Mechanistic Characterization of *Toxoplasma gondii* Thymidylate Synthase-Dihydrofolate Reductase: Evidence for a TS Intermediate and TS Half-Sites Reactivity†

Eric F. Johnson¥, Wolfgang Hinz¥, Chloé E. Atreya¥, Frank Maley‡, Karen S. Anderson¥§

From ¥ Yale University School of Medicine Department of Pharmacology, 333 Cedar Street, New Haven, Connecticut 06520, and the ‡New York State Department of Health, Empire State Plaza, Box 509, Albany, New York 12201.

§ Author to whom correspondence should be addressed.

Tel: (203) 785-4526

Fax: (203) 785-7670

E-mail: karen.anderson@yale.edu

†This work was supported by National Institute of Health AI44630 and American Cancer Society Grant RPG-98-027-01-CDD to Karen S. Anderson, National Institute of Health CA44355 to Frank Maley, and National Institute of Health Medical Scientist Training Program GMO7205 to Chloé E. Atreya.

**Running Title:** Mechanistic Characterization of *T. gondii* TS-DHFR

**SUMMARY**

This study describes the use of rapid transient kinetic methods to characterize the bifunctional TS-DHFR enzyme from *Toxoplasma gondii*. In addition to elucidating the detailed kinetic Scheme for this enzyme, this work provides the first direct kinetic evidence for the formation of a TS intermediate and for half-sites TS reactivity in human and *Escherichia coli* monofunctional TS and in *Toxoplasma gondii* and *Leishmania major* bifunctional TS-DHFR. Comparison of the *Toxoplasma gondii* TS-DHFR catalytic mechanism to that of the *Leishmania major* enzyme reveals the mechanistic differences to be predominantly in DHFR activity. Specifically, TS ligand induced domain-domain communication involving DHFR activation is observed only in the
**Leishmania major** enzyme and, while both DHFR activities involve a rate-limiting conformational change, the change occurs at different positions along the kinetic pathway.

**INTRODUCTION**

Thymidylate synthase (TS\(^1\)) and dihydrofolate reductase (DHFR) are essential metabolic enzymes and established targets for anti-cancer and anti-microbial drugs (1-3). While, in most species including humans, TS and DHFR activities reside on separate monofunctional enzymes, several protozoan parasites have these activities expressed on a single polypeptide chain that comprises a bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR\(^2\)) enzyme (4-8). One of these protozoa, *Toxoplasma gondii*, is prevalent and problematic in the US (8). Opportunistic toxoplasmosis is often associated with the onset of the clinical AIDS syndrome and is a primary cause of suffering and death in AIDS patients (6). Enzymes unique to the parasite, including the bifunctional TS-DHFR, are optimal targets for the development of new antiparasitic drugs.

As illustrated in **Scheme 1**, TS catalyzes the only *de novo* source of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). The reaction uses (6R)-L-5,10-methylenetetrahydrofolate (CH\(_2\)H\(_4\)F) as a cofactor for the one carbon transfer reaction generating 7,8-dihydrofolate (H\(_2\)F) in the process. DHFR regenerates the fully reduced form of the folate (6R)-5,6,7,8-Tetrahydrofolate (H\(_4\)F) from H\(_2\)F using NADPH as a cofactor and generating NADP\(^+\) in the process. H\(_4\)F can then be primed for subsequent one carbon transfer reactions in the cell. In the presence of dUMP, NADPH,
and CH$_2$H$_4$F, the bifunctional TS-DHFR enzymes catalyze the conversion of CH$_2$H$_4$F directly to H$_4$F.

**Scheme 2** illustrates the detailed catalytic mechanism that has been proposed for the TS reaction (9). Following ordered substrate binding, in which dUMP binds first, a conformational change takes place whereby the C-terminal tetrapeptide of TS closes over the substrates to create an active site cavity shielded from solvent (step 1) (1,10-14). It has been suggested that this is followed by the formation of an iminium ion involving the bridge methylene and N-5 of CH$_2$H$_4$F (step 2) (15).

It is this highly reactive electrophilic iminium ion that has been proposed to be the reactive form of the cofactor. In fact, the structure of a TS mutant lacking a C-terminal valine and crystallized with CH$_2$H$_4$F and 5-fluorodeoxyuridine monophosphate (FdUMP) in which (6R)-L-5-hydroxymethyltetrahydrofolate (HO-CH$_2$H$_4$F) is bound at the active site provided structural evidence supporting the formation of this iminium ion during TS catalysis (10). Kinetic isotope effect studies suggest that it is bound CH$_2$H$_4$F, potentially in its iminium ion form, that accumulates at the active site (9). Several steps in rapid equilibrium ensue leading up to an overall rate-limiting step involving hydride transfer to form the products dTMP and H$_2$F (steps 3-6) (9).

All known TS enzymes, with the exception of a class of recently discovered flavin-dependent tetrameric TS (ThyX) enzymes from several non-sybiotic microbes, exist as a TS homodimer (16). It has been suggested that TS is a half-sites active enzyme in which only one TS site of dimeric TS productively binds substrates (17-19). Until now, however, definitive kinetic evidence was lacking and the issue of whether TS is actually a half-sites reactive enzyme has remained unresolved (9).
There is structural and kinetic evidence to suggest that bifunctional TS-DHFRs from certain protozoa including *Leishmania major* and *T. gondii* exhibit electrostatic substrate channeling in which H2F is directly transferred between the TS and DHFR active sites without release into bulk solvent (5,20-22). Previous kinetic studies from our group provided support for substrate channeling in TS-DHFR from *L. major* (23). These rapid transient kinetic studies on *L. major* also provided direct kinetic evidence for domain-domain communication in *L. major* TS-DHFR in which ligation of the TS active site with FdUMP and CH2H4F to form the covalent FdUMP-CH2H4F-TS-DHFR covalent complex resulted in activation of DHFR chemistry from 14s\(^{-1}\) to 120s\(^{-1}\) (23).

In this work, we set out to address fundamental mechanistic questions about the TS-DHFR from *T. gondii*; a detailed understanding of which might aid in novel anti-parasite drug development against this medically relevant organism. Specifically, we asked:

What are the rate-limiting steps in the reactions catalyzed by this bifunctional enzyme? Can we gain any new insight into the catalytic mechanism of TS from this bifunctional enzyme? How does this bifunctional TS-DHFR compare with the previously characterized TS-DHFR from *L. major* for which a structure is known?

In this report, we describe a transient kinetic analysis using rapid chemical quench and stopped-flow methods to provide the first in-depth characterization of the reaction pathway for the bifunctional TS-DHFR from *T. gondii*. In addition to elucidating the detailed mechanism for the *T. gondii* TS-DHFR enzyme, this study provides the first direct kinetic evidence for the formation of a TS intermediate and for half-sites TS reactivity. Subsequent analysis of *L. major* TS-DHFR, *Escherichia coli* TS, and human
TS provided evidence for an intermediate and for half-sites TS reactivity in these enzymes as well.

