′) anneal to form a duplex with *Hin*dIII and *Xho*I compatible overhangs (underlined). A stop codon (bold and underlined) was placed immediately after the Arg 253 codon and an *Xho*I site (bold) following the stop codon. ON2 was ligated with *Xho*I/*Hin*dIII digested pSLDHFR to yield pMT2.

T. gondii TS Coding Cassette. The *Bgl*II−*Not*I fragment from pMT1, which encoded residues 87−295 of the isolated TS domain, was cloned into *Bgl*II−*Not*I digested pSL1190 to yield pSLTS. The 5′ end of the TS gene was amplified by PCR using pMT1 as a template. Two synthetic primers DNAs ON3 (5′- CATGCCATGGGCCATGAAGAATTC-CAG-3′) and ON4 (5′-GTCTCCGACCTCTCGGT-3′) were used for the PCR amplification. The ON3 primer introduced an *Nco*I site (underlined) at the ATG codon. ON4 primes in the reverse direction downstream from the *Bgl*II site. The resulting DNA fragment was digested with *Nco*I and *Bgl*II and cloned into pSLTS, yielding pMT3.

Growth Conditions for Expression. Bacteria were grown in LB supplemented with 100 μg/mL ampicillin. A fresh overnight culture from a single colony was used to inoculate 2 L of medium. The culture was grown at 37 °C until A_{600} reached 0.5−0.6, and then IPTG was added to a final concentration of 1 mM. The culture was allowed to grow at 37 °C for an additional 3 h prior to harvesting by centrifugation at 10000*g* for 10 min at 4 °C. When expressing the TS domain, the culture was grown at 30 °C until A_{600} reached 0.5−0.6, and then the temperature was shifted to 24 °C and IPTG was added to a final concentration of 0.4 mM. The culture was allowed to grow at 24 °C for 8 h before harvesting by centrifugation at 10000*g* for 10 min at 4 °C.

Purification of DHFR-TS. Cell paste from 2 L of culture was suspended in 25 mL of buffer A (10 mM KH_2PO_4, pH 7.0, 1 mM EDTA) containing a mixture of six protease inhibitors (Meek et al., 1985), and the cell suspension was subjected to two cycles of a French press (18,000 psi). After centrifugation at 30000*g* for 30 min at 4 °C to remove cell debris, the extract was circulated through a MTX-Sepharose column (1.5 × 10 cm) at a flow rate of 1 mL min^{-1} for 3 h at 4 °C. The column had been pre-equilibrated with buffer A that contained 0.2 M KCl. Approximately 92% of the enzyme activity bound to the column. The column then was washed with buffer A containing 0.5 M KCl (∼200 mL) until no protein was detectable in the effluent, and then it was re-equilibrated with buffer A containing 0.2 M KCl (∼50 mL). *T. gondii* DHFR-TS was eluted with 50 mL of 2 mM H_2folate in buffer A containing 0.2 M KCl. Initially, 25 mL of the elution buffer was equilibrated on the column for 20 min, and then the rest of the buffer was added. Fractions containing DHFR-TS activity (20 mL) were pooled, and H_2-folate was removed by chromatography on a DE52 column (2.5 × 50 cm) equilibrated with buffer B (50 mM TES, 0.1 mM EDTA, pH 7.0). DHFR-TS was eluted with buffer B containing 25 mM KCl; H_2folate remained bound to the column. Fractions that showed DHFR-TS activity were pooled (10 mL). The ratio of the absorbance at 280 nm to that at 320 nm was measured to confirm that the enzyme was free of H_2folate.

Purification of the DHFR Domain from Inclusion Bodies. Cell paste from 1 L of culture was suspended in 10 mL of buffer A, and the cell suspension was subjected to two cycles of a French press (18 000 psi). After centrifugation at 30000*g* for 30 min at 4 °C, the pellet was washed twice with buffer A. Inclusion bodies were purified by reported

procedures (Lin & Cheng, 1991). Unfolding and refolding were performed at 4 °C, as follows. Purified inclusion bodies from 1 L of culture were dissolved in 10 mL of buffer C (20 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 10 mM DTT, 20% glycerol) containing 0.2 M KCl and 6 M guanidine HCl. The mixture was stirred slowly at 4 ° C for at least 1 h to achieve complete unfolding. Refolding was achieved by dropwise addition of the unfolded protein into 20 volumes of buffer C containing 0.2 M KCl. After 3 h, the insoluble material was pelleted by centrifugation at 10,000 X g for 20 min, and the clear supernatant was loaded into a MTX-Sepharose column (1.5 × 5 cm) that had been pre-equilibrated with buffer C containing 0.2 M KCl. The enzyme purification was performed as described above.

Partial Purification of the TS Domain. Cell extract from 4 L of culture (20 g of cells) was loaded directly onto a DE52 column (2.5 × 50 cm) equilibrated in buffer D (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 2 mM DTT) containing 100 mM KCl. The column was eluted with the same buffer; fractions of 6 mL were collected, and those containing TS activity were pooled. The TS domain did not bind to the DE52 column equilibrated with buffer D containing 100 mM KCl, while the bulk of nucleic acids did. The DE52 eluate (45 mL) was diluted 2-fold with buffer D and then applied to a Q-Sepharose column (2.5 × 20 cm) previously equilibrated with buffer D containing 50 mM KCl at a rate of 1 mL min^{-1}. After the column was washed with 500 mL of buffer D containing 50 mM KCl, the protein was eluted with a 500 mL linear gradient of 0−500 mM KCl in buffer D at a flow rate of 1 mL min^{-1}. Fractions of 6 mL were collected, and those containing TS activity were pooled.

