Kinetic Characterization of Bifunctional Thymidylate Synthase-Dihydrofolate Reductase (TS-DHFR) from Cryptosporidium hominis

A PARADIGM SHIFT FOR TS ACTIVITY AND CHANNELING BEHAVIOR*§

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This study presents a kinetic characterization of the recently crystallized bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR) enzyme from the apicomplexan parasite, Cryptosporidium hominis. Our study focuses on determination of the C. hominis TS-DHFR kinetic mechanism, substrate channeling behavior, and domain-domain communication. Unexpectedly, the unique mechanistic features of C. hominis TS-DHFR involve the highly conserved TS domain. At 45 °C, C. hominis TS activity is 10–40-fold faster than other TS enzymes studied and a new kinetic mechanism was required to simulate C. hominis TS behavior. A large accumulation of dihydrofolate produced at TS and a lag in product formation at DHFR were observed. These observations make C. hominis TS-DHFR the first bifunctional TS-DHFR enzyme studied for which there is clear evidence against dihydrofolate substrate channeling. Furthermore, whereas with Leishmania major TS-DHFR there are multiple lines of evidence for domain-domain communication (ligand binding at one active site affecting activity of the other enzyme), no such effects were observed with C. hominis TS-DHFR.

Protozoal parasites such as Cryptosporidium hominis, Plasmodium falciparum, Toxoplasma gondii, and Leishmania major are unusual in that their thymidylate synthase (TS)1 and dihydrofolate reductase (DHFR) enzymes exist on the same polypeptide as part of a single bifunctional TS-DHFR enzyme.2 TS and DHFR are critical enzymes and established drug targets. TS represents the only means of de novo synthesis of 2'-deoxythymidylic acid (dTMP) for DNA synthesis, via reductive methylation of 2'-deoxyuridylic acid (dUMP) with methylene tetrahydrofolate (CH$_2$H$_4$folate), producing dihydrofolate (H$_2$folate) in the process. DHFR catalyzes the reduction of H$_2$folate by NADPH to generate tetrahydrofolate (H$_4$folate), used for one carbon unit transfer reactions in several biochemical processes, including thymidylate, purine, and amino acid biosynthesis.

Almost a decade of research on bifunctional TS-DHFR from protozoal parasites has relied on the only available TS-DHFR crystal structure, that from the kinetoplastid protozoa L. major. The recent solution of the crystal structures of TS-DHFR enzymes from two clinically relevant apicomplexan protozoa, P. falciparum and C. hominis, however, has revealed unanticipated deviations from the kinetoplastid model (2, 3). An obvious next question is whether these significant structural differences translate as significant mechanistic differences between parasite classes. We sought to address this question through a detailed kinetic characterization of TS-DHFR from C. hominis, again yielding surprising results.

A major conclusion of the recent solution of the apicomplexan TS-DHFR structures is that there exist two families of bifunctional TS-DHFR structures: a short linker family with an N-terminal tail, as in the kinetoplastids (a class that includes Leishmania and the trypanosomes); and a long linker family with a donated or crossover helix, as in the apicomplexan parasites (which include P. falciparum, C. hominis, and T. gondii) (3). Leishmania major TS-DHFR contains only 2 junctional amino acids between the TS and DHFR domains (short linker) and has a 22-amino acid N-terminal tail that extends from DHFR and wraps the TS domain. By contrast, the apicomplexan protozoa have extensive linker regions but lack an N-terminal tail that contacts TS. The functional TS-DHFR domains are 89 amino acids in P. falciparum, 58 amino acids in C. hominis, and 72 amino acids in T. gondii. TS exists as a dimeric enzyme; in the apicomplexan protozoa the junctional region makes extensive contacts with the DHFR domain of the opposite half of the TS-DHFR dimer, bringing the two DHFR domains closer together.

