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Probing the Role of Parasite-specific, Distant, Structural Regions on Communication and Catalysis in the Bifunctional Thymidylate Synthase- Dihydrofolate Reductase from *Plasmodium falciparum*

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Abstract

Plasmodium falciparum thymidylate synthase-dihydrofolate reductase (TS-DHFR) is an essential enzyme in nucleotide biosynthesis, and a validated molecular drug target in malaria. Because *P. falciparum* TS and DHFR are highly homologous to their human counterparts, existing active-site antifolate drugs can have dose-limiting toxicities. In humans, TS and DHFR are two separate proteins. In *P. falciparum*, however, TS-DHFR is bifunctional, with both TS and DHFR active sites on a single polypeptide chain of the enzyme. Consequently, *P. falciparum* TS-DHFR contains unique distant or 'non-active' regions which might modulate catalysis: 1) an N-terminal tail; and 2) a 'linker' region tethering DHFR to TS, and encoding a 'crossover helix' that forms critical electrostatic interactions with the DHFR active site. The role of these non-active sites in the bifunctional *P. falciparum* TS-DHFR is unknown. We report the first in-depth, pre-steady state, kinetic characterization of the full-length, WT *P. falciparum* TS-DHFR enzyme, and probe the role of distant, non-active regions through mutational analysis. We show that the overall rate-limiting step in the WT *P. falciparum* TS-DHFR enzyme is TS catalysis. We further show that if TS is in an 'activated' (liganded) conformation, the DHFR rate is 2-fold activated, from 60 s⁻¹ to 130 s⁻¹ in the WT enzyme. The TS rate is also reciprocally activated by ~1.5-fold if DHFR is in an activated, ligand-bound conformation. Mutations to the linker region affect neither catalytic rate nor domain-domain communication. Deletion of the N-terminal tail – although in a location remote to the active site - decreases DHFR single and the bifunctional TS-DHFR rate by a factor of 2. The two-fold activation of the DHFR rate by TS ligands remains intact, although even the activated N-terminal mutant has just half the DHFR activity of the WT enzyme. However, the reciprocal communication – between TS active site and DHFR ligands - is impaired in N-terminal mutants. Surprisingly, deletion of the analogous N-terminal tail in *Leishmania major* TS-DHFR causes a 3-fold *enhancement* of the DHFR rate from ~14 s⁻¹ to ~40 s⁻¹. In summary, our results demonstrate a complex interplay of domain-domain communication and non-active site modulation of catalysis in *P. falciparum* TS-DHFR. Furthermore, each parasitic TS-DHFR is activated by unique mechanisms, modulated by their non-active site regions. Finally, our studies suggest the N-terminal tail of *P. falciparum* TS-DHFR is a highly selective, novel target for potential antifolate development in malaria.

Keywords

Dihydrofolate reductase-thymidylate synthase (DHFR-TS); *Plasmodium falciparum*; antifolates; presteady state kinetics; non-active site

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Malaria is a parasitic disease that kills over 3,000 a day and infects some 300 million people per year. The causative agents of malaria are *Plasmodium* spp. parasites, bred in warm stagnant waters, and spread in humans by the bite of the *Anopheles* mosquito. While many species of *Plasmodium* are endemic to different regions, it is *Plasmodium falciparum* which accounts for over 90% of malaria-related deaths. Despite advances in healthcare, mortality from malaria has increased by nearly 25% over the last decade in sub-Saharan Africa, due primarily to an increase in drug-resistant parasites. High-dose therapy is often limited by the toxicity of the anti-malarials [1]. New, targeted therapies are urgently needed.

Thymidylate synthase-dihydrofolate reductase (TS-DHFR) is a critical metabolic enzyme and validated drug target in *P. falciparum* [2]. As illustrated in Figure 1a, TS catalyzes the conversion of methylene tetrahydrofolate ($\text{CH}_2\text{H}_4\text{-folate}$) to dihydrofolate ($\text{H}_2\text{-folate}$) while methylating 2'-deoxyuridine monophosphate (dUMP) to 2'-deoxythymidine monophosphate (dTTP) [2]. DHFR subsequently converts $\text{H}_2\text{-folate}$ to $\text{H}_4\text{-folate}$ while oxidizing NADPH to NADP^+ . Both $\text{H}_2\text{-folate}$ and $\text{H}_4\text{-folate}$ are important metabolites in the nucleotide biosynthesis pathway; consequently, active-site inhibitors of *P. falciparum* DHFR (e.g. pyrimethamine, cycloguanil) have been used successfully in antifolate therapy in malaria [3].

TS-DHFR is a bifunctional enzyme. Most higher eukaryotes, including humans and prokaryotes, express TS and DHFR as two discrete enzymes. However, several human parasites like *P. falciparum* encode TS and DHFR on a single polypeptide chain, with DHFR N-terminal to TS [2–4]. Previous studies have highlighted novel characteristics of these bifunctional enzymes, with significant variation in different parasites [5–7]. One such interesting property is domain-domain communication; i.e. if the TS active site is liganded in the *Leishmania major* TS-DHFR, a ten-fold activation of the DHFR rate results [6]. Conformational changes in TS and DHFR are thought to modulate this domain-domain communication [4]. Notably, this activation is absent in the *Cryptosporidium hominis* enzyme [5], and has not previously been explored in *P. falciparum* TS-DHFR.

Because TS and DHFR are evolutionarily well-conserved, there is high homology with the corresponding enzymes from other eukaryotes (including humans). However, flanking the TS and DHFR active sites in parasitic TS-DHFR's are distinct, distant, structural or "non-active site" regions, which have no homology between organisms [8–10]. Our work thus focuses on two such non-active site regions in *P. falciparum* TS-DHFR: the crossover helix of the linker region, and the N-terminal tail (Figure 1b).