Enzyme Assays. TS activity was assayed spectrophotometrically as described (Pogolotti et al., 1986). The formation of H_2folate was monitored at 340 nm ($\epsilon_{340} = 6400$ M^{-1} cm^{-1}) in 50 mM TES, pH 7.8, 75 mM 2-mercaptoethanol, 1 mM EDTA, and 5 mM H_2CO. DHFR activity was monitored at 25 °C by the decrease in absorbance at 340 nm associated with product formation ($\epsilon_{340} = 12\,300$ M^{-1} cm^{-1}) (Hillcoat et al., 1967) in 50 mM TES, pH 7.0, 75 mM 2-mercaptoethanol, and 1 mg/mL BSA. For both TS and DHFR, one unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of product min^{-1} at 25 °C. For determination of the pH−initial velocity profiles, a three-component constant ionic strength buffer was used (Williams & Morrison, 1981). The concentration of active DHFR upon refolding was determined by titration with MTX (Sirawaraporn et al., 1993b). The concentration of MTX was determined spectrophotometrically (Seeger et al., 1949).

For determination of the steady-state kinetic parameters, the concentration of one substrate was varied between $0.5K_m$ and $10K_m$, with the other substrate fixed at 100 μM; reactions were initiated by addition of the enzyme. K_m values for H_2-folate and NADPH were determined using a 10 cm cuvette. Kinetic constants were determined from a nonlinear square fit to the Michaelis−Menten equation using Kaleidagraph (Abelbeck Software) on a Macintosh computer.

Inhibition of DHFR by TMP and Pyr was measured by varying the concentration of inhibitor in a reaction mixture containing 2.5 nM enzyme and 25 μM H_2folate. The equation for competitive inhibition from Segel (1975) was used to calculate K_i.

Coupled Assay. The DHFR-TS coupled system was assayed by monitoring the decrease in NADPH absorbance at 340 nm in the absence of added H_2folate (Meek et al., 1985). The assay mixture containing 50 mM TES, pH 7.8, 75 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM H_2CO, 30 μM NADPH, 28 μM CH_2-H_4folate, 1 mg/mL BSA, and specified amounts of enzyme was incubated at 25 °C until no change in absorbance at 340 nm was detectable (about 2−5 min). The reaction was initiated by addition of 10 μl of 10 mM dUMP (final concentration 100 μM), and the absorbance was monitored at 340 nm ($\Delta\epsilon_{340} = 6000$ M^{-1} cm^{-1}). The coupled system is complicated by the fact that CH_2H_4folate inhibits DHFR activity in the bifunctional enzyme ($K_i = 100$ μM); therefore, we used a low concentration of CH_2H_4folate, 28 μM, and a regeneration system for the cofactor.

Individual TS and DHFR rates were obtained under conditions of the coupled assay, with the following modifications: for DHFR, dUMP was omitted and 100 μM H_2folate was included; for TS, NADPH was omitted and the formation of H_2folate was monitored spectrophotometrically.

The effect of inhibitors in the coupled assay was analyzed by including different concentrations of the inhibitor in the assay mixture and monitoring the decrease in NADPH absorbance as in the regular coupled assay. Rates were measured after reaching the steady-state concentration of H_2-folate.

Protein Analysis. Protein concentrations in the crude extract were determined by the Bradford method using BSA as a standard (Bradford, 1976). The concentration of the purified enzyme was determined spectrophotometrically at 280 nm by the method of Edelhoch (1967), using a molar extinction coefficient for *T. gondii* DHFR-TS of 170 000 M^{-1} cm^{-1}. SDS−PAGE was performed as previously described (Read & Northcote, 1981).

Analytical Gel Filtration. Gel filtration of *T. gondii* DHFR-TS was performed on a Superose 12HR 10/30 column. One hundred microliters of the purified enzyme (4 mg/mL) in buffer B, pH 7.0, containing 200 mM KCl, was injected and the column was eluted with the same buffer at a flow rate of 0.2 mL min^{-1}. The column was calibrated with molecular mass standards of 43−200 kDa.

RESULTS

Expression Vectors for DHFR-TS. The *T. gondii* DHFR-TS gene was subcloned under control of the T7 promoter in pET-15b (Figure 1). First, the complete DHFR-TS coding sequence was assembled in pTgDHFR-TS by combining sequences from two partial cDNA clones, λgt11-c6 and pPCR31−13 (Donald & Roos, 1993; Roos, 1993). This construct complements DHFR and TS-deficient *E. coli* mutants. A synthetic oligonucleotide duplex, ON1, was cloned in the unique *Age*I site at nt 9 of the DHFR gene and the *Apa*I site in the polylinker of pTgDHFR-TS. The resulting plasmid, pMT1, contains within the ON1 fragment a unique *Bsp*MI site which can be cleaved to yield an *Nco*I compatible overhang at the ATG start codon. The coding cassette was excised with *Nco*I and *Bam*HI and cloned into *Nco*I−*Bam*HI digested T7 promoter vector, pET-15b, to yield pMTDHFR-TS.