MATERIALS AND METHODS

Enzymes. A clone harboring the p02CLSA-4 plasmid expressed in an E. coli Rue 10 expression vector was used following previously described methods to obtain bifunctional L. major TS-DHFR of high purity (23-25). The TS-DHFR was further purified using a Pharmacia Superdex® 75 HiLoad® (26/60) gel filtration column to rid of residual H2F contaminant. T. gondii TS-DHFR was similarly prepared from E. coli BL21-DE3 cells (Stratagene) freshly transformed with PET15b plasmid containing the coding sequence for T. gondii TS-DHFR. The T. gondii TS-DHFR plasmid was a generous gift from Dr. David S. Roos. Enzyme concentrations were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 67,800 M⁻¹cm⁻¹ for L. major TS-DHFR, 78,800 M⁻¹cm⁻¹ for T. gondii TS-DHFR, 43,800 M⁻¹cm⁻¹ for human TS, and 52,150 M⁻¹cm⁻¹ for E. coli TS.

Chemicals. All buffers and other reagents employed were of the highest commercial purity. Millipore ultrapure water was used for all solutions. 7,8-Dihydrofolate (H2F) was chemically prepared by the reduction of folate with sodium hydrosulfite (26). (6R,S)-5,6,7,8-Tetrahydrofolate (H4F) was obtained from Schirks Laboratories, Switzerland. Radiolabeled H2F was synthesized by sodium hydrosulfite reduction of tritium-labeled [3',5',7,9-³H] folic acid obtained from Moravek Biochemicals (Brea, CA). Radiolabeled and unlabeled CH₂H₄F were prepared by enzymatic conversion of radiolabeled and unlabeled H₂F respectively to form (6R,S)-5,6,7,8-Tetrahydrofolate (H₄F) and subsequent condensation with formaldehyde (27). Both H2F and CH₂H₄F were purified by DE-52
(Whatman Co.) anion exchange chromatography as previously described (28). $^{14}$C-labeled dUMP was obtained from Moravek Biochemicals (Brea, CA). The concentrations of $H_2F$ ($\varepsilon_{282}=28,000 \text{ M}^{-1}\text{cm}^{-1}$), $H_4F$ ($\varepsilon_{297}=28,000 \text{ M}^{-1}\text{cm}^{-1}$), and $\text{CH}_2\text{H}_4F$ ($\varepsilon_{290}=32,000 \text{ M}^{-1}\text{cm}^{-1}$) were determined spectrophotometrically (29,30). NADPH ($\varepsilon_{340}=6220 \text{ M}^{-1}\text{cm}^{-1}$), NADP$^+$ ($\varepsilon_{260}=18000 \text{ M}^{-1}\text{cm}^{-1}$), dUMP ($\varepsilon_{260}=10000 \text{ M}^{-1}\text{cm}^{-1}$), and dTMP ($\varepsilon_{260}=8400 \text{ M}^{-1}\text{cm}^{-1}$) were purchased from Sigma, and their concentration determined using reported molar extinction coefficients. Experiments were carried out at 25 °C in 50 mM Tris buffer (pH 7.8) containing 1mM EDTA, 25mM MgCl$_2$, and 10mM DTT. Buffer solutions were purged with argon prior to use.

**Stopped-Flow Measurements.** Stopped-flow measurements were performed using a Kintek SF-2001 apparatus (Kintek Instruments, Austin, TX) as previously described (23). The data were collected over a given time interval using a PC and software provided by Kintek Instruments. In experiments designed to measure dissociation rate constants, the trapping ligand was used at a concentration of $>5$ fold excess over that of the bound ligand to allow analysis as a pseudo-first order rate constant. Coenzyme FRET was utilized in DHFR experiments and in all ligand association and dissociation experiments involving NADPH. For these experiments, a monochrometer was set to 287 nm on the input and changes in NADPH FRET were monitored with an output filter at 450 nm. For TS and TS-DHFR experiments, changes in absorbance at 340 nm were monitored. For all other ligand binding experiments, changes in fluorescence with excitation at 287 and emission at 340 nm were monitored. Due to ordered binding to TS in which nucleotide binds first, followed by folate; $H_2F$ binding to the DHFR domain could be isolated from that to the TS domain by performing the experiments in the absence of nucleotide (dUMP
or dTMP). Likewise, binding of H$_2$F to TS could be separately assessed by pre-incubating enzyme with a DHFR-saturating concentration of H$_2$F (> 5µM) prior to mixing with a large excess of nucleotide and an equimolar concentration of H$_2$F. The combination of rapid chemical quench and stopped-flow methods allowed for an accurate interpretation of fluorescence and absorbance signals.

*CCD- Array Stopped-Flow Measurements.* CCD-Array Stopped-flow measurements were performed using a Kintek SF-2001 apparatus (Kintek Instruments, Austin TX) and detected with an Ocean Optics PC2000 CCD linear silicon array detector. Absorption measurements were taken for 2.1 seconds (integration time of 3 ms) from 220-450 nm (over 649 elements) and the data was analyzed using a PC and Specfit/32™ Software (Spectrum Software Associates).

*Rapid Chemical Quench.* Rapid chemical quench experiments were performed using a Kintek RFQ-3 Rapid Chemical Quench Apparatus (Kintek Instruments, Austin, TX). The reactions were initiated by mixing enzyme solution (15 µL) with radiolabled substrate (15 µL, approximately 20,000 dpm). In all cases, the concentrations of enzyme and substrates cited in the text are those after mixing and during the reaction. Reactions utilizing radiolabled folates were terminated by quenching with 67 µL of 0.78 N KOH, 10% sodium ascorbate, and 200 mM 2-mercaptoethanol. Ascorbate and 2-mercaptoethanol were added to prevent oxidative degradation of H$_4$F after quenching and resulted in a pH of 12.6 for the base quench solution. Since CH$_2$H$_4$F is more stable under basic conditions, its solutions were maintained at a basic pH (9.5) until mixing with enzyme solution, providing a final pH of 7.8 during the reaction. TS reactions utilizing radiolabeled dUMP were quenched with 67 µL of 0.4 N HCl. The quenched reaction
solutions were directly collected into argon-purged Waters Wisp autosampler vials, immediately vortexed, and analyzed by HPLC in combination with radioactivity-flow detection. The substrates and products were then quantified as described below. All samples that were not immediately analyzed were stored at -80 °C until just prior to analysis to minimize degradation. To confirm complete quenching of the enzymatic reactions, controls in which substrate was added to a premixed solution of enzyme and quench solution was included with each experiment.

**HPLC Analysis.** The substrates and products were quantified by radio-HPLC using a BDS-Hypersil C18 reverse phase column (250 mm × 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 ml/min. For separation of folates, an isocratic separation using a solvent system of 10% methanol in 180 mM triethylammonium bicarbonate (pH 7.8) was used. The elution times were as follows: H₄F, 7.5 min; TS intermediate, 12.5 min; H₂F, 14 min, CH₂H₄F, 16 min. For separation of dUMP and dTMP, an isocratic separation using a solvent system of 200 mM triethylammonium bicarbonate was used. The elution times were as follows: dUMP, 11 min; dTMP, 18 min. The HPLC effluent from the column was monitored continuously using a Flo-One radioactivity-flow detector (Packard Instruments, Downers Grove, IL). The analysis system was automated using a Waters 712B WISP (Milford, MA) autosampler.