The linker, a long region connecting TS and DHFR, is 89 and 52 aa in *P. falciparum* and *C. hominis*, respectively. The *L. major* TS-DHFR has no linker (Figure 1b). Although there is no sequence homology between the linkers of *P. falciparum* and *C. hominis*, both encode a 15-aa crossover helix, one face of which forms electrostatic interactions with the backside of the DHFR active site [8–10]. The crossover helices in *C. hominis* and *P. falciparum* TS-DHFR vary in their amino acid compositions and interactions. In *C. hominis*, the face of the crossover is composed of hydrophilic and hydrophobic residues (like S195, D198, L202, I206, R210), which interact with several important residues in the DHFR backbone (like Y132, N42, F35, F172 and E31). However, remarkably, the crossover helix in *P. falciparum* TS-DHFR (residues 283 to 295) has numerous acidic residues (residues 283 to 289 are either Asp or Glu, followed by F290, V291, Y292, F293, N294, and F295), which form electrostatic interactions with the many positively charged residues (primarily Lys) in the backside of the DHFR active site (shown in Figure 2b). Mutational disruption of the electrostatic interactions of the crossover helix in *C. hominis* TS-DHFR diminishes DHFR catalytic rates by 10-fold if the entire crossover helix is substituted with Alanines, or by 3-fold with Alanine-substitution of only the face of the helix which forms interactions with the DHFR active site [11]. However, the kinetic

consequences of disrupting the electrostatic interactions crossover helix in *P. falciparum* TS-DHFR have not yet been studied. The interactions between the crossover helix face and the DHFR active site that can potentially be disrupted by mutagenesis are shown in Figure 2b.

The N-terminal tail comprises the first few amino acids preceding the DHFR domains in certain TS-DHFR's. *P. falciparum* TS-DHFR has a 6 aa long N-terminal tail, oriented away from the DHFR, in a position remote from active site [9] (Figures 2a and c). The N-terminal tail is absent in *C. hominis* TS-DHFR, and in *L. major*, it is 22 aa long and is of unknown function (Figure 1b). The *L. major* N-terminal tail has no homology to its counterpart in *P. falciparum*, and wraps around the surface of the bifunctional enzyme making extensive direct contacts with the TS domain [10]. Studies done on the monofunctional *P. falciparum* DHFR and TS enzymes suggest that deletions of the N-terminal region decrease activity of DHFR and co-expressed TS [12,13]. However, the role of the N-terminal tail in modulating catalysis in the full-length, bifunctional TS-DHFR enzyme has not yet been studied.

To investigate the kinetics of the WT *P. falciparum* TS-DHFR and the role of its non-active site regions, we used pre-steady state kinetic analysis. Transient kinetic methodology allows one to follow chemical catalysis individually at both TS and DHFR active sites, to investigate chemical rates of all three reactions (TS, DHFR and TS-DHFR) individually, to monitor intermediates as they shift from one active site to another, and to investigate the importance of communication between these active sites.

Our first objective was a detailed transient kinetic analysis of the wildtype (WT) *P. falciparum* TS-DHFR enzyme. Specifically, using rapid chemical quench and stopped flow fluorescence, we asked whether communication occurs between the TS and DHFR domains of the enzyme. Our second objective was to determine whether the electrostatic interactions of the crossover helix play an important role in catalysis and communication in *P. falciparum* TS-DHFR. The “face” of the crossover helix which interacts with the DHFR active site is comprised of five residues; we substituted only these five residues with Alanines to form the “Ala-FACE” helix mutant, or replaced the entire crossover helix with Alanines to form the “all-Ala” helix mutant of *P. falciparum* TS-DHFR. Finally, our third objective was to understand the effects of an N-terminal tail mutation on the pre-steady state kinetics the bifunctional, full-length *P. falciparum* TS-DHFR enzyme. The results of deleting residues 2 to 5 of N-terminal tail in *P. falciparum* (“D4”) were compared to those of an analogous deletion of residues 2 to 22 of the N-terminal tail in *L. major* (“N22”). We used the same nomenclature for the *P. falciparum* N-terminal mutants as described previously in the literature [12].

This is the first systematic, transient kinetic characterization of the WT *P. falciparum* TS-DHFR enzyme, and of the non-active site regions of any bifunctional parasitic TS-DHFR. We demonstrate that elegant communication exists between the TS and DHFR active sites of *P. falciparum* TS-DHFR. Also, we show that mutations of the linker crossover helix do not disrupt the catalytic rates in this enzyme. A four amino-acid deletion of the N-terminal tail, however, affects the chemical rates of the TS, DHFR and TS-DHFR reactions in *P. falciparum* TS-DHFR. Surprisingly, analogous mutations in the *L. major* N-terminal tail increase the DHFR chemical rate of the enzyme, suggesting differential regulation between bifunctional TS-DHFR's from different organisms. Our studies of the *P. falciparum* TS-DHFR enzyme highlight the unique regulation of the kinetic mechanisms of bifunctional parasitic TS-DHFR enzymes, especially since *P. falciparum* encodes both an N-terminal tail and a linker with crossover helix. Since TS-DHFR is also a validated drug target, understanding the role of these distinct structural regions may also aid in rational drug design for the development of more targeted and less toxic therapies for this deadly disease.

METHODS

Reagents, Chemicals and Bacterial strains

We used reagents of the highest available commercial grade. Millipore ultrapure water was used in the preparation of all solutions. β -nicotinamide adenine dinucleotide 2'-phosphate (NADPH), dUMP (2'-deoxyuridine 5'-monophosphate disodium salt), 5-Fluoro-2'-deoxyuridine 5'-monophosphate sodium salt (FdUMP), sodium hydrosulfite, chloramphenicol and methotrexate agarose were purchased from Sigma Aldrich (St Louis, MO). 1,4-Dithiothreitol, isopropyl β -D-thiogalactopyranoside (IPTG) and ampicillin (D- α -aminobenzylpenicillin) were purchased from American Bioanalytical. Luria Broth (LB) and Terrific Broth (TB) were obtained from Invitrogen (Carlsbad, Ca) and VWR International (WestChester, PA), respectively.

The tritiated H₂folate was prepared also by reducing commercially available [3',5',7,9-³H] folic acid (Moravek Biochemicals, Brea, CA) with sodium hydrosulfite, and purified using a triethylammonium bicarbonate (TEAB) gradient on a DE-52 resin [14,15]. CH₂H₄folate (methylene) was prepared first by enzymatic conversion of H₂folate to (6R,S)-5,6,7,8-tetrahydrofolate, and then by a condensation reaction in the presence of formaldehyde. Radiolabeled CH₂H₄folate was prepared from tritiated-H₂ folate, with the synthesis otherwise identical to that of the unlabelled CH₂H₄folate. Both unlabelled and tritiated CH₂H₄folate were purified using a TEAB gradient on a DE-52 resin (Whatman) [14,15]. The purity of the radiolabelled substrates was confirmed using HPLC analysis (see below). Calcium-competent *Escherichia coli* strains BL21 (DE3) pLysS were purchased from Invitrogen (Carlsbad, CA).