Expression Vectors for DHFR and TS Domains. The individual DHFR and TS domains of the bifunctional protein were cloned under control of the T7 promoter. Arg 253 was

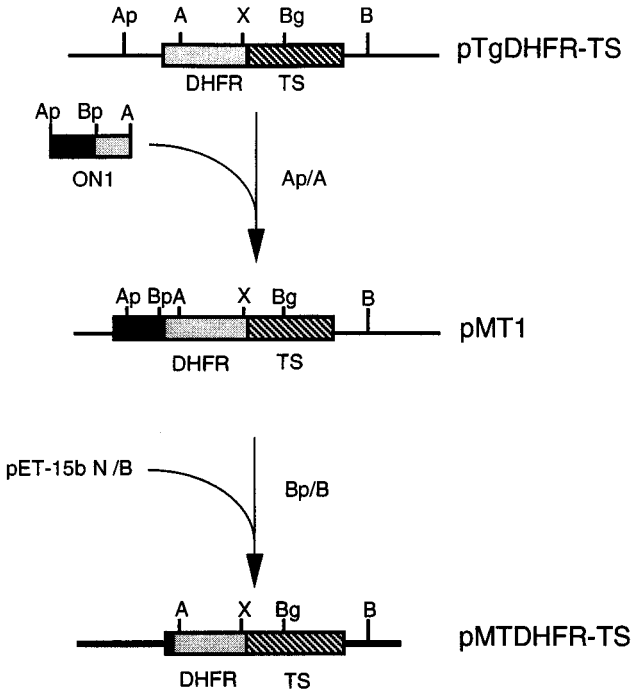


FIGURE 1: Assembly of the bifunctional *T. gondii* DHFR-TS. Restriction enzymes and sites are designated: Ap, *Apa*I; A, *Age*I; B, *Bam*HI; Bg, *Bgl*II; Bp, *Bsp*MI; N, *Nco*I; X, *Xho*I. Restriction enzymes beside arrows designate digestion steps. Converging arrows represent ligation/transformation steps. ON1 is a synthetic oligonucleotide duplex encoding the first three amino acids of DHFR and inserting a *Bsp*MI site to provide an *Nco*I overhang at the start codon. ON1 was inserted into pTgDHFR-TS, creating a coding cassette in pMT1. The entire resulting coding sequence was then cloned into pET-15b.

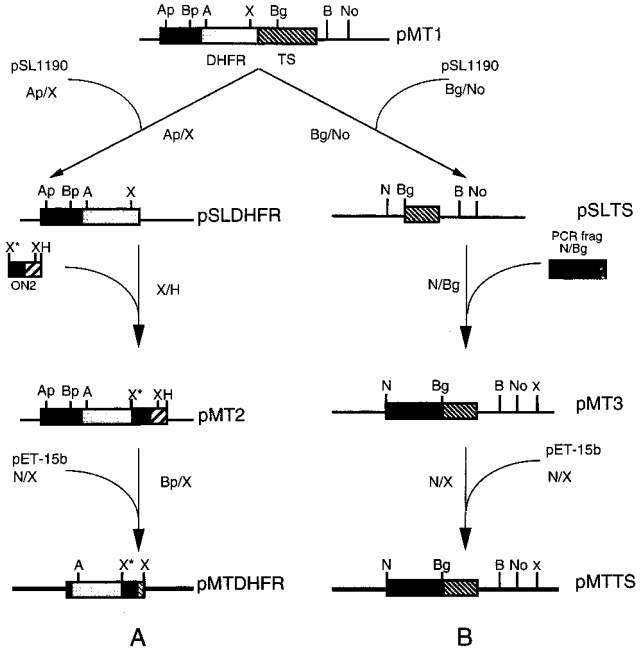


FIGURE 2: Assembly of the *T. gondii* DHFR and TS domains. Abbreviations and symbols are as in Figure 1. Additional restriction enzymes: H, *Hin*dIII; No, *Not*I; X*, destroyed *Xho* I site. (I) DHFR domain: ON2 is a synthetic oligonucleotide duplex which inserts a stop codon at the C-terminus. This synthetic duplex and the rest of the DHFR coding sequence were assembled in pSL1190. The entire DHFR coding sequence was then cloned into pET-15b. (II) TS domain: A PCR fragment was designed to create the N-terminus of the isolated TS domain. This fragment and the rest of the TS coding sequence were assembled in pSL1190. The entire TS coding sequence was then cloned into pET-15b.