**Data Analysis:** Rapid chemical quench single turnover and burst data were fit to single exponential and burst equations respectively using the curve fitting program Kaleidagraph. Stopped-flow measurements provided estimates for the association and dissociation rate constants (k<sub>on</sub> and k<sub>off</sub>) and for reaction rate constants. Comparison of
rapid chemical quench and stopped-flow reaction time-courses allowed for the assignment of observed stopped-flow rates to chemical steps or conformational changes.

Spectrophotometric TS Assay: The $K_m$ of CH$_2$H$_4$F was determined using a steady-state spectrophotometric kinetic assay. $T. gondii$ TS-DHFR enzyme (25 nM) was preincubated with dUMP (100 µM) prior to mixing with CH$_2$H$_4$F (5 to 350 µM) and absorbance was monitored at 340 nm using a Hewlett Packard 8452A spectrophotometer. Initial rates were determined in triplicate using the software provided with the instrument and these rates were converted to units of specific activity using the reported extinction coefficient for the reaction ($\Delta\varepsilon_{\text{rxn}} = 6.4$ mM$^{-1}$cm$^{-1}$). Data in this paper is presented as the average of triplicate determination with error bars representing the standard deviation.

Kinetic Simulation: The KINSIM kinetic simulation program was used to model kinetic data presented in this paper (31,32). The data were fit by a trial and error process, maintaining the constraints of constants measured in this study. The focus of this simulation was to validate the minimal kinetic mechanism elucidated in this study. The model and estimated rate constants are described in Chart 1. Half-sites TS reactivity was modeled by defining TS (E) and DHFR (Z) as unique species and defining the modeling parameters such that $Z = 2E = \text{concentration of TS-DHFR used}$.

$T. gondii$ Homology Model: A homology model of $T. gondii$ TS-DHFR was built using the Swiss PDB program in conjunction with the Swiss Model homology modeling link available at the Swiss PDB website. The C-terminal 315 amino acids (residues 295 to 610) and residues 115 to 166 were modeled using the PDB file for the $L. major$ TS-DHFR structure. The N-terminal 52 amino acids were modeled using the PDB file for $Pneumocystis carinii$ DHFR (PDB entry 1CD2), which was the highest homology DHFR
relative to the N-terminal portion of the *T. gondii* DHFR domain for which a structure is available.

**RESULTS**

*Overview of the TS-DHFR Reaction.* Bifunctional TS-DHFR enzymes catalyze three basic reactions (Scheme 1), the TS reaction, the DHFR reaction, and a bifunctional TS-DHFR reaction. Moreover, each of these reactions can be further subdivided into two classes of events: binding and dissociation of ligands and those events involved in catalysis. We will first address those events involving binding and dissociation of substrates and products, and we will consider an example experiment used to measure rates for each. Those events involved in chemical catalysis will then be elucidated, including the occurrence of conformational changes, formation of intermediates, and the identification of rate-limiting steps. We begin with the characterization of the DHFR reaction since it is relatively straightforward and has features used to help interpret the TS reaction. We will then consider the TS and TS-DHFR reactions, providing a complete TS-DHFR reaction mechanism and new insight into the mechanism of TS catalysis.

*Kinetics of Ligand Binding.* The second-order rate constants for the binding of ligands to TS-DHFR were determined by measuring the ligand concentration-dependence of the observed binding rate. The apparent first-order rates for the binding of dUMP, dTMP, H$_2$F to the TS domain and for H$_2$F, H$_4$F, NADPH, and NADP$^+$ to the DHFR domain were measured using stopped-flow fluorescence. A representative stopped-flow fluorescence trace for the binding of NADPH to the bifunctional enzyme is shown in **Figure 1a.** The trace is biphasic and fits a double exponential equation with a fast NADPH concentration-dependent phase of 83.0 ± 3.9 s$^{-1}$ and a slow NADPH concentration-
independent phase of $5.6 \pm 0.5 \text{ s}^{-1}$. The fast phase represents the apparent first-order binding rate and conforms to the equation: $k_{\text{obs}} = k_{\text{on}}[L] + k_{\text{off}}$, in which $k_{\text{obs}}$, $k_{\text{on}}$, $k_{\text{off}}$ are the apparent first-order observed binding rate, association rate constant, and dissociation constant respectively. The plot of $k_{\text{obs}}$ versus $[L]$ for NADPH binding is shown in Figure 1b. Accordingly, the binding and dissociation rate constants are $9.0 \pm 0.4 \text{ } \mu \text{M}^{-1}\text{s}^{-1}$ and $39.8 \pm 3.0 \text{ s}^{-1}$ for the formation and dissociation of the E:NADPH complex respectively at $25 \, ^\circ\text{C}$ and pH 7.8. The slow NADPH concentration-independent phase is likely to represent a conformational change following NADPH binding. A summary of the association rate constants measured for the various ligands with bifunctional TS-DHFR enzyme is shown in Table 1.

Kinetics of Ligand Dissociation. The rate constants for the dissociation of ligands from TS-DHFR were measured by ligand competition experiments. A representative stopped-flow trace for the measurement of the dissociation of H$_2$F from the E:H$_2$F complex using methotrexate as the trapping ligand is shown in Figure 2. The resulting trace was fit to a single exponential equation with a rate of $9.0 \pm 0.1 \text{ s}^{-1}$, corresponding to the dissociation rate constant, $k_{\text{off}}$, for H$_2$F release from the DHFR domain of TS-DHFR. A summary of the dissociation constants obtained from these experiments is shown in Table 2.

The DHFR Reaction. The first experiment examining the DHFR activity of the *T. gondii* TS-DHFR enzyme is a pre-steady-state burst experiment. As shown in Figure 3a a burst in DHFR catalysis is observed at a rate of $180 \pm 20 \text{ s}^{-1}$. This is followed by slow steady-state product accumulation at a rate of $5.7 \pm 0.6 \text{ s}^{-1}$, corresponding to a rate-limiting step preceding subsequent turnover. The observed burst amplitude was $31.1 \pm$
1.4 μM, within error of the enzyme concentration used, suggesting that essentially 100% of the DHFR sites are active. The second experiment is a single turnover stopped-flow fluorescence experiment, the time-course for which is shown in Figure 3b. Since enzyme-bound NADPH but not NADP⁺ exhibits coenzyme fluorescence resonance energy transfer, the time-course for the fluorescence at 450 nm represents the conversion of NADPH to NADP⁺ at the active site, and hence the rate of catalysis. The data was fit to a single exponential equation with a rate \( k_{chem} \) of 180 ± 2.7 s⁻¹ consistent with the rate of chemistry observed in the burst reaction. There was no increase in the rate of catalysis under conditions in which the enzyme concentration was doubled, indicating that substrate binding was not limiting.