Enzyme Mutagenesis, Expression and Purification

The plasmid expressing the TM4/8.2 strain of wildtype *P. falciparum* TS-DHFR is a generous gift from Drs. Penchit Chitnumsub and Yongyuth Yuthavong (BIOTEC, National Science and Technology Development Agency, Thailand) [16]. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), we mutated *P. falciparum* TS-DHFR residues 283 to 295 to Ala to create an "all-Alanine" crossover helix, and residues 284, 285, 288, 289, and 292 to Ala to make the "Ala-FACE" crossover helix. We also deleted residues 2 through 5 to make the N-terminal tail mutant "D4", and residues 2 through 6 to make the mutant "D5". Primers were synthesized and gel purified by the Yale Keck Facility according to protocols available online. The mutated plasmids were amplified using calcium-competent DH5 α *Escherichia coli* cells, and then purified using the Qiaprep Spin Manipule Kit (Qiagen, Valencia, CA). Plasmids were sequenced by the Yale Keck Facility (New Haven, CT) to confirm the mutagenesis, and stored at -20°C. Concentration of DNA was determined spectrophotometrically using absorbance at 260nm. The plasmid expressing the TS-DHFR enzyme from *L. major* was the generous gift of C.-C.Kan and D. Matthews (Agouron Pharmaceuticals, La Jolla, CA). Its N-terminal residues 2 to 22 were deleted using QuikChange (Stratagene, La Jolla, CA) to create the N-terminal mutant "N22". The plasmid was cloned into a pET11b for expression in *E. coli* BL21 cells. All plasmids were sequenced by the Yale Keck Facility to confirm mutagenesis.

The *P. falciparum* WT TS-DHFR was expressed and purified as previously described [16]. The protocol for expression of Ala-FACE and all-Ala helix mutants was identical to that for wild type *P. falciparum* TS-DHFR. The protocols for expression and purification of the D4 and D5 N-terminal mutants were also identical to that for the WT enzyme, except that 350 mM KCl (instead of 1 M KCl) was used to wash the unbound enzyme off of the methotrexate affinity resin. The active-site concentrations of wild type and mutant enzymes were equivalent, as determined by pre-steady state burst amplitudes of their DHFR reactions (see below). The

method for expression and purification of *L. major* TS-DHFR WT and N23 deletion mutant were as described previously [6].

Assessment of Enzyme Concentration and Activity

Enzyme concentration was determined spectrophotometrically by following absorbance at 280nm. The extinction coefficient for *P. falciparum* TS-DHFR is $83740 \text{ M}^{-1}\text{cm}^{-1}$, and for *L. major* TS-DHFR is $69955 \text{ M}^{-1}\text{cm}^{-1}$. DHFR steady-state activity was assessed by reacting enzyme with H_2 folate and NADPH, and following absorbance at 340 nm, which decreases as NADPH is depleted to form NADP^+ . An extinction coefficient of $-12.8 \text{ mM}^{-1}\text{cm}^{-1}$ was used for the DHFR reaction. TS steady-state activity was assayed by incubating the enzyme with CH_2H_4 folate and dUMP, and following the increase in absorbance at 340nm with the production of dTMP and H_4 folate. An extinction coefficient of $-6.4 \text{ mM}^{-1}\text{cm}^{-1}$ was used for the TS reaction [17].

Rapid Chemical Quench Experiments

We performed rapid chemical quench experiments on a Kintek RFQ-3 Rapid Chemical Quench Apparatus (Kintek Instruments, Austin, TX) to enable us to study enzymatic experiments on a millisecond time scale. All reactions were performed at 25°C . The reactions involved mixing 15 μL of enzyme solution with 15 μL of substrate solution. The substrates were tritiated (as described above), which enabled subsequent high performance liquid chromatography (HPLC) analysis to follow the reaction substrates and products (see below for details). The enzyme solution consisted of a mixture of enzyme, non-limiting substrate, and 2x reaction buffer (50 mM MgCl_2 , 1 mM EDTA, 50 mM Tris-HCl pH=7.8). The substrate solution consisted of approximately 20,000 dpm of the appropriate tritiated substrate. The DHFR single turnover experiment consisted of mixing an “enzyme solution” of enzyme, NADPH and 2x reaction buffer with a “substrate solution” of tritiated H_2 folate. The TS single turnover experiment consisted of mixing an “enzyme solution” of enzyme, dUMP, and 2x reaction buffer with a “substrate solution” of tritiated CH_2H_4 folate. Finally, the TS-DHFR bifunctional single turnover experiment consisted of mixing an “enzyme solution” of enzyme, dUMP, NADPH and 2x reaction buffer with a “substrate solution” of tritiated CH_2H_4 folate. All reactions were quenched by addition of 67 μL of 0.78M KOH solution. In each reaction, the final concentration of reaction buffer was 1x. All concentrations for enzyme and substrate listed in the text are final concentrations after mixing.

High Performance Liquid Chromatography Analysis

The tritiated reaction products from the rapid chemical quench reactions were analyzed by high performance liquid chromatography (HPLC) with radioactivity and ultraviolet flow detectors. A BDS-Hypersil C18 reverse phase column ($250 \times 4.6 \text{ mm}^2$, Keystone Scientific, Bellefonte, PA) was used for separation. We used a solvent consisting of 10% Methanol in 180 mM triethylammonium bicarbonate (pH=7.8), in isocratic separation mode at a flow rate of 1 mL/min, for optimal separation of our substrates and products. Under these conditions, tritiated H_4 folate eluted at 7 minutes, tritiated H_2 folate at 14 minutes, and tritiated CH_2H_4 folate at 15 minutes.

Stopped Flow Experiments

We used a Kintek SF-2001 Stopped Flow apparatus (Kintek Instruments, Austin, TX) to probe communication between the TS and DHFR domains. Reactions were performed under burst conditions, in which substrate concentration is in slight excess over enzyme concentration. For the DHFR reaction, the “enzyme solution” – consisting of enzyme, NADPH, +/- CH_2H_4 folate, +/- FdUMP, and 2x reaction buffer - was mixed with the “substrate solution” of H_2 folate. For the TS reaction, the “enzyme solution” - consisting of enzyme, dUMP, +/- NADPH – was

mixed with the “substrate solution” of CH₂H₄folate. For the DHFR reaction, we excited the reaction at 287 nm and followed the FRET (fluorescent resonance energy transfer) at 450 nm. For the TS reaction, we excited the reaction at 287 nm and followed the fluorescent emission at 340 nm.