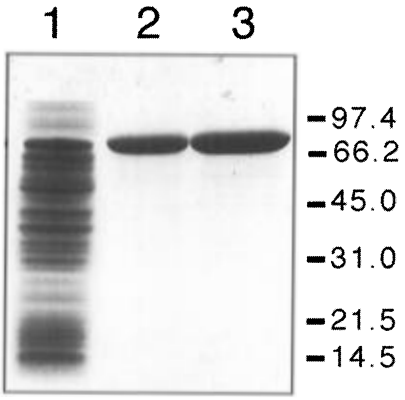


FIGURE 3: SDS−PAGE showing purification of the recombinant *T. gondii* DHFR-TS. Lane 1, crude extract; lane 2, MTX-Sepharose pool; lane 3, DE52 pool.

chosen as the C-terminus of the DHFR domain based on the following considerations. Comparison of the three-dimensional structures of monofunctional DHFRs (Bolin et al., 1982; Davies et al., 1990) and the *Leishmania major* bifunctional enzyme (Knighton et al., 1994) indicated the presence of a conserved β strand near the C-terminus of DHFR. An alignment of the amino acid sequences of 28 DHFRs (Reche et al., 1994) showed that many monofunctional DHFRs have C-termini three residues after the conserved β-strand. In *T. gondii* DHFR-TS the third residue after the β-strand corresponds to Arg 253. The expression vector for the isolated DHFR domain was constructed utilizing a unique *Xho*I site which occurs at nt 701, five codons upstream from the selected C-terminus (Figure 2A). First, the *Apa*I−*Xho*I fragment from pMT1 was cloned into pSL1190 to yield pSLDHFR. The synthetic oligonucleotide duplex, ON2, which restored the last five codons of DHFR, was then cloned into pSLDHFR to yield pMT2. ON2 was designed to destroy the *Xho*I site within the gene and to introduce a stop codon plus a new *Xho*I site at the 3′ end of the coding sequence. The *Bsp*MI−*Xho*I fragment of pMT2 (nt −1 to 760) containing the entire DHFR coding sequence was cloned into pET-15b to create pMTDHFR, which contained the DHFR gene under control of the T7 promoter.

His 232 was chosen as the N-terminus for the TS domain. In alignments of 29 monofunctional and bifunctional TS sequences, His-Glu-Glu (residues 232−234) is a moderately conserved motif followed by highly conserved sequences (Carreras & Santi, 1995). There is considerable variation in the length and sequence of the TS N-terminus upstream from the selected His. Therefore, Met-Gly codons were added to provide a start codon with an *Nco*I site. The expression vector for the isolated TS domain was constructed by first assembling a coding cassette in pSL1190 (Figure 2B). The *Bgl*II−*Not*I fragment from pMT1 plus a PCR fragment encoding the new 5′ end of the domain were cloned in successive steps to yield pMT3. The *Nco*I−*Xho*I fragment (nt −1 to 1304) containing the entire TS coding sequence was cloned into pET-15b to create pMTTS, which contained the TS gene under the control of the T7 promoter.

Expression and Purification of DHFR-TS. pMTDHFR-TS was used to transform *E. coli* BL21(DE3) and *E. coli* BL21(DE3) pLysS cells, and soluble cell extracts from IPTG induced cultures were analyzed by both SDS−PAGE and enzyme activity measurements. Substantial DHFR and TS activities were observed in both transformed strains (Table

Table 1: Expression of *T. gondii* DHFR-TS

source	specific activity (units/mg)	
	DHFR	TS
BL21(DE3) pET-15b	0.01	0.06
BL21(DE3) pTgDHFR-TS	0.68	0.4
BL21(DE3)pLysS pTgDHFR-TS	0.66	0.5

1), and the extracts showed a new 70 kDa protein on SDS−PAGE that was estimated to constitute more than 10% of the soluble protein (Figure 3).

DHFR-TS was purified to apparent homogeneity by affinity chromatography on MTX-Sepharose followed by DE52 chromatography to remove H_2folate (Figure 3). Approximately 42 mg of pure DHFR-TS was obtained from 4 L of culture (Table 2). Both TS and DHFR activities were stable during the purification procedure. The purified protein could be stored in buffer B containing 20% glycerol at −80 °C for up to 6 months without loss of either activity.

Characterization of DHFR-TS. The purified enzyme migrated as a single 68 kDa band on SDS−PAGE (Figure 3) and eluted from Superose 12 with an apparent molecular mass of 140 kDa. We concluded that the native protein was a dimer of identical subunits.

We measured the effect of pH, ionic strength, and urea concentration on the specific activity of DHFR and TS in the bifunctional enzyme. The pH optima in constant ionic strength buffer were 6.4 and 7.5 for DHFR and TS activities, respectively, with half-life maximal activity at pH 7.6 for DHFR and at 9.0 for TS. The specific activity of TS decreased with increasing NaCl or KCl concentration, with approximately 50% reduction in activity at 0.5 M salt. In contrast, DHFR was activated 2−3-fold by increasing NaCl or KCl concentration with a broad peak at 0.5 M salt. DHFR activity increased 5-fold, and TS activity decreased 4-fold with 1 M urea (data not shown).

Kinetic parameters were measured for the DHFR and TS activities in the bifunctional protein (Table 4). K_m values for DHFR were 0.6 ± 0.1 and 0.5 ± 0.1 μM, for H_2folate and NADPH, respectively, and k_{cat} was 13.0 ± 0.1 s^{-1}. TS K_m values were 33 ± 4 and 4 ± 1 μM for CH_2H_4folate and dUMP, respectively, and k_{cat} was 7.5 ± 0.1 s^{-1} (Table 4).

IC_{50} values for TMP and Pyr were determined in a reaction containing 25 μM H_2folate. K_i values were calculated to be 190 and 7.5 nM, respectively, for TMP and Pyr (Table 4).