**T. gondii DHFR Activation Experiment.** Experiments designed to examine whether there was domain-domain communication involving DHFR activation in *T. gondii* TS-DHFR, as previously observed with the *L. major* enzyme, were performed. Both DHFR single turnover and burst experiments were similar to those described above, except that the enzyme was preincubated with FdUMP and CH₂H₄F to form the covalent FdUMP-CH₂H₄F-TS-DHFR covalent complex. In contrast to the DHFR activation observed with *L. major* TS-DHFR (14s⁻¹ to 120s⁻¹), no significant change in the *T. gondii* DHFR rate was observed (data not shown).

**The TS and TS-DHFR Reactions – A Burst in TS Activity?** The first experiment examining the TS activity of the *T. gondii* TS-DHFR enzyme was a pre-steady-state burst experiment in which enzyme was preincubated with excess \(^{14}\text{C}\)-dUMP prior to mixing with a large excess of CH₂H₄F. The time-course for the reaction is shown in Figure 4a. The reaction occurs at a linear steady-state rate with no burst in \(^{14}\text{C}\)-dUMP
consumption or $[^{14}C]$-dTMP formation. The absence of a burst suggests that chemistry or a preceding step is limiting in the TS reaction for this enzyme. A second TS burst experiment was conducted in which CH$_2$H$_4$F was the radiolabeled substrate. The time-course for the consumption of $[^3]$H-CH$_2$H$_4$F is shown in **Figure 4b**. In contrast to the linear, steady-state consumption of dUMP, this reaction occurs with a burst in $[^3]$H-CH$_2$H$_4$F consumption. A third TS burst experiment was conducted using stopped-flow absorbance. As shown in **Figure 4c**, a burst in absorbance at 340 nm consistent with the rapid chemical quench burst experiment is observed.

*The Observation of a TS Intermediate.* As shown in **Figure 5 (bottom)**, the burst in TS substrate consumption is coupled to the formation of a species whose retention time under the HPLC conditions used does not allow for complete separation from the product of the reaction, $[^3]$H-H$_2$F. Coupling the TS and DHFR reactions allows one to monitor the bifunctional TS-DHFR reaction and thereby monitor the direct conversion of $[^3]$H-CH$_2$H$_4$F to $[^3]$H-H$_4$F. As shown in **Figure 5 (top)**, when a TS-DHFR burst reaction is conducted, the burst in $[^3]$H-CH$_2$H$_4$F consumption is still coupled to the formation of the species seen in the TS reaction. This is followed by slow accumulation of $[^3]$H-H$_4$F without significant accumulation of $[^3]$H-H$_2$F.

Further evidence of a TS intermediate was obtained by CCD-array stopped-flow measurements where a shift in the isosbestic point was observed. The spectra of the *E. coli* TS reaction under varying enzyme and substrate concentrations are shown in **Figure 6**. The first experiment was conducted under burst conditions: *E. coli* TS (25 µM) was preincubated with dUMP (500 µM) before mixing with CH$_2$H$_4$F (200 µM). The resulting spectra contained an isosbestic point at 322.8 nm (**Figure 6a**). A shift in the isosbestic
point to 337.0 nm was observed when the reaction was repeated using 40 µM CH₂H₄F (all other conditions identical) (Figure 6b). Under single turnover conditions (25 µM TS and 10 µM CH₂H₄F) the isosbestic point was observed at 338.1 nm (Figure 6c). To determine the isosbestic point for the quantitative conversion of CH₂H₄F to H₂F, the reaction was conducted under steady-state conditions in which E. coli TS (2.5 µM) was preincubated with dUMP (500 µM) prior to mixing with CH₂H₄F (200 µM). The resulting isosbestic point was observed at 322.7 nm (Figure 6d). Similar CCD-array stopped-flow TS experiments were conducted using bifunctional T. gondii and L. major TS-DHFR. Similar shifts in the isosbestic point of the TS reaction spectra for the bifunctional enzymes were observed (data not shown).

Isolation of TS Catalytic Rates. To isolate the rates of TS catalytic events, a TS-DHFR burst experiment was conducted, the time course for which, is shown in Figure 7a. The reaction occurs with a burst in [³H]-CH₂H₄F consumption at a rate of 130 ± 10 s⁻¹ coupled to the rapid accumulation of an intermediate species up to a steady-state concentration corresponding to the burst amplitude for the reaction. The burst in [³H]-CH₂H₄F consumption is followed by a rate-limiting steady-state phase of 6.2 ± 0.6 s⁻¹ in which [³H]-H₂F is formed without significant [³H]-H₂F accumulation.

Evidence for Half-sites TS Reactivity. Another vital piece of information provided by the burst experiment shown in Figure 7a is the burst amplitude, representing the concentration of TS active-sites for this reaction. The burst amplitude is 58.9 ± 11.4 µM, or roughly 50% the concentration of TS-DHFR used, and of DHFR active sites, consistent with half-sites TS reactivity.
To examine the CH$_2$H$_4$F concentration-dependence of the burst rate and amplitude in the TS burst reaction, a second series of TS bursts, similar to that shown in Figure 4c, was performed by stopped-flow absorbance at 340 nm. In this series, the bifunctional *T. gondii* TS-DHFR enzyme (25 µM) was pre-incubated with a large excess of dUMP (1 mM) prior to mixing with excess CH$_2$H$_4$F (40-1500 µM). Plotting the observed burst rate versus CH$_2$H$_4$F concentration suggested that the relationship was a linear one, consistent with rate-limiting CH$_2$H$_4$F association ($k_{on} \approx 1$ µM$^{-1}$s$^{-1}$), up to the maximum burst rate of 105 ± 4 s$^{-1}$ (data not shown) rather than a hyperbolic one that would be consistent with rapid equilibrium and a weak $K_d$. By contrast, the observed burst amplitude versus CH$_2$H$_4$F concentration displayed a hyperbolic relationship suggesting that the apparent $K_d$ for CH$_2$H$_4$F binding was 18 ± 5 µM (Figure 8a). This is consistent with the steady-state $K_m$ of 17.0 ± 2.0 µM determined for the reaction (Figure 8b).

A TS-DHFR single turnover series was also performed in order to determine the rate of the rate-limiting chemical step in the TS reaction ($k_{chem}$) and to examine the TS-DHFR concentration-dependence on the observed single turnover rate. In this series, the bifunctional TS-DHFR enzyme (12.5-100 µM) was preincubated with a large excess of dUMP and NADPH prior to mixing with limiting [$^3$H]-CH$_2$H$_4$F. Figure 8c and 8d show that the reaction time-course for this series displays a hyperbolic dependence on the concentration of enzyme used. The enzyme concentration at which the observed rate is half maximal ($K_{d, apparent}$) for this series is 45 ± 8 µM and the maximum rate ($k_{chem}$) is 5.5 ± 0.4 s$^{-1}$.