RESULTS

Expression of *P. falciparum* WT and Mutant TS-DHFR enzymes

We were able to produce 0.28 mg of WT enzyme, 0.29 mg of Ala-FACE enzyme and 0.14 mg of D4 pure protein per liter of *E. coli* culture. The DHFR steady-state rates for the WT, Ala-FACE and D4 enzymes were $16.7 \pm 0.164 \text{ s}^{-1}$, $17.6 \pm 0.258 \text{ s}^{-1}$ and $0.496 \pm 0.00421 \text{ s}^{-1}$, respectively. The bifunctional TS-DHFR steady-state rates for the WT, Ala-FACE and D4 enzymes were $1.77 \pm 0.0795 \text{ s}^{-1}$, $1.875 \pm 0.0693 \text{ s}^{-1}$ and $2.53 \pm 0.537 \times 10^{-2} \text{ s}^{-1}$, respectively. We also mutated the *P. falciparum* TS-DHFR plasmid to encode an All-Ala crossover helix. The full-length, all-Ala enzyme consistently co-purified with a 39 kDa band, which was confirmed by mass spectrometry to be a fragment of *P. falciparum* TS-DHFR (and not an *E. coli* TS or DHFR enzyme). Despite using high concentrations of protease inhibitors and expediting the protein purification, degradation of the All-Ala mutant into this 39 kDa fragment could not be prevented. Under these conditions, only 0.01 mg of pure all-Ala helix mutant could be expressed per liter of *E. coli* culture. When we tried to express a mutant with the crossover helix completely deleted, the protein was consistently degraded intracellularly. The interactions disrupted by the Ala-FACE and N-terminal deletion mutants are highlighted in Figure 2b and c.

We also tried to express the “D5” mutant of the N-terminal tail in which residues 2 through 6 of *P. falciparum* WT TS-DHFR were deleted. Protein expression was too low to allow a detailed kinetic characterization of the protein; however, the expressed enzyme reproducibly showed DHFR activity by spectrophotometric analysis.

Single Turnover Experiments for DHFR Reaction for *P. falciparum* TS-DHFR

To examine the DHFR activity of the WT, Ala-FACE and D4 mutants, single enzyme turnover experiments were conducted using rapid chemical quench approach. The formation of tritiated H₄-folate was monitored over time by HPLC analysis of the reaction products. We pre-incubated 80 μM of the appropriate enzyme and a saturating concentration of NADPH (500 μM), and then mixed with 6.5 μM tritiated H₂-folate. All reactions were carried out to completion but only the earlier time points are shown here for clarity (Figure 3a). The data were fit to a single exponential equation, and the rate constants for the WT and Ala-FACE mutant were similar at $80 \pm 5.2 \text{ s}^{-1}$ and $64 \pm 4.4 \text{ s}^{-1}$, respectively. The rate constant for the D4 mutant was ~ 2 -fold slower than WT at $38 \pm 2.4 \text{ s}^{-1}$.

Single Turnover Experiments for the TS Reaction for *P. falciparum* TS-DHFR

To examine the TS activity for the WT, Ala-FACE and D4 mutants, single enzyme turnover experiments were conducted again using a rapid chemical quench approach. The formation of tritiated H₂-folate was followed over time by HPLC analysis of the reaction products. In these experiments, 80 μM of the appropriate enzyme was preincubated with a saturating concentration of dUMP (500 μM), and then mixed with 6.5 μM tritiated CH₂H₂-folate. All reactions were carried out to completion but only a subset of the data is shown here (Figure 3b). The data were fit to a single exponential equation, and the rate constants for the WT and Ala-FACE mutant were similar at $1.2 \pm 0.22 \text{ s}^{-1}$ and $1.4 \pm 0.28 \text{ s}^{-1}$, respectively. The rate constant for the D4 N-terminal tail mutant was $0.84 \pm 0.083 \text{ s}^{-1}$, demonstrating that the TS activity of the enzyme for the D4 is very similar to that of the WT enzyme. These results are summarized in Table 1.

Single Turnover Experiments for the Bifunctional Reaction for *P. falciparum* TS-DHFR

To examine the TS-DHFR bifunctional activity for the WT, Ala-FACE and D4 mutants, single enzyme turnover experiments were conducted with a similar rapid chemical quench approach. In this case, the formation of tritiated H₄-folate was monitored over time by HPLC analysis of the reaction products. We pre-incubated 80 μM of the appropriate enzyme with a saturating concentration of dUMP (500 μM) and NADPH (500 μM), and then mixed with 6.5 μM tritiated CH₂H₄-folate. The reactions were carried out to completion, but only a subset of the data is shown here for clarity (Figure 3c). The data were fit to a single exponential equation, and the rate constants for the WT and Ala-FACE mutant were similar at $1.2 \pm 0.085 \text{ s}^{-1}$ and $1.4 \pm 0.091 \text{ s}^{-1}$, respectively. The rate constant for the D4 N-terminal tail mutant for this same reaction is only half that, at $0.60 \pm 0.15 \text{ s}^{-1}$. These results are summarized in Figure 3c and Table 1.

Pre-steady state burst experiments of the DHFR reaction for *P. falciparum* TS-DHFR

NADPH bound to the DHFR active site produces a fluorescent resonant energy transfer (FRET) at 450nm. As H₂-folate is mixed, the NADPH bound at the active site is oxidized to NADP⁺, producing a decrease in fluorescence. Thus, stopped flow fluorescence studies can be used to follow consumption of NADPH at the DHFR active site on a millisecond time scale. The purpose of these particular studies was to confirm the rapid chemical quench DHFR chemical rates determined by following the production of H₄-folate. In these reactions, 7.5 μM of the corresponding enzyme were preincubated with 500 μM NADPH, and then mixed with 50 μM H₂-folate in the stopped flow. Excitation was at 287nm and emission at 450nm. The data were fit to a single exponential burst equation (Figure 4 a), and the rate constants are reported in Table 2. The chemical rates determined from the stopped flow burst experiments are very similar to those obtained by rapid chemical quench single turnover. Importantly, these results also confirm that the D4 mutant has a DHFR rate constant that is about half that of the WT and Ala-FACE enzymes.