Expression and Purification of the DHFR Domain. E. coli BL21(DE3) and *E. coli* BL21(DE3) pLysS cells were transformed with pMTDHFR. Cell extracts from IPTG induced cultures grown at 37 °C were analyzed using SDS−PAGE. In both strains, the expressed DHFR represented more than 50% of the total protein but was present in the insoluble pellet. Induction of the cells at 25 °C did not increase DHFR activity in the soluble fraction. We isolated active DHFR by unfolding and refolding the enzyme from inclusion bodies as described by Lin and Cheng (1991) and Sirawaraporn et al. (1993). Different procedures for unfolding and refolding were investigated (Table 3). In the final procedure, the protein was unfolded in 6 M guanidine HCl and refolded by 20-fold dilution into a buffer containing 20% glycerol. After refolding, DHFR was obtained that was about 80% homogeneous by SDS−PAGE.

The refolded DHFR domain was purified by MTX-Sepharose affinity chromatography. The purified enzyme migrated as a single band with an apparent molecular mass of ∼30 kDa. The specific activity of the purified protein was 1.8 units/mg, and active site titration with MTX indicated 100% activity.

Characterization of the DHFR Domain. The K_m values for the isolated DHFR domain were 0.4 and 0.6 μM for NADPH and H_2folate, respectively, essentially identical to the values obtained for the bifunctional enzyme (Table 4).

IC_{50} values for TMP and Pyr were determined in a reaction containing 25 μM H_2folate. K_i values were 195 nM for TMP and 8 nM for Pyr, identical to the values of the bifunctional DHFR-TS (Table 4).

Expression and Purification of the TS Domain. χ2913recA(DE3) cells were transformed with pMTTS, and the cell extracts from IPTG induced cultures grown at 37 °C were analyzed by SDS−PAGE. The expressed product was mainly present in the insoluble pellet. Induction of the cells with 0.4 mM IPTG at 24 °C resulted in expression of the protein in the soluble fraction. TS was purified 5-fold by successive DE52 and Q-Sepharose chromatography steps. After this purification, TS was about 60% homogeneous as judged by SDS−PAGE. The partially purified preparation had a specific activity of 0.3−0.4 unit mg^{-1} and was suitable for channeling experiments.

Coupled Assay Kinetics. A coupled assay (Scheme 1) was used to monitor the DHFR-catalyzed reduction of the H_2-folate formed by TS (Meek et al., 1985). As H_2folate is formed in the TS reaction, it is converted into H_4folate by the DHFR catalyzed oxidation of NADPH, which can be monitored at 340 nm.

Under steady-state conditions for the TS reaction, eq 1

$$[NADP^+] = v_1 t + (v_1/v_2)K_m(e^{-v_2 t/K_m} - 1) \qquad (1)$$

(Easterby, 1973; Meek et al., 1985; Rudolph et al., 1979) describes the time course of $NADP^+$ formation.

In this equation, K_m is the K_m for H_2folate, v_1 is the rate (μM min^{-1}) of TS under conditions of the coupled assay, and v_2 is the rate (μM min^{-1}) of DHFR using near-saturating concentrations of substrates. In a plot of [$NADP^+$] versus time, extrapolation of the linear portion of a plot of [$NADP^+$] vs time to the horizontal axis (K_m/v_2) provides the lag time that precedes attainment of the steady-state concentration of H_2folate. Extrapolation to the vertical axis of the curve

Table 2: Purification of the Recombinant *T. gondii* DHFR-TS[a]

step	protein (mg/mL)	volume (mL)	total protein (mg)	DHFR activity		TS activity		purification	
				units/mg	total units	units/mg	total units	(%)	fold[b]
crude extract	24.4	25	611	0.3	182	0.1	61	100	
MTX-Sepharose	4.3	16	69	2.6	138	0.7	48	75	8.7
DE52-Sepharose	8.4	5	42	2.8	120	1.4	59	66	9.5

[a] Prepared from 4000 mL of culture. [b] Based on DHFR activity.

Table 3: Conditions for Unfolding and Refolding of *T. gondii* DHFR

unfolding buffer	method of refolding	refolding buffer	specific activity of refolded *T. gondii* DHFR (units/mg)[b]
8 M urea in buffer C[a] + 0.1 M KCl	10-fold dilution	buffer A + 0.1 M KCl	0.4−0.5
8 M urea in buffer C + 0.2 M KCl	10-fold dilution	buffer A + 0.2 M KCl	0.3−0.5
6 M guanidine HCl in buffer C+ 0.2 M KCl	10-fold dilution	buffer A + 0.2 M KCl	0.7−0.8
6 M guanidine HCl in buffer C +0.2 M KCl	20-fold dilution	buffer A + 0.2 M KCl	0.6−0.9

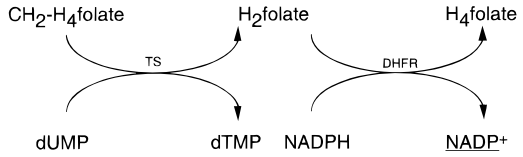
[a] Buffer C consists of 20 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 20% glycerol, and 5 mM DTT. [b] Protein concentration was measured by the Bradford assay (Bradford, 1976).