Are these large structural differences correlated with mechanistic or functional differences? To address this question we have applied a transient kinetic approach to the study of TS-DHFR from C. hominis. Transient kinetic analysis is invaluable for mechanistic studies because this technique enables direct monitoring of chemical catalysis at each active site and measurement of individual steps in a kinetic pathway. Transient kinetics also allows one to define the reaction kinetics of intermediate formation as well as to monitor transit of an intermediate from one active site to the other. We focused our efforts in three areas: determination of the C. hominis TS-DHFR kinetic mechanism, substrate channeling, and communication between the TS and DHFR domains.

TS is more highly conserved than DHFR. When the C. hominis TS-DHFR nucleic acid sequence was ascertained, it was noted that there exist unique residues in the DHFR domain...
analogous to mutations conferring resistance to anti-folate therapies in other species. It was postulated that these intrinsic differences may explain why C. hominis is refractory to the anti-folate drugs that are standard therapy for other apicomplexan infections. In the case of the TS domain, a change in a single conserved residue of unknown significance was mentioned (4). The C. hominis crystal structure revealed a 9-stranded rather than the usual 8-stranded β sheet in the DHFR domain, and a TS domain strongly resembling that from most eukaryotes (3). These findings, combined with the fact that we had previously observed mechanistic differences between T. gondii and L. major DHFR but not TS (5), led us to speculate that unique features in the C. hominis mechanism would most likely involve the DHFR domain.

Our second objective was to explore the possibility of substrate channeling. An attractive hypothesis is that a having a bifunctional TS-DHFR enzyme is that H2folate produced at TS is directly channeled to the DHFR active site, without equilibration in bulk solution (1, 6). Indeed, there is evidence for substrate channeling with both L. major and T. gondii TS-DHFR. In the case of these bifunctional enzymes, unlike with the combination of monofunctional TS + DHFR, H2folate produced at the TS active site does not substantially accumulate in solution and there is no lag in product formation at DHFR, suggesting that the TS product does not leave the protein and enter bulk solution before binding at DHFR (5, 7, 8). Steady-state spectroscopic analysis, used to detect the presence or absence of a lag in NADPH production via the DHFR-catalyzed reduction of H2folate formed at TS, also supports substrate channeling by both L. major and T. gondii TS-DHFR (9, 10).

In the case of L. major TS-DHFR, the crystal structure lends itself to the possibility of substrate channeling; there is a path of solvent-exposed basic residues between the TS and DHFR active sites. It was proposed that these residues compose a 40-Å “electrostatic highway” for directional substrate channeling of negatively charged H2folate (1, 6). Conversely, basic residues are relatively scattered over the P. falciparum and C. hominis TS-DHFR protein surfaces making electrostatic channeling improbable. Furthermore, the TS active site of one-half of the apicomplexa dimer is orthogonal to the DHFR active site of the same peptide chain, making an alternate, dynamic channeling hypothesis, whereby a conformational change brings the active sites in closer proximity (6), less feasible. We sought to determine whether or not there was mechanistic evidence for substrate channeling by C. hominis TS-DHFR.

Finally, the most striking differences between the L. major and apicomplexa structures exist in non-active site regions; the N-terminal tail in the case of L. major, and the long-linker region in the case of the new structures. Do these structural differences in non-active site regions result in differences in domain-domain communication, or conformational changes induced by ligand binding at one active site that affect activity at the other active site of the bifunctional enzyme? There is evidence with L. major TS-DHFR for domain-domain communication: unlike T. gondii DHFR where activity is maximal even when TS is in an unliganded state (5), with L. major, formation of a dUMP-CH₂H₄folate-enzyme ternary complex at TS accelerates the DHFR rate 20-fold (7). There is further evidence of reciprocal communication in L. major in that an additional conformational change at TS occurs in the presence of the DHFR ligand, NADPH. Moreover, addition of the DHFR folate inhibitor, methotrexate, results in a 4-fold slower L. major TS single enzyme turnover rate (7). Complementary ligand-binding studies, reported here, have been conducted with C. hominis and T. gondii TS-DHFR to probe communication between domains.