DHFR Activation reaction in the presence of TS ligands FdUMP and CH₂H₄-folate

We also monitored the DHFR activity in the presence of the two TS ligands, CH₂H₄-folate and FdUMP (a dead-end inhibitor of TS), for WT and mutant *P. falciparum* TS-DHFR. We preincubated 7.5 μM of the corresponding enzyme with 500 μM NADPH, 100 μM FdUMP and 25 μM CH₂H₄-folate, and then mixed with 50 μM H₂-folate in the stopped flow. Excitation was at 287nm and emission at 450nm. The data were fit to a single exponential burst equation (Figure 4b) and summarized in Table 2. In the presence of TS ligands, the chemical rate is almost doubled in all three WT, Ala-FACE and D4 enzymes, and the D4 rate remains about half that of the WT enzyme.

Pre-steady state burst experiments of the TS reaction in the absence and presence of the DHFR ligand NADPH

We also asked the reciprocal question, of whether the TS rate is activated by the DHFR ligand NADPH. Using the stopped flow, we followed the change in intrinsic fluorescence at the TS active site upon substrate binding and conversion to product. Excitation was at 287nm and emission at 340nm. We preincubated 7.5 μM of the corresponding enzyme with 100 μM dUMP and mixed this with 25 μM CH₂H₄-folate. For the experiment in the presence of NADPH, 7.5 μM of the corresponding enzyme were preincubated with 100 μM dUMP and 500 μM NADPH, and then mixed with 25 μM CH₂H₄-folate in the stopped flow. The data were fit to a single exponential burst equation. The TS rates, in the absence of DHFR ligand NADPH, for WT, Ala-FACE and D4 mutants were $9.94 \pm 0.203 \text{ s}^{-1}$, $8.58 \pm 0.249 \text{ s}^{-1}$ and $10.1 \pm 0.507 \text{ s}^{-1}$, respectively. However, the TS rates in the presence of NADPH were $15.3 \pm 0.428 \text{ s}^{-1}$, $13.2 \pm 0.324 \text{ s}^{-1}$ and $10.3 \pm 0.541 \text{ s}^{-1}$, for the WT, Ala-FACE and D4 mutants, respectively. Thus,

the DHFR ligand NADPH seems to seem activate the TS burst rate in WT and Ala-FACE enzymes, but not in the D4 mutant.

***L. major* N22 DHFR single turnover and Pre-steady state DHFR Burst**

We also expressed the *L. major* WT bifunctional TS-DHFR enzyme, and a mutation in which only its N-terminal tail is deleted (N22). Though expression of N22 was about 0.07 mg pure protein per liter of *E. coli* culture, DHFR single turnover and pre-steady burst experiments could be performed. As described above, 45 μM *L. major* WT or N22 TS-DHFR were preincubated with 500 μM NADPH, and then mixed with 5.5 μM tritiated H_2 -folate in the rapid chemical quench. The production of tritiated H_4 -folate was monitored over time by HPLC analysis. The rate constant for DHFR is $40.2 \pm 4.60 \text{ s}^{-1}$ in the N22 mutant (Figure 5a), compared to the WT *L. major* DHFR catalytic rate of $14.5 \pm 1.37 \text{ s}^{-1}$ (data not shown). The DHFR rate constant for the WT *L. major* enzyme is similar to previously published data [6]. However, the fast chemical rate was unexpected in the N22 mutant. To confirm this fast rate, a presteady state, DHFR burst experiment of the N22 enzyme was also performed using stopped flow analysis, as outlined above for the *P. falciparum* enzyme. We preincubated 7.5 μM enzyme and 500 μM NADPH, and mixed with 50 μM H_2 -folate. Excitation was at 287 nm and emission at 450 nm, and the data were fit to a single exponential. The DHFR burst rate constant for the N22 mutant was $45.21 \pm 0.853 \text{ s}^{-1}$ (Figure 5 b), which is in good agreement with our single turnover rate.

DISCUSSION

Kinetics of the Wildtype *P. falciparum* TS-DHFR enzyme

This is the first report of the single turnover rate constants for the TS, DHFR and bifunctional TS-DHFR reactions for *P. falciparum* TS-DHFR. We have shown that, in WT *P. falciparum* TS-DHFR, the DHFR chemical rate (70 s^{-1}) is significantly faster than TS. TS is the rate-limiting step in the both the TS reaction and the bifunctional TS-DHFR reaction with a chemical rate $\sim 1 \text{ s}^{-1}$ (Table 1), an observation consistent with the known kinetic mechanisms for other parasitic TS-DHFR's [5,6].

This is also the first report of domain-domain communication in *P. falciparum* TS-DHFR. We show that, if the TS active site is liganded in the *P. falciparum* TS-DHFR, the DHFR chemical rate is nearly doubled to $\sim 130 \text{ s}^{-1}$ (Table 2). This communication between TS and DHFR active sites is important for three reasons. First, the activation of *P. falciparum* DHFR is analogous to the regulation of DHFR activity in the *L. major* TS-DHFR enzyme, but very different from that of *C. hominis* and *T. gondii* TS-DHFR's, which are activated regardless of whether ligands are bound at the TS active site. Second, the rate of the activated *P. falciparum* and *L. major* DHFR's are about 130 s^{-1} and 120 s^{-1} [6], respectively, while the rate constants of DHFR from *C. hominis* and *T. gondii* are about 130 s^{-1} and 180 s^{-1} , respectively. This suggests that these four parasitic TS-DHFR enzymes have similar *in vitro* DHFR chemical rates, but different methods of activation. And third, since presumably the *P. falciparum* TS-DHFR is in a liganded state in the cell, this "activated" DHFR rate is probably a more accurate representation of the chemical rate. Our data also show a reciprocal communication occurs between TS and DHFR in the WT enzyme, as TS rate is activated in the presence of the DHFR ligand, NADPH.