Table 4: Kinetic and Inhibition Constants for DHFR-TS and the DHFR Domain

	kinetic parameters						IC_{50}[a]		K_i[c]	
recombinant enzyme	K_m for H_2folate (μM)	K_m for NADPH (μM)	k_{cat} (s^{-1})	K_m for CH_2H_4folate (μM)	K_m for dUMP (μM)	k_{cat} (s^{-1})	TMP (μM)	Pyr (μM)	TMP (nM)	Pyr (nM)
DHFR-TS	0.6 ± 0.1	0.5 ± 0.1	13.0 ± 0.1	33 ± 4	4 ± 1	7.5 ± 0.1	7.9	0.29	190 ± 5	0.2 ± 0.1[e]
DHFR domain	0.60 ± 0.02	0.4 ± 0.1	ND[b]	—[d]	—[d]	—[d]	8.0	0.30	195 ± 5	0.2 ± 0.1

[a] The concentration of H_2folate is 25 μM in the assay mixture. [b] Not determined. [c] Calculated using equation for inhibition by a competitive inhibitor $IC_{50} = (1 + [S]/K_m)K_i$. Segel (1975) pp 105 (eq III-5). [d] Not applicable. [e] The K_i for Pyr was obtained by fitting the data to the equation for tight inhibitors Segel (1975) pp 105 (eq III-63).

Scheme 1



(v_1K_m/v_2) provides the steady-state concentration of H_2folate. A simulated curve is shown at Figure 4A.

The applicability of eq 1 under our experimental conditions was confirmed using mixtures of different individual monofunctional enzymes. The mixture of *Lactobacillus casei* TS (LcTS) (Pinter et al., 1988) with either *Cryptococcus neoformans* DHFR (CnDHFR) (Sirawaraporn et al., 1993a) or *T. gondii* DHFR domain or the mixture of *T. gondii* TS with the *T. gondii* DHFR domain were used in the coupled assay. With each mixture tested, both the lag time of $NADP^+$ formation and the steady-state concentration of H_2-folate were in good agreement with those calculated from eq 1 (Table 5). Figure 4A shows the close correspondence of the experimental curve for LcTS and CnDHFR with the curve simulated from eq 1.

Using the K_m value for H_2folate and the individual rates of the DHFR and TS components of the bifunctional enzyme determined under coupled assay conditions, eq 1 predicted 30 or 46 s lag times for different concentrations of the bifunctional DHFR-TS (Table 5). However, no lag time could be detected in two different preparations of the bifunctional enzyme with slightly different DHFR/TS ratios (Table 5 and Figure 4B).

The coupled assay was performed using varying concentrations of NaCl to test the proposal that electrostatics may be involved in substrate channeling of DHFR-TS (Knighton et al., 1994). At 0.5 M NaCl, where the rate of TS was 0.10 μM min^{-1} and the rate of DHFR was 2.3 μM min^{-1}, the predicted lag time for noninteracting enzymes was 15 s. Under these conditions, we detected a lag time of 10 s. Although this result is in accord with involvement of electrostatic effects in kinetic channeling, the interpretation is encumbered by the fact that DHFR activity is stimulated by increasing ionic strength, while TS activity is inhibited. The effect of urea and guanidine were also tested. These agents also activated DHFR activity and inhibited TS. It is not possible to measure an effect on channeling.

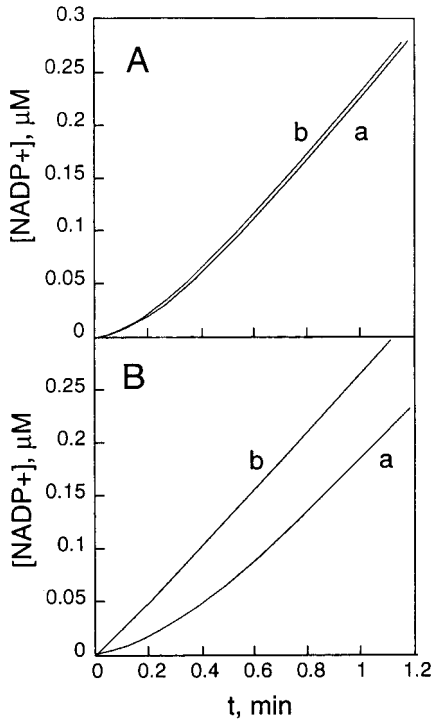


FIGURE 4: Experimental and simulated time courses for $NADP^+$ formation in the coupled assay. Reactions were performed as described under Materials and Methods, and simulations were generated from to eq 1. (A) Curve a is the observed time course when LcTS ($v_{(TS)} = 0.40$ μM min^{-1}) and CnDHFR ($v_{(DHFR)} = 1.62$ μM min^{-1}) are added to the cuvette. Curve b is the time course calculated using the same rates and $K_m = 0.48$ μM (Sirawaraporn et al., 1991). (B) Curve a is the observed time course using the bifunctional enzyme from *T. gondii*, where $v_{(TS)} = 0.28$ μM min^{-1} and $v_{(DHFR)} = 1.12$ μM min^{-1}. Curve b is the time course calculated from these values and $K_m = 0.6$ μM.