Morbidity and mortality because of apicomplexan protozoa is of increasing significance. In the United States, C. hominis and T. gondii have become particularly problematic in the era of AIDS, antinecancer chemotherapy, and organ transplantation (11, 12). No effective treatments currently exist for cryptosporidiosis, caused by C. hominis infection and producing chronic diarrhea (often lasting 4–12 months) and the wasting disease of AIDS (13–15). Thus there is an urgent need for new drugs to treat apicomplexan infections, as well as for the identification of new chemotherapeutic targets. Detailed mechanistic and structure-function analysis represent an important step toward the rational design of novel therapies.

MATERIALS AND METHODS

Chemicals and Reagents—All buffers and other reagents employed were of the highest chemical purity. Millipore ultrapure water was used for all solutions. CH₃H₂folate and H₂folate were purchased from Schircks Laboratories (Switzerland); H₂folate was synthesized by reduction of folic acid with sodium borohydride. Tritium-labeled H₂folate and CH₂H₄folate were synthesized as previously described using tritiated folic acid as a starting material (5, 16). The [3H, 5, 7, 9-2H]folic acid, as well as [14C]-labeled dUMP and DUMP (5-fluoroodeoxyuridine-5'-monophosphate, [2-14C]), were obtained from Moravek Biochemicals (Brea, CA). NADPH, dUMP, methotrexate, and trimethoprim were purchased from Sigma; the concentration of NADPH was determined by using a molar extinction coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm.

Protein Expression and Purification—C. hominis TS-DHFR was purified from a single colony of pTrc99A-rHCp (the “genotype 1” TS-DHFR gene derived from a human parasite isolate) in Escherichia coli strain PA414 (dhfr⁻) on an LB ampicillin/kanamycin Petri plate kindly provided by Dr. Richard G. Nelson and Dr. Amy C. Anderson. The T. gondii TS-DHFR coding sequence was inserted into a PET15b expression vector in a generous gift of Dr. David S. Roos. These clones were used to obtain protein of high purity by previously described methods for purification (7, 17). The protein was further purified using an Amersham Biosciences Superdex 75 Highload (26/60) gel filtration column to remove residual H₂folate. The purified C. hominis enzyme contains negligible H₂folate and ~5% of DHFR active sites have NADPH bound.

Determination of Enzyme Concentration and Activity Assays—The TS-DHFR protein concentration was estimated spectrophotometrically at 280 nm using an extinction coefficient of 78,800 M⁻¹ cm⁻¹ for T. gondii and 80,722 M⁻¹ cm⁻¹ for C. hominis. The DHFR activity was determined by following the decrease in absorbance at 340 nm that accompanies the conversion of substrates NADPH and H₂folate to products NADP⁺ and H₂folate as described previously. The TS activity was monitored by following the increase in absorbance at 340 nm that accompanies the conversion of substrates dUMP and CH₂H₄folate to dTMP and H₂folate (18).

Rapid Chemical Quench Experiments—The rapid quench experiments were performed using a Kinetek RQF-3 Rapid Chemical Quench Apparatus (Kinetek Instruments, Austin, TX). The single enzyme turnover reaction was initiated by mixing the 15 μl of enzyme solution (enzyme + 2× reaction buffer: 1 mM EDTA, 50 mM MgCl₂, 50 mM Tris, pH 7.8) with the tritiated substrates (15 μl), approximately 20,000 dpm; in all, concentrations of enzyme and substrates cited in the text are the TS-DHFR single enzyme turnover reaction was monitored by addition of tritiated CH₂H₄folate to enzyme + NADPH and DUMP. The DHFR reaction was monitored by addition of tritiated H₂folate to enzyme + NADPH. The TS reaction was also monitored under burst conditions: 1.5 μM enzyme + 250 μM CH₂H₄folate mixed with 90 μM [14C]dUMP. The enzymatic reactions were terminated by quenching with 67 μl of 0.78 N KOH to give a final concentration of 0.54 M KOH (7). TS reactions utilizing radiolabeled DUMP were quenched with 67 μl of 0.4 N HCl. The rate constants for individual single-turnover rapid chemical or burst quench experiments were estimated by fitting the data to a single exponential or burst curve using the curve fitting program, Kaleidagraph.