This communication between active sites is likely mediated by conformational changes to the enzyme active site initiated by ligand-binding, a well-studied phenomenon in both the *E. coli* TS and DHFR enzymes [18–22]. In fact, when we performed the DHFR reactions in the presence of only FdUMP (without CH_2H_4 -folate), the DHFR chemical rates were not activated (data not shown). The published crystal structure of *P. falciparum* TS-DHFR has a doubly-liganded DHFR active site (bound with NADPH and WR99210, a triazine DHFR inhibitor

[23,24]) but only a singly-liganded TS active site (bound with dUMP but no folate ligand) [9]. It would be interesting to see if our kinetic data on activation and communication are reflected in significant structural differences in a TS-DHFR where the TS is doubly liganded. If the DHFR active site is conformationally different in the presence of a fully-liganded TS, then these activation data could have considerable implications for structure-based drug design against *P. falciparum* TS-DHFR.

The crossover helix mutant of *P. falciparum* TS-DHFR enzyme

Our results show that the crossover helix does not play a role in modulating catalysis in *P. falciparum* TS-DHFR. We designed several mutations of the crossover helix (residues 284 to 295) in the linker region of the *P. falciparum* TS-DHFR enzyme, and probed these mutants for changes in the chemical rates of the TS, DHFR and bifunctional TS-DHFR reactions. The sequence encoding the crossover helix has no homology to the linker region of other parasitic TS-DHFR's, but the helix itself is conserved in the *C. hominis* and *T. gondii* TS-DHFR enzymes. In *P. falciparum*, the crossover helix is very negatively charged, and interacts with positively charged residues of the backside of the active site of DHFR (as shown in Figure 2b) [9]. The importance of this helix, already suggested by its numerous interactions with the DHFR domain, was compounded by unpublished data from W. Sirawaraporn demonstrating that the shortest region of a linker-TS construct that could interact with DHFR started at residue 282, immediately N-terminal to the crossover helix. In *C. hominis*, mutations of the crossover helix have been shown to reduce DHFR activity [11].

Thus, we designed three mutations of the crossover helix: 1) deletion of the entire helix (Δ -helix); 2) substitution of all residues of the helix with Alanines (All-ALA); and 3) substitution of only those residues on the face of the helix which interact with the backside of the DHFR active site (Ala-FACE). The Δ -helix and All-ALA mutations underwent significant intracellular proteolysis, and could not be expressed in sufficient quantity for detailed kinetic analysis.

The Ala-FACE enzyme had TS, DHFR, and TS-DHFR chemical rates that were very similar to WT, under both single turnover and pre-steady state burst conditions (Table I–Table II). Also, the Ala-FACE enzyme also had a similar activation of the DHFR chemical rate by TS ligands (Table 2), and the TS rate by the DHFR ligand NADPH. Taken together, these results suggest that the crossover helix perhaps plays a role in stabilizing the interaction between TS and DHFR in the *P. falciparum* bifunctional enzyme, but does not affect catalytic rate directly. Significant disruption of the electrostatic interactions of the helix, or deletion of the helix, might expose previously internalized regions of the enzyme to cytosolic proteases. Since disruption of crossover helix interactions in *C. hominis* significantly decreases DHFR catalytic rate, similar results for the *P. falciparum* enzyme had initially been expected. However, in *P. falciparum*, the much higher prevalence of acidic residues in, and the larger number of electrostatic interactions made by, the helix with the DHFR backbone perhaps renders this crossover helix more critical in maintaining structural integrity in this large enzyme.

The D4 N-terminal mutant of *P. falciparum* TS-DHFR enzyme

The N-terminal tail of the *P. falciparum* protein comprises only residues 1 to 6, and yet two previous studies have suggested it plays an important role in DHFR activity and the interaction between TS and DHFR domains [12,13]. Shallom *et al.* demonstrated that the integrity of the N-terminal region of *P. falciparum* DHFR is essential for TS to be active [13]. Wattanarangsang *et al.* made sequential deletions of the N-terminal region of the DHFR domain, and found that the number of amino acids deleted from the N-terminal tail corresponded with decreasing DHFR activity. They also suggest that TS will remain active in the presence of a conformationally-intact DHFR, even though the DHFR enzyme has significantly impaired

activity [12]. One must note that neither of these previous studies was carried out on the full-length, bifunctional *P. falciparum* TS-DHFR enzyme, or involved pre-steady state kinetic analysis. They involved expressing monofunctional *P. falciparum* DHFR and linker-TS constructs, and monitoring their interaction with complementation assays in *E. coli*.

Our results confirm that deleting the N-terminal tail in *P. falciparum* TS-DHFR decreases DHFR catalytic rate (Figure 3 and Figure IV). Furthermore, our data newly suggest that the rate of the bifunctional TS-DHFR reaction is 2-fold slower in the D4 mutant than in wildtype, although TS chemical rate in D4 mutant is about two-thirds that of the wildtype enzymes (Table 2, Figure 3c). We also show that 2-fold activation of DHFR rate by TS ligands remains intact in the D4 mutant (Table 2), but is impaired in the activation of TS by the DHFR ligand NADPH.

It is imperative to note that the bifunctional D4 mutant is significantly more active than might have been suggested by the previous studies on the monofunctional DHFR [12].

Wattananarangsarn *et al.* show the D4 DHFR mutant to be 80-fold less active than WT DHFR when activity is determined spectrophotometrically from crude extract, and over 400-fold less active when DHFR activity is determined by bacterial complementation assay [12]. In our studies, the D4 mutant DHFR rate is only 2-fold decreased compared to WT under single turnover conditions, and about 30-fold decreased under steady state conditions. Also, we show that the full-length D4 mutant has impaired bifunctional TS-DHFR activity under both single turnover and steady state conditions (Figure 3b). We attribute the differences between the monofunctional and bifunctional D4 enzyme studies to the elegant communication between TS and DHFR domains in the intact enzyme. Our results also suggest that the DHFR ligand NADPH does not activate the TS activity, which might explain why the bifunctional TS-DHFR chemical rate is somewhat slower than the TS single turnover rate alone. The differences in our pre-steady state *in vitro* data, compared to previous studies on N-terminal mutants, may also be accounted for by the different microenvironment of the intracellular environment.