Inhibition of DHFR in the Coupled Assay. Evidence for kinetic channeling may be obtained by studies of the effects of competitive inhibitors on the DHFR reaction in the coupled assay. The premise is that if a higher local concentration of H_2folate is provided to DHFR in the channeled system, the apparent inhibitory effects of competitive inhibitors should decrease. Using the independently

Table 5: Coupled DHFR-TS Assay Using Monofunctional TS and DHFR Enzymes and the Bifunctional *T. gondii* DHFR-TS

source	experimental no.	rate (μM min^{-1})		lag time of $NADP^+$ formation (s)		steady-state [H_2folate] (μM)	
monofunctional		TS	DHFR	calcd	exptl	calcd	exptl
Lc TS + Cn DHFR	A	0.82	1.27	20	22 ± 2	0.28	0.27 ± 0.03
	B	0.40	1.62	18	20 ± 2	0.29	0.28 ± 0.03
	C	0.82	2.42	10	12 ± 1	0.15	0.16 ± 0.02
Lc TS + Tg DHFR[b]	A	0.78	1.12	15	16 ± 2	0.40	0.41±0.04
TgTS[c] + TgDHFR	A	0.18	0.64	56	60 ± 4	0.17	0.15 ± 0.01
bifunctional							
Tg DHFR-TS[d]	A	0.28	1.12	30	*a*	0.15	*a*
	B	0.29	0.79	46	*a*	0.22	*a*
	C	0.48	1.20	30	*a*	0.22	*a*

[a] Not detectable. [b] *T. gondii* DHFR domain. [c] *T. gondii* TS domain. [d] Bifunctional *T. gondii* DHFR-TS.

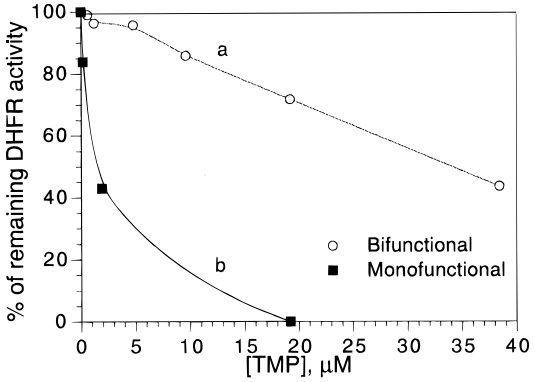


FIGURE 5: Inhibition of monofunctional and bifunctional DHFR from *T. gondii* by TMP in the coupled assay. Reactions were performed as described under Materials and Methods. Curve a is the inhibition seen when using the bifunctional enzyme from *T. gondii*, ($v_{(TS)}$ = 3.6 μM min^{-1} and $v_{(DHFR)}$ = 14. 4 μM min^{-1}). Curve b is the is the inhibition seen when LcTS ($v_{(TS)}$ = 3.6 μM min^{-1}) and *T. gondii* DHFR domain ($v_{(DHFR)}$ = 14.2 μM min^{-1}) were used in the coupled assay. We are unaware of previous reports of the seemly obvious use of competitive inhibitors for this purpose.

determined K_i values and the concentration of exogenous inhibitor required to inhibit DHFR by 50%, the "apparent concentration", $[S]_{app}$, of the substrate in the coupled system may be calculated from eq 2 (Segel, 1975). The $[S]_{app}$

$$[S]_{app} = [(IC_{50}/K_i) - 1]K_m \quad (2)$$

reflects the local concentration of the substrate, H_2folate, available to DHFR.

The IC_{50} values for TMP and Pyr were measured in the coupled assay using either the bifunctional enzyme or the DHFR domain plus LcTS adjusted such that the resulting rates were the same as the rates in the bifunctional enzyme (Figure 5). The inhibition of the DHFR activity in the bifunctional enzyme was significantly lower than in the monofunctional DHFR. Using eq 2 and the K_i value in Table 4, we calculated that increases in the apparent H_2folate concentration were 15- and 20- fold higher with the bifunctional protein than with the mixtures of monofunctional enzymes for TMP and Pyr, respectively.

DISCUSSION

Of the recombinant DHFR-TSs thus far reported, *T. gondii* DHFR-TS was best suited for in-depth studies. First, the expression of soluble, active DHFR-TS in *E. coli* was the highest of any bifunctional enzyme (Gamarro et al., 1995; Reche et al., 1994; Sirawaraporn et al., 1990) and provides an abundant source of protein for structural and functional studies. Second, the TS specific activity is higher than those other reported recombinant DHFR-TSs (Gamarro et al., 1995; Reche et al., 1994; Sirawaraporn et al., 1990). Third, unlike other recombinant DHFR-TSs, both activities of the *T. gondii* bifunctional enzyme were stable under conditions of purification and storage. Finally, the DHFR/TS ratio was well suited for studies of kinetic coupling of the two activities.

The expressed enzyme represented about 10% of the soluble protein and was purified to apparent homogeneity in high yield by MTX-Sepharose affinity chromatography. SDS−PAGE and gel filtration studies indicated that the protein was a homodimer with subunit molecular mass of ca. 68 kDa, in agreement with the size predicted from the DNA sequence.

Steady-state kinetic measurements of the activities of the bifunctional protein showed K_m and k_{cat} values similar to those reported for other bifunctional DHFR-TSs (Gamarro et al., 1995; Reche et al., 1994; Sirawaraporn et al., 1990, 1991, 1993b). The antimicrobial antifolates, TMP and Pyr, were good inhibitors of *T. gondii* DHFR with Pyr showing the most potent inhibition. These results are in accord with *in vivo* effects (Eyles & Coleman, 1955) and with measurements made on enzyme in crude extracts of organisms (Chio & Queener, 1993).