High Performance Liquid Chromatography (HPLC) Analysis—Tritiated products of the rapid quench experiments were quantified by HPLC in combination with a radioactivity flow detector as detailed previously (8). The HPLC separation was performed using a BDS-Hypersil C18 reverse phase column (250 × 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 ml/min. An isocratic separation
Using a solvent system of 10% methanol in 180 mM triethylammonium bicarbonate at pH 8.0 was employed. The elution times were as follows: H$_4$folate, 9 min; H$_2$folate, 18 min; CH$_2$H$_4$folate, 20 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, 21.5 min.

**Stopped-flow Absorbance/Fluorescence Measurements**—Stopped-flow measurements were performed using a Kintek SF-2001 apparatus (Kintek Instruments, Austin, TX) as detailed previously (7). In the absorbance experiments designed to measure the burst in CH$_2$H$_4$folate consumption in the TS reaction, absorbance at 340 nm was monitored when 25 μM enzyme was preincubated with 1 mM dUMP and buffer (1 mM EDTA, 50 mM MgCl$_2$, 50 mM Tris, at pH 7.8) and then mixed with 500 μM CH$_2$H$_4$folate. For TS, the protein conformational change upon substrate binding was followed by setting the monochrometer to 287 nm on the input and monitoring the change in intrinsic enzyme fluorescence with an output filter at 340 nm (7.5 μM enzyme was preincubated with 100 μM FdUMP and buffer and then mixed with 50 μM CH$_2$H$_4$folate). For DHFR, coenzyme fluorescence resonance energy transfer experiments were carried out with 290 nm excitation and an output filter at 450 nm. In DHFR burst experiments, 7.5 μM enzyme was preincubated with 50 μM H$_2$folate and buffer and then mixed with 500 μM NADPH. In single enzyme turnover experiments, 50 μM enzyme was preincubated with 500 μM NADPH and buffer and then mixed with 10 μM H$_2$folate. The data were collected over a given time interval using a PC and software provided by Kintek Instruments. An average of 4–7 runs was used for data analysis: rate constants were obtained by fitting the data to a single or double exponential by nonlinear regression analysis.

In the fluorescence experiments designed to measure dissociation rate constants, the trapping ligand (L2) was used at a concentration of ≥5-fold excess over that of L1 to allow for analysis as a pseudo-first order rate constant. Fluorescence changes were monitored following excitation at 287 nm and emission at 340 nm. In experiments involving enzyme and PDDF, however, the monochromator was set at 287 nm on the input and the fluorescence resonance energy transfer was monitored with an interference filter at 380 nm to determine $k_{on}$ and a 340 nm cut-off filter to measure $k_{off}$. The data were collected over a given time interval using a PC and software provided by Kintek Instruments. In most cases an average of four runs were used for data analysis: rate constants were obtained by fitting the data to a single or double exponential by nonlinear regression analysis.

**Kinetic Simulation**—The KinTekSim kinetic simulation program (version 3.0.3) was used to model kinetic data presented here (7, 19). As a starting point, C. hominis rate constants measured in this study were substituted into the minimal kinetic mechanism derived for T. gondii TS-DHFR (5). The resulting model and estimated rate constants are delineated in Chart 1.

**Steady-State Spectroscopic Channeling Assays**—The steady-state TS-DHFR reaction was monitored spectroscopically for a lag in NADP$^+$ formation at DHFR using a procedure detailed previously (8, 9). The predicted change in absorbance is very small, therefore a 10-cm path length quartz observation cell was used to enhance sensitivity. The

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