Why does the N-terminal tail, although remote from the active site, affect catalysis? Since D4DHFR can still be activated in the presence of TS ligands (Figure 4), and since substrate channeling from TS to DHFR domains remains unperturbed in the D4 mutant (data not shown), we suggest that the D4 mutant is affecting co-ordinated, domain-domain motions which occur during catalysis and ligand-binding in DHFR. Such movements have been clearly demonstrated in *E. coli* DHFR [18,22] and TS enzymes [19–21]. Close examination of the structure of *P. falciparum* TS-DHFR shows that the N-terminal tail is distant from the active site, but can interact with helices in Insert II (a 35aa region unique to Plasmodial DHFR's) and the $\alpha\beta$ loop which encodes part of the active site [9] (Figure 2c). Thus, the interaction between these loops and the N-terminal tail may be essential in the co-ordinated motions of DHFR during catalysis. Since the structure of the fully-liganded, *P. falciparum* TS-DHFR enzyme has not yet been solved, there may be additional, or distinct, interactions between the N-terminal tail and regions linked to the DHFR active site, which are not fully appreciable with the present structure.

It should be noted, however, that our data cannot exclude solvation differences between wildtype and mutant enzymes to account for the difference in their activities. We would expect such differences to be negligible, given that only four amino acids were deleted to make the D4 mutant. However, such potential solvation differences may especially become important in ligand design if the N-terminal tail of *P. falciparum* TS-DHFR is targeted in rational drug design [25–27].

A comparison of mutant forms of the enzyme with WT requires normalization of the active functional protein. Our previous studies on other enzymes have established that equivalent active site concentrations using pre-steady-state burst experiments offers a reliable means for

comparing WT and mutant bifunctional TS-DHFR proteins. The steady state determination of TS specific activity has also been suggested as a reliable indicator of functional protein [28], but cannot be used to compare mutants with impaired TS function.

The N22 N-terminal mutant of the *L. major* TS-DHFR enzyme

We wanted to determine whether deleting the 22aa N-terminal tail in *L. major* TS-DHFR would also decrease the DHFR catalytic rate. Surprisingly, we found that the N22 mutation had a faster single turnover DHFR rate, an observation confirmed with pre-steady state burst studies on the stopped flow (Figure 5a–b). Since the N-terminal tail in *L. major* makes extensive contacts with the TS domain (Figure 5c) [10], perhaps it is playing an autoinhibitory role, and is dislocated during the co-ordinated motions which accompany catalysis in TS and DHFR enzymes. The pre-steady state DHFR burst experiment in the presence of TS ligands for the N22 mutant shows that the enzyme has lost its DHFR burst when TS is activated (data not shown), although it is already known that the WT *L. major* enzyme demonstrates clear domain-domain communication between DHFR and TS [6]. Therefore, we conclude that deleting the *L. major* TS-DHFR N-terminal tail leads to a faster DHFR rate of chemistry, and a disruption in the activation of DHFR by TS. Furthermore, the N-terminal tail plays opposite roles in *L. major* and *P. falciparum* TS-DHFR's. Perhaps because of the extensive contacts it makes with the TS domain, the N-terminal may play an autoinhibitory role, and must be dislocated for the enzyme to be active (Figure 5c).

Summary and Implications for Parasitic Bifunctional TS-DHFR's and Inhibitor Design

This is the first systematic, in-depth, pre-steady state kinetic analysis of the non-active site regions of any bifunctional TS-DHFR enzyme, especially one from *P. falciparum*. Our results show that, in the WT enzyme, TS catalyzes the rate-limiting step, and communication occurs between both the TS and the DHFR domains. Mutations to the face of the crossover helix in the linker region affect neither the rate of catalysis at either active site, nor inter-domain communication. Deletion of the N-terminal tail of *P. falciparum* TS-DHFR decreases the DHFR single turnover rate by one-half, and the bifunctional TS-DHFR rate by about one-third. While DHFR rate can still be activated 2-fold in the presence of TS ligands, reciprocal activation of TS by DHFR ligands is impaired in the D4 mutant. However, an analogous deletion in the N-terminal tail of the *L. major* TS-DHFR N-terminal tail leads to a three-fold activated DHFR rate and impaired DHFR-to-TS communication.

Even though there is overall structural similarity in parasite TS-DHFR's, general conclusions about enzyme mechanism cannot be drawn: TS-DHFR enzymes from different parasites are regulated very differently; and there is minimal kinetic basis for previous phylogenetic classification of these TSDHFR enzymes by homologous structural features [29]. The N-terminal tail can have both an activating and an auto-inhibitory role. The crossover helix in the linker may increase catalytic activity or have minimal effect on rate of chemistry at DHFR [7]. Each one of these enzymes is unique and elegant in its design. The impact of these non-active mutations needs to be further elucidated in cell culture models of *P. falciparum* and *L. major* parasites. More studies are needed to dissect the interaction of the DHFR N-terminal tail with its neighboring helices in the DHFR domain.

Implications for Novel Therapeutics

In many well-characterized enzymes like the HIV reverse transcriptase and the HIV-1 protease, the importance of non-active site regions in drug design and drug resistance is already well-appreciated [30,31]. Even in human and *E. coli* DHFR's, the non-active site regions have long been known to play an important role in therapeutics and regulation of expression [32,33]. Antifolates are used in the clinic for treatment of malaria and leishmaniasis, and, in both cases, toxicity is a limiting factor in high-dose treatment [34]. Thus, a better understanding of the

non-active site regions, with no homologous counterparts in the corresponding human enzymes, could lead to the development of more targeted therapy. In *P. falciparum* TS-DHFR, inhibitors directed towards the N-terminus could be used in combination with active-site inhibitors for a synergistic inhibition of the malaria TS-DHFR. Our study, examining the functional contributions of non-active site regions in *P. falciparum* and *L. major* TS-DHFR, thus has implications in the design of novel, selective, less toxic therapies for these parasitic diseases.

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The following abbreviations were used

TS-DHFR, thymidylate synthase-dihydrofolate reductase (this is a functional designation as dihydrofolate is produced at TS and utilized at DHFR; this enzyme is also commonly referred to as DHFR-TS in the literature because the DHFR domain is N-terminal to TS); dUMP,

deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; CH₂H₄folate, methylene tetrahydrofolate; H₂folate, dihydrofolate; H₄folate, tetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography.

Figure 1. The Reaction Scheme and the Non-active Site Regions of Bifunctional Parasitic TS-DHFR enzymes

a) TS catalyzes the conversion of $\text{CH}_2\text{H}_4\text{folate}$ and dUMP to dTMP and H_2folate . H_2folate is converted to H_4folate at the DHFR active site while an NADPH is oxidized to NADP^+ .

b) Diagrammatic representation of organism-specific differences in non-active site regions of TS-DHFR. The N-terminal tail (yellow) of the DHFR active site (blue) can be of variable length, or completely absent. This is also true of the linker or junctional region (green), which links the DHFR and TS (red) active sites.