We have also cloned and expressed the DHFR and TS domains of the *T. gondii* bifunctional protein. The C-terminus for the DHFR domain and the N-terminus for the TS domain were chosen after considering sequence alignments and three-dimensional structural data on the monofunctional and bifunctional counterparts from other species. The isolated DHFR and TS domains were expressed as inclusion bodies after growth and induction at 37 °C. Reduced temperature and IPTG concentration resulted in expression of the soluble TS domain, and this represents the first reported expression of an active TS domain from a bifunctional enzyme. After unfolding and refolding DHFR from inclusion bodies the homogeneous, fully active DHFR domain could be obtained after affinity chromatography on MTX-Sepharose. The steady-state kinetic parameters of the DHFR domain and its inhibition by the antifolates TMP and Pyr were essentially identical to those of the DHFR in the bifunctional protein.

It has been suggested that a possible biological advantage of the bifunctional DHFR-TS over the monofunctional counterparts might be substrate channeling of the H_2folate produced by TS to the DHFR active site (Meek et al., 1985). Indeed, kinetic evidence for substrate channeling of H_2folate

in the *L. major* DHFR-TS has been reported (Meek et al., 1985). To obtain evidence for kinetic channeling in the *T. gondii* bifunctional enzyme, we studied the kinetic behavior of DHFR-TS using an assay which couples the two activities. When DHFR and TS are present in a reaction containing all substrates except H_2folate, the oxidation of NADPH to $NADP^+$ can only occur via concomitant reduction of the H_2-folate endogenously produced by TS. Using purified preparations of monofunctional DHFR and TS, including the *T. gondii* TS and DHFR domains, measurements of steady-state concentrations of H_2folate and the lag times required to achieve steady state agreed with simulations based on noninteracting coupled enzymes (eq 1). However, with the bifunctional DHFR-TS there was no lag time in production of $NADP^+$ under conditions where the simulated noninteracting systems predicted lag times of 30−46 s. Since we could have detected a lag time as low as 10 s, at least 80% of the H_2folate produced by TS was transferred to the DHFR site faster than it was released into bulk solvent.

The effects of competitive inhibitors of DHFR in the coupled assay on the bifunctional versus monofunctional systems provided a novel approach for investigating kinetic channeling. The concentrations of monofunctional enzymes were adjusted to possess the same TS and DHFR rates as the rates in the bifunctional enzyme. In the absence of substrate channeling, both systems would provide the same steady concentrations of H_2folate, and the effect of the inhibitor should be the same. In contrast, substrate channeling would provide a higher local concentration of H_2-folate to DHFR, and hence a higher concentration of analog would be required to inhibit the enzyme. Assuming that the K_i of the inhibitor in the coupled assay is the same as that determined in the standard DHFR assay, an "effective" concentration of H_2folate available to DHFR in the coupled assay can be calculated (eq 2). The effective concentration of H_2folate calculated by this method was 15−20-fold higher in the bifunctional system than monofunctional system, providing additional evidence for substrate channeling.

Having obtained evidence for kinetic channeling of H_2-folate in the bifunctional DHFR-TS, we considerate to possible mechanisms for the phenomenon. In the crystal structure of *Leishmania* DHFR-TS (Knighton et al., 1994) the TS and DHFR folate binding sites are too far apart (40 Å) for direct transfer of H_2folate without a major conformational change of the protein, and there is no protected channel available for transport of H_2folate from TS to DHFR (Hyde et al., 1988). However, there is a positive electrostatic potential surface around and between both the TS and DHFR folate binding sites which has been suggested to play a role in kinetic channeling (Knighton et al., 1994; Stroud, 1994). It has been proposed that the glutamic acid moiety of the H_2folate binds to the positively charged surface of the enzyme and is guided from one active site to the other. The positive surface is conserved in the DHFR domains of *T. gondii* and other bifunctional enzymes from protozoa, suggesting a functional role for these regions. If electrostatics are an important factor in kinetic channeling, an increase in the ionic strength could disrupt the interactions between H_2folate and the positively charged surfaces of the bifunctional protein. With salt concentration of 0.5 M NaCl, channeling was disrupted and a lag time for a noninteracting system was observed. Thus, although the mechanism of channeling is not completely understood, it does appear that the electrostatic surface in bifunctional DHFR-TSs may play a role in this phenomenon.

In summary, heterologous expression of both the full-length *T. gondii* DHFR-TS and monofunctional DHFR and TS domains has been accomplished. Both kinetic and inhibition studies provide direct evidence for channeling of the H_2folate product from TS to the DHFR domain. In many respects, the *T. gondii* enzyme offers distinct advantages over other systems for further studies of bifunctional DHFR-TSs. High yields of stable, active protein are available for the biochemical and structural studies needed for rational drug design. The effects of antimicrobial antifolates on recombinant *T. gondii* DHFR activity (of the bifunctional protein) are comparable to *in vivo* studies and results using crude enzyme preparations from isolated parasites. Finally, the accessibility of *T. gondii* to molecular genetic manipulation (Donald & Roos, 1995) provides the means to test hypotheses based on heterologous expression and modeling studies *in vivo*.

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