*A Kinetic Model for the T. gondii TS-DHFR Reaction.* The mechanistic information obtained in this study was used to formulate a minimal kinetic mechanism for the
bifunctional TS-DHFR from \textit{T. gondii} (\textbf{Scheme 3}). The TS reaction is depicted in \textbf{Scheme 3a} and the DHFR reaction in \textbf{Scheme 3b}. This mechanism along with the rates obtained in this study was then used to simulate reaction time-courses using the program KINSIM. The KINSIM model and rate constants used are described in \textbf{Chart 1}. The resulting simulations were consistent with experimental data for the TS-DHFR reactions (\textbf{Figure 7b}).

\textit{Bursts in \textit{L. major} TS and TS-DHFR.} The TS and TS-DHFR bursts observed in \textit{T. gondii} TS-DHFR represent a marked mechanistic difference from our previous study of \textit{L. major} TS-DHFR. The \textit{L. major} TS and TS-DHFR reactions were therefore re-analyzed under identical conditions to the \textit{T. gondii} reactions described above. The resulting time-courses suggested that CH$_2$H$_4$F binding was rate-limiting under the experimental conditions employed in our previous study in which no burst in TS activity was observed. A representative TS-DHFR burst time-course for \textit{L. major} TS-DFHR is shown in \textbf{Figure 9a}. This experiment suggest that, like for \textit{T. gondii} TS-DHFR, rapid conversion of CH$_2$H$_4$F to intermediate occurs and that, like for \textit{T. gondii}, this is followed by an overall rate-limiting chemical step associated with dTMP formation. As with \textit{T. gondii} TS-DHFR, the burst amplitude of the TS-DHFR burst reactions and the steady state concentration of intermediate that accumulates during the time-course are approximately equal to one-half the TS-DHFR concentration used. TS burst experiments were conducted with \textit{L. major} TS-DHFR at corresponding concentrations and were consistent with the TS-DHFR reaction (data not shown).

\textit{Bursts in Monofunctional Human and \textit{E. coli} TS.} The observation of a catalytic burst in the TS reaction of the \textit{L. major} and \textit{T. gondii} TS-DHFR bifunctional enzymes also
suggested a marked mechanistic difference from previous reports on monofunctional TS enzymes (9). To verify this difference, monofunctional Human and *E. coli* TS enzymes were analyzed under identical burst conditions to those used for the bifunctional enzymes. Surprisingly, the time-courses for the monofunctional TS enzymes also displayed a burst in CH$_2$H$_4$F consumption consistent with the rapid formation of a TS intermediate preceding a rate-limiting step in TS chemistry. **Figure 9b and 9c** show the time-courses for the monofunctional *E. coli* and human TS enzymes respectively. As with the bifunctional TS-DHFR enzymes, the monofunctional TS burst amplitudes were consistent with half-sites TS reactivity.

*Homology Model of T. gondii TS-DHFR.* To obtain a possible structural explanation for the difference in DHFR activities observed, specifically the lack of TS-ligand-induced DHFR activation in *T. gondii* TS-DHFR that is observed in *L. major*, a homology model of *T. gondii* TS-DHFR was built as described in materials and methods. As shown in **Figure 10**, the homology model suggests that the *T. gondii* TS-DHFR enzyme lacks an N-terminal tail, linking the DHFR and TS domains, that is present in the crystal structure of *L. major* TS-DHFR.

**DISCUSSION**

In this work, we have characterized the complete kinetic scheme for bifunctional TS-DHFR from *T. gondii*. In addition to providing the detailed enzymatic mechanism for this important chemotherapeutic target, this study compares the mechanism with that from bifunctional TS-DHFR from *L. major* for which a structure is known, highlighting both similarities and differences in mechanism. Finally, the use of rapid transient
kinetics methods in this study provides the first direct kinetic evidence for half-sites TS reactivity and for the accumulation of an intermediate during TS catalysis.

*An Overview of the T. gondii TS-DHFR Reaction.* For the *T. gondii* TS reaction (scheme 3a), the overall rate-limiting step occurs during chemistry at a rate \( k_{chem} \) of 5.5 s\(^{-1}\). This is consistent with previous studies with other TS enzymes which have suggested that a hydride transfer step occurring in the final step of TS catalysis is rate-limiting (9). For the *T. gondii* DHFR reaction (Scheme 3b), chemistry occurs at a relatively fast rate \( k_{chem} \) of 180 s\(^{-1}\), compared with an overall rate-limiting conformational change \( k_{ss} \) at a rate of 5.6 s\(^{-1}\) that occurs immediately after NADPH binding.

*Comparison of L. major and T. gondii DHFR mechanisms.* As might be predicted on the basis of relative sequence homology, the differences between *L. major* and *T. gondii* TS-DHFR reside primarily in the DHFR mechanisms. The first major difference is the lack of DHFR activation in the *T. gondii* enzyme. The homology model of *T. gondii*, when compared to the crystal structure of *L. major*, suggests a possible explanation for this difference. We postulate that the 23 amino acid tail of *L. major* that is absent in the *T. gondii* homology model may serve to inhibit DHFR activity in a TS conformation-specific manner. This domain-domain communication mechanism may serve as a TS activity sensor activating DHFR activity during TS catalysis, further coupling the sequential TS and DHFR activities.

A second notable difference between the *L. major* and *T. gondii* DHFR mechanisms is found in the location of the rate-limiting step for each. In both cases, the rate-limiting step involves a conformational change; however, this step occurs in different places along the respective DHFR pathways. As shown in Scheme 4, the rate-limiting conformational...
change for the *T. gondii* enzyme occurs after NADPH binding, whereas it takes place after NADP⁺ release from E:H₄F:NADP⁺ in the *L. major* enzyme.

A final subtle, but noteworthy, difference between the DHFR mechanisms for these species is that the product release pathway for *L. major* DHFR is kinetically restricted to one path, whereas product release in *T. gondii* can occur via multiple kinetically competent paths (Scheme 4). This is not to say that multiple product release pathways are not utilized by the *L. major* enzyme, but that only one of the pathways is kinetically competent in that it contains no one rate slower than the overall rate-limiting step for the enzyme. In contrast, all the possible product release pathways in the *T. gondii* enzyme are kinetically competent and therefore likely to be equally utilized.

**Evidence for Half-site TS Reactivity.** It has been suggested that TS is a half-sites reactive enzyme in which only one TS monomer of dimeric TS is catalytically active at a time. This proposal is based on structural evidence that suggests that TS binds ligands asymmetrically to each monomer’s active site and mutagenesis studies in which an active-site and non-active-site TS dead mutant are combined to form a heterodimer with fully restored TS activity (17-19). To our knowledge, however, this study provides the first direct kinetic evidence for half-sites reactivity in TS enzymes.

The pre-steady-state burst amplitude observed for the TS reaction in *T. gondii* TS-DHFR, which reflects the concentration of TS active sites, is approximately one-half the concentration of TS used, while the corresponding DHFR burst experiment suggests that the enzyme contains essentially 100% DHFR active sites. In addition, analysis of the concentration dependence of TS activity under single turnover and burst conditions suggests that this half-sites reactivity is a result of asymmetric substrate binding.
Specifically, the observation that the apparent $K_d$ for the single turnover series in which the enzyme is in excess and determines binding is approximately twice the $K_d$ for the burst series in which CH$_2$H$_4$F determines binding suggests that only one-half of the TS present in solution can productively bind CH$_2$H$_4$F. Taken together, these studies provide the first direct kinetic evidence in support of half-sites TS reactivity, in which only one TS monomer binds substrate productively.