Figure 2. The Non-active Sites Regions of *P. falciparum* TS-DHFR enzymes, highlighting the interactions disrupted in the Ala-FACE and D4 mutants

a) The Pf TS-DHFR (PDB entry: 1J3I) structure is colored with DHFR domains in blue, TS in red, with the linker region highlighted in green, and the N-terminal tail in yellow.

b) The interactions between the crossover helix (green) and the backside DHFR active site (burgundy) disrupted by the Ala-FACE mutant. The residues shown are Asp 284, Glu 285, Asp 288, Asp 289 and Tyr 292 in the crossover helix, and Lys 69, Lys 72, Tyr 158, Lys 160, Lys 180, Lys 181 and Tyr 183 in the DHFR backbone. The substrates WR99210 and NADPH are shown in navy blue to mark the active site. For clarity, only a single DHFR monomer and its adjacent TS domain are shown, and also the residues Asn 231 to Asp 222 and Lys 227 are not shown.

c) Interactions of the residues of the N-terminal tail (shown in yellow) with Insert II (shown in orange) and with the α E- β E loop (shown in purple). The residues highlighted are Glu 3 and Val 5 in the N-terminal tail, Tyr 90 in Insert II and Ile 50 in the α E- β E loop. The substrates WR99210 [23] and NADPH are shown in navy blue to mark the active site. Only a single DHFR domain is shown for clarity.

Figure 3. Single turnover experiments for WT, Ala-FACE and D4 mutants of *P. falciparum* TS-DHFR show the N-terminal tail mutant slows the all three reactions, TS, DHFR and TS-DHFR. The data for WT enzyme are (red, circle), for Ala-FACE enzyme (blue, square) and for the D4 N-terminal mutant (green, diamond) are plotted by reaction. The reactions were conducted using rapid chemical quench apparatus under single turnover conditions at 25°C.

a) The DHFR experiment was conducted with 80 μM enzyme and 500 μM NADPH mixed with 6.5 μM tritiated H_2 -folate.

b) The TS experiment was conducted with 80 μM enzyme and 500 μM dUMP mixed with 6.5 μM tritiated CH_2H_4 -folate.

c) The bifunctional TS-DHFR experiment was conducted with 80 μM enzyme, 500 μM NADPH, and 500 μM dUMP mixed with 6.5 μM tritiated H_2 -folate. In all cases, the data were fit to a single exponential equation, and the rate constants from these are summarized in Table 1.

Figure 4. DHFR rate in *P. falciparum* TS-DHFR is activated in the presence of TS ligands, dUMP and CH₂H₄-folate

DHFR burst experiments for WT, Ala-FACE and D4 N-terminal mutants conducted in the presence or absence of TS ligands. Using stopped flow fluorescence, we performed these experiments under burst conditions, with fluorescence excitation at 287 nm and the emission at 450 nm.

Column a) shows data from DHFR burst experiments conducted in the absence of TS ligands. The conditions were used were 7.5 μ M enzyme and 500 μ M NADPH, mixed with 50 μ M H₂-folate.

Column b) shows data from DHFR burst experiments conducted in the presence of TS ligands, FdUMP and CH₂H₄-folate. The conditions were used were 7.5 μ M enzyme, 500 μ M NADPH, 100 μ M FdUMP, and 25 μ M CH₂H₄folate, mixed with 50 μ M H₂-folate.

WT data points are shown in red, Ala-FACE data points, in blue and D4 data points, in green. In all cases, the data were fit to a single exponential equation, and the rate constants from these are summarized in Table 2.

Figure 5. DHFR reactions of the *L. major* N-terminal deletion mutant, N22, show that the N-terminal tail is autoinhibitory in the *L. major* TS-DHFR enzyme

a) Single turnover, rapid chemical quench experiments for N22 conducted using 45 μM enzyme and 500 μM NADPH mixed with 5.5 μM tritiated H_2 -folate.

The data were fit to a single exponential equation to provide a rate constant of $40.2 \pm 4.6 \text{ s}^{-1}$. In comparison, the DHFR single turnover rate for WT *L. major* TS-DHFR had a rate constant of $14.5 \pm 1.37 \text{ s}^{-1}$ (data not shown).

b) N22 mutant DHFR burst experiment, performed on the stopped flow, was conducted using 7.5 μM enzyme and 500 μM NADPH, mixed with 50 μM H_2 -folate. The fluorescence

excitation was at 287 nm and the emission was at 450 nm. The data were fit to a single exponential burst equation to provide a rate constant of $45.21 \pm 0.853 \text{ s}^{-1}$.

c) The *L. major* TS-DHFR structure is colored with DHFR domains in blue, TS in red, and the N-terminal tail in yellow, which wraps around and makes extensive contacts with the TS domain. The substrates, methotrexate, NADPH, FdUMP and 10-propargyl-5,8-dideazafolate (PDDF) are shown in gray.

Table 1

Summary of Single turnover results for DHFR, TS and TS-DHFR reactions from Rapid Chemical Quench Experiments^a

Enzyme	DHFR rate constant (s ⁻¹)	TS rate constant (s ⁻¹)	Bifunctional TS-DHFR rate constant (s ⁻¹)
WT	80 ± 5.2	1.2 ± 0.22	1.2 ± 0.085
Ala-FACE	64 ± 4.4	1.4 ± 0.28	1.4 ± 0.091
D4	38 ± 2.4	0.84 ± 0.083	0.60 ± 0.15

^aThe data were fit to a single-exponential equation, and the conditions used were as summarized in the legend for Figure 3.

Table 2

Summary of DHFR rate constants from Stopped Flow FRET experiments under burst conditions for WT, Ala-FACE and D4 mutants^a

	+ no TS ligands	+ FdUMP + CH ₂ H ₄ folate
WT DHFR burst rate (s⁻¹)	61.0 ± 7.52	135 ± 9.18
Ala-FACE DHFR burst rate (s⁻¹)	68.0 ± 8.14	161 ± 7.09
D4 DHFR burst rate (s⁻¹)	28.9 ± 4.56	63.2 ± 2.93

^aThe data were fit to a single-exponential burst equation, and the conditions used were as summarized in the legend for Figure 4.