It is worth noting that a burst amplitude corresponding to 50% enzyme concentration might also be explained by two other scenarios: (i) 50% misfolded enzyme or (ii) a 50-50 equilibrium between two forms of the enzyme prior to chemistry. The former is unlikely since a 50% burst amplitude was observed with multiple enzyme preps and with TS from various species, and the latter scenario is unlikely on the basis of the results of the current as well as previous studies, which are consistent with half-site reactivity resulting from asymmetric binding of TS ligands.

**Evidence for a TS Intermediate.** It has also been proposed that TS catalysis involves the formation of an iminium ion form of CH$_2$H$_4$F and that this is the reactive form of the cofactor. Moreover, there is structural evidence involving a mutant of TS lacking a C-terminal valine crystallized with CH$_2$H$_4$F and FdUMP in which (6R)-L-5-hydroxymethyltetrahydrofolate (HO-CH$_2$H$_4$F) was found to be bound at the active site suggesting the formation of the putative iminium ion during TS catalysis (10). However, this study provides the first direct kinetic evidence for the accumulation of a TS intermediate during TS catalysis.

The presence of a burst in substrate (CH$_2$H$_4$F) consumption without a corresponding burst in product formation and the observation of a rapidly formed transient species not
attributable to substrate or product in HPLC analyses provide the first direct kinetic evidence for the accumulation a TS intermediate.

The accumulation of a TS intermediate is further supported by the shift in the isosbestic point for the TS reaction in the stopped-flow CCD experiments. Specifically, under steady-state conditions or burst conditions where CH$_2$H$_4$F is in large excess over enzyme, a single isosbestic point is observed at $\sim$322 nm for the TS reaction. Under conditions where enzyme concentration becomes significant relative to CH$_2$H$_4$F, such as under single turnover conditions, there is a shift in the isosbestic point to $\sim$337 nm. Stadman et al have shown that the wavelength of an isosbestic point may change under varying experimental conditions provided (i) either the molar absorbtivity of the substrate changes under the varying experimental conditions or (ii) the fraction of the substrate that is converted to multiple products changes (33). The observed shift in the isosbestic point during the TS reaction is consistent with the latter, in which there is formation of an enzyme-bound intermediate species that becomes increasingly significant as the reaction conditions approach single turnover conditions.

Considering the proposed TS mechanism (Scheme 2), the results of previous TS mechanistic studies, and the results of this study, we suggest that this TS intermediate is the putative iminium ion form of the cofactor. Specifically, there is a burst in CH$_2$H$_4$F consumption without a corresponding burst in dUMP consumption, and previous kinetic isotope effect studies with *E. coli* TS suggest that either the CH$_2$H$_4$F cofactor or its iminium ion form, but not any other intermediate species, accumulates at the active site during TS catalysis (9). On the basis of these kinetic isotope effect studies and the
proposed mechanism, it is likely that the rate of iminium ion formation is limited by the conformational change represented by step 1 in Scheme 2.

*Half-sites TS reactivity and Intermediate Formation in L. major TS-DHFR and Monofunctional TS enzymes.* The direct observation of half-sites TS reactivity and the formation of a transient TS intermediate were novel observations. Accordingly, it was of interest to see whether similar kinetics would be observed in other TS enzymes. Analysis of the bifunctional TS-DHFR from *L. major* and of monofunctional *E. coli* and human TS suggest half-site reactivity and intermediate formation also occurs with TS from these species.

While this study has provided new insight into the *T. gondii* TS-DHFR mechanism and into the TS mechanism in general, several questions remain unresolved. First, it remains to be established whether *T. gondii* TS half-site reactivity may be coordinated, with the activity alternating between monomers of a TS dimer or whether this activity is uncoupled and random. An additional possibility as suggested by studies with the R126E/C146W heterodimeric mutant *E. coli* TS enzyme is that only one subunit is required for activity with the other simply serving as a scaffold for the active subunit (18). Second, while DHFR activation is not observed with *T. gondii* TS-DHFR, a detailed study of potential domain-domain communication in this enzyme remains to be conducted. It is possible that more subtle communication, such as changes in substrate affinity (cooperativity), between the TS and DHFR domains might occur in this enzyme. Finally, it would be of interest to compare the detailed *T. gondii* TS-DHFR mechanism to that of human TS and DHFR. Subtle differences, such as the identity and location of
rate-limiting steps or conformations unique to the bifunctional enzyme, might provide crucial insights for the development of novel therapeutics specific for *T. gondii*.

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Footnotes:

1 The abbreviations used are: TS, thymidylate synthase; DHFR, dihydrofolate reductase; TS-DHFR, bifunctional thymidylate synthase-dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; CH₂H₄F, (6R)-L-5,10-methylene tetrahydrofolate; H₂F, 7,8-dihydrofolate; H₄F

2 The bifunctional enzyme is sometimes referred to as DHFR-TS since DHFR comprises the N-terminal portion of the protein. This paper will use the TS-DHFR designation as mechanistically, the TS reaction precedes the DHFR reaction.

Figure Legends:

Figure 1: Stopped-flow Binding Experiment Measuring the Association Rate of NADPH to T. gondii TS-DHFR. Figure 1a: Stopped-flow fluorescence trace observed upon mixing TS-DHFR (1 μM) with NADPH (6 μM). Figure 1b: Plot of concentration dependent rate ($k_{obs}$) versus NADPH concentration.
Figure 2: A Representative Stopped-flow Trace for the Measurement of the Dissociation of H$_2$F from the E:H$_2$F Complex. The bifunctional TS-DHFR enzyme (5 µM) was preincubated with H$_2$F (10 µM) prior to mixing with excess methotrexate (50 µM).

Figure 3: Determination of DHFR Catalytic Rates. Figure 3a: DHFR burst experiment in which \textit{T. gondii} TS-DHFR (30 µM) was preincubated with a large excess of NADPH (500 µM) before mixing with excess [$^3$H]-H$_2$F (100 µM). Time points in the burst phase are the average of triplicate determinations with error bars representing the standard deviation. Time points in the steady state phase are either singlet or duplicate measurements. The inset in Figure 3a shows an enlarged view of the burst phase. Figure 3b: DHFR single turnover stopped-flow FRET experiment in which \textit{T. gondii} TS-DHFR (50 µM) was preincubated with a large excess of NADPH (250 µM) prior to mixing with limiting H$_2$F (25 µM) and the decrease in fluorescence at 450 nm monitored over time.

Figure 4: TS Burst Experiments. Figure 4a: TS burst experiment in which \textit{T. gondii} TS-DHFR (25 µM) was preincubated with excess [$^{14}$C]-dUMP (90 µM) prior to mixing with a large excess of CH$_2$H$_4$F (500 µM). Figure 4b: TS burst experiment in which \textit{T. gondii} TS-DHFR (40 µM) was preincubated with a large excess of dUMP (500 µM) prior to mixing with excess [$^3$H]-CH$_2$H$_4$F (120 µM). Figure 4c: Stopped-flow absorbance TS burst experiment in which the bifunctional TS-DHFR enzyme (25 µM) was pre-incubated with a large excess of dUMP (1 mM) prior to mixing with CH$_2$H$_4$F (500 µM).
Figure 5: HPLC Analysis of TS Burst Reaction – Evidence for a TS Intermediate.

HPLC analysis of the TS (Figure 5 bottom) and TS-DHFR (Figure 5 top) burst reactions. Both reactions were analyzed at t = 100 ms. The peak at 4 min is a contaminant (cont.) formed during chemical synthesis of $^3$H-CH$_2$H$_4$F.

Figure 6: CCD Array Stopped-Flow Spectra. Figure 6a: *E. coli* TS (25 µM) was preincubated with excess dUMP (0.5 mM) and mixed with CH$_2$H$_4$F (200 µM). The inset is a 3-D representation of the data to illustrate the isosbestic point. Figure 6b: *E. coli* TS (25 µM) was preincubated with excess dUMP (0.5 mM) and mixed with CH$_2$H$_4$F (40 µM). Figure 6c: *E. coli* TS (25 µM) was preincubated with excess dUMP (0.5 mM) and mixed with CH$_2$H$_4$F (10 µM). The inset is a magnification of the area surrounding the isosbestic point. Figure 6d: *E. coli* TS (2.5 µM) was preincubated with excess dUMP (0.5 mM) and mixed with CH$_2$H$_4$F (200 µM). All reactions were scanned for 2.1 seconds with a 3 ms integration time. The spectra are shown as viewed down the time axis. The solid black lines indicate the isosbestic points.

Figure 7: The TS-DHFR Burst Reaction. Figure 7a: *T. gondii* TS-DHFR (120 µM) was preincubated with a large excess of dUMP (500 µM) and NADPH (500 µM) prior to mixing with excess $[^3]$H]-CH$_2$H$_4$F (240 µM). The reaction proceeds with a burst in $[^3]$H]-CH$_2$H$_4$F (▼) consumption at a rate of $130 \pm 10$ s$^{-1}$ and with a burst amplitude of $58.9 \pm 11.4$ µM; rapid accumulation of intermediate (◆) at $125 \pm 12$ s$^{-1}$ to a steady-state concentration of $62.1 \pm 1.4$ µM; and a slow, steady state accumulation of H$_4$F (●) at a
rate of $6.2 \pm 0.6\ s^{-1}$. **Figure 7b:** KINSIM model of TS-DHFR burst reaction superimposed on experimental data. Experimental [CH$_2$H$_4$F] (▼); [Intermediate] (◆); [H$_4$F] (●).

**Figure 8:** Evidence for Half-sites TS Reactivity. **Figure 8a:** Hyperbolic dependence of the burst amplitude on CH$_2$H$_4$F concentration for a series of stopped-flow burst experiments. **Figure 8b:** Steady-state K$_m$ determination for CH$_2$H$_4$F (K$_m = 17.0 \pm 2.0\ \mu M$.) **Figure 8c:** TS-DHFR single turnover series in which *T. gondii* TS-DHFR [12.5 µM (■), 25 µM (●), 50 µM (▲), or 100 µM (▼)] was preincubated with a large excess of dUMP (500 µM) and NADPH (500 µM) prior to mixing with limiting [³H]-CH$_2$H$_4$F (5.6 µM). **Figure 8d:** Hyperbolic dependence of the observed single turnover rate on enzyme concentration (K$_d$ apparent $= 45 \pm 8\ \mu M$.) For 8a and 8b, error bars represent the standard deviation of triplicate determination. For 8d, error bars represent the error associated with each single exponential fit.

**Figure 9:** *L. major, E. coli,* and Human TS Burst Reactions: **Figure 9a:** *L. major* TS-DHFR burst (52.6 ± 3.8 µM amplitude) in which TS-DHFR (120 µM) was preincubated with a large excess of NADPH (500 µM) and dUMP (500 µM) prior to mixing with [³H]-CH$_2$H$_4$F (240 µM). **Figure 9b:** *E. coli* TS burst (10.6 ± 0.8 µM amplitude) in which *E. coli* TS (30 µM) was preincubated with large excess of dUMP (500 µM) prior to mixing with [³H]-CH$_2$H$_4$F (60 µM). **Figure 9c:** Human TS burst (13.7 ± 0.6 µM amplitude) in
which TS-DHFR (30 µM) was preincubated with large excess of dUMP (500 µM) prior to mixing with [³H]-CH₂H₄F (60 µM).

**Figure 10: *T. gondii* Homology Model:** A homology model of *T. gondii* TS-DHFR (bottom; blue) was built as described in Materials and Methods. Comparison of this model with the crystal structure of *L. major* TS-DHFR (top; red) demonstrates that a 23 amino acid N-terminal tail linking the DHFR and TS domains in the *L. major* Structure (tail shown in yellow) is absent in the *T. gondii* structure (N-terminal 3 amino acids shown in yellow).

**TABLES:**

| Ligand    | Enzyme Species | $k_{on}$ (µM⁻¹s⁻¹) ¹ |
|-----------|----------------|-----------------------|
| NADPH     | E:H₄F          | 2.9                   |
| H₄F       | E              | 27 s⁻¹ ²              |
| NADP⁺     | E              | 2.6                   |
| NADP⁺     | E:H₄F          | 2.2                   |
| NADPH     | E              | 9.0                   |
| H₂F       | E              | 5.7                   |
| CH₂H₄F    | E              | 2.0 ³               |
| dUMP      | E              | 13.4                  |
| dTMP      | E              | 10.9                  |
| H₂F       | E:dTMP:H₂Fₖ_{DHFR} ⁴ | 2.0                  |

**Table 1: Kinetics of Association of Ligands for *T. gondii* TS-DHFR at pH 7.8 and 25 °C.** ¹The error associated with the determination of each rate constant is <10% in all cases. ²The observed rate of H₄F binding was independent of concentration and likely reflects a conformational change following rapid binding. ³Since it was not possible to directly measure the rate of CH₂H₄F binding, this rate was estimated based on the CH₂H₄F-concentration dependence of the TS reaction and by modeling. ⁴H₂F binding to the TS domain was isolated from binding to the DHFR domain by taking advantage of TS ordered binding in which dTMP binds first. H₂F was preincubated with TS-DHFR prior to mixing with an equivalent of H₂F and a large excess of dTMP.
| Ligand  | Enzyme Species | Trapping Ligand | $k_{off}$(s$^{-1}$)$^a$ |
|---------|----------------|-----------------|--------------------------|
| H$_2$F  | E:H$_2$F       | MTX             | 9.0                      |
| NADP$^+$| E:NADP$^+$     | NADPH           | 95                       |
| NADPH   | E:NADPH        | NADP$^+$        | 7.0                      |
| NADPH   | E$_0$:NADPH    |                 | 40$^b$                   |
| NADP$^+$| E:H$_4$F:NADP$^+$ | NADPH       | 7.0                      |
| H$_4$F  | E:H$_4$F       | MTX             | 39                       |
| H$_4$F  | E:H$_4$F:NADP$^+$ | MTX        | 17                       |
| NADPH   | E:H$_4$F:NADPH | NADP$^+$        | 87                       |
| CH$_2$H$_4$F | E:CH$_2$H$_4$F |                 | 40$^c$                   |
| dUMP    | E              | dTMP            | 60                       |

Table 2: Dissociation Kinetics of Ligands for *T. gondii* TS-DHFR at pH 7.8 and 25 °C. $^a$The error associated with the measurement of each rate constant is <10% in all cases. $^b$k$_{off}$ as determined from the binding curve (fig. 1b). The rate of 7 s$^{-1}$ for NADPH dissociation from E:NADPH likely reflects the rate of release from a different TS-DHFR conformer than that involved in the binding experiment. $^c$The off rate of CH$_2$H$_4$F was estimated from the product of K$_d$ and $k_{on}$ and by modeling.
FIGURES

Figure 1

1a

Fluorescence (450 nm)

0 0.1 0.2 0.3 0.4

Time (s)

1b

$K_{\text{obs}}$ (s$^{-1}$)

0 3 6 9 12 15

[NADPH] (µM)

Figure 2

Fluorescence (340 nm)

0.62 0.63 0.64 0.65 0.66

0 0.25 0.5 0.75 1

Time (s)
Figure 3

Figure 4
Figure 7

**Figure 7a**

Concentration (µM) vs. Time (s)

**Figure 7b**

Concentration (µM) vs. Time (s)
Figure 8

8a

Burst Amplitude (A.U.)

[CH2H4F] (μM)

8b

V_0/[E]_tot (s⁻¹)

[CH_2H_4F] (μM)

8c

[H4F] (μM)

Time (s)

8d

k_{obs} (s⁻¹)

[TS-DHFR] (μM)
Figure 9

[Graphs showing the consumption of [CH2H4F] over time for L. Major and T. Gondii TS-DHFR and Homology Model.]

Figure 10

**TS Domain**  **DHFR Domain**

[L. Major TS-DHFR Homology Model]
Scheme 1

CH$_2$H$_4$F $\xrightarrow{\text{TS}}$ H$_2$F $\xrightarrow{\text{DHFR}}$ H$_2$F $\xrightarrow{\text{DHFR}}$ H$_4$F

dUMP $\xrightarrow{\text{TS}}$ dTMP $\xrightarrow{\text{DHFR}}$ NADPH $\xrightarrow{\text{DHFR}}$ NADP$^+$
### Scheme 3a: The TS Reaction

| Reaction | Forward Rate | Reverse Rate |
|----------|--------------|---------------|
| $E + dUMP + CH_2H_4F$ | 13.4 µM$^{-1}$s$^{-1}$ | 60 s$^{-1}$ |
| $E.dUMP + CH_2H_4F$ | 2.0 µM$^{-1}$s$^{-1}$ | 36 s$^{-1}$ |
| $E.dUMP.CH_2H_4F$ | $k_{iso} = 130$ s$^{-1}$ | 5 s$^{-1}$ |

$k_{iso} = 130$ s$^{-1}$

| Reaction | Forward Rate | Reverse Rate |
|----------|--------------|---------------|
| $E + dTMP + H_2F$ | 10.9 µM$^{-1}$s$^{-1}$ | 100 s$^{-1}$ |
| $E.dTMP + H_2F$ | 2.0 µM$^{-1}$s$^{-1}$ | 200 s$^{-1}$ |
| $E.dTMP.H_2F$ | $k_{ss} = 5.5$ s$^{-1}$ | |

### Scheme 3b: The DHFR Reaction

| Reaction | Forward Rate | Reverse Rate |
|----------|--------------|---------------|
| $E + dTMP + H_2F$ | $k_{iso} = 130$ s$^{-1}$ | 5 s$^{-1}$ |
| $E.dTMP + H_2F$ | 13.4 µM$^{-1}$s$^{-1}$ | 60 s$^{-1}$ |
| $E.dTMP.H_2F$ | 9.0 µM$^{-1}$s$^{-1}$ | 40 s$^{-1}$ |

$k_{iso} = 130$ s$^{-1}$

| Reaction | Forward Rate | Reverse Rate |
|----------|--------------|---------------|
| $E + dTMP + H_2F$ | $k_{chem} = 180$ s$^{-1}$ | 17 s$^{-1}$ |
| $E + NADP^+ + H_4F$ | 2.0 µM$^{-1}$s$^{-1}$ | 200 s$^{-1}$ |
| $E + NADP^+ + H_4F$ | 200 s$^{-1}$ | 200 s$^{-1}$ |
| $E.NADP^+.H_4F$ | 10.9 µM$^{-1}$s$^{-1}$ | 100 s$^{-1}$ |
| $E.NADP^+.H_4F$ | 9.0 s$^{-1}$ | 95 s$^{-1}$ |

$k_{chem} = 180$ s$^{-1}$
Scheme 4a: T. gondii DHFR

Scheme 4b: L. major DHFR

*Rate-limiting Step  **Not a Kinetically Competent Step
### Chart 1

| Reaction          | Rate Constant | Units               |
|-------------------|---------------|---------------------|
| U + E ↔ UE        | \( k_1 \)     | \( 13.4 \, \mu M^{-1} \, s^{-1} \) |
| UE + M ↔ UME      | \( k_2 \)     | \( 60 \, s^{-1} \) |
| UME ↔ UIE         | \( k_3 \)     | \( 2 \, \mu M^{-1} \, s^{-1} \) |
| UIE + QDE ↔ QDE   | \( k_4 \)     | \( 40 \, s^{-1} \) |
| QDE ↔ QE + D      | \( k_5 \)     | \( 130 \, s^{-1} \) |
| QE + E ↔ Q + E    | \( k_6 \)     | \( 5 \, s^{-1} \) |
| Q + P + D ↔ ZPD   | \( k_7 \)     | \( 0 \, s^{-1} \) |
| ZPD ↔ ZNT         | \( k_8 \)     | \( 10 \, s^{-1} \) |
| ZNT ↔ ZN + T      | \( k_9 \)     | \( 2 \, \mu M^{-1} \, s^{-1} \) |
| ZN + T ↔ Z + N    | \( k_{10} \)   | \( 180 \, s^{-1} \) |
| Z + N ↔ U         | \( k_{11} \)   | \( 0.001 \, s^{-1} \) |

- U = dUMP
- E = TS active site
- M = CH₂H₄F
- I = Intermediate
- Q = dTMP
- D = H₂F
- Z = DHFR active site
- P = NADPH
- N = NADP⁺
- T = H₄F
Mechanistic characterization of Toxoplasma gondii thymidylate synthase-dihydrofolate reductase: evidence for a TS intermediate and TS half-sites reactivity

Eric F. Johnson, Wolfgang Hinz, Chloe E. Atreya, Frank Maley and Karen S. Anderson

*J. Biol. Chem.* published online August 20, 2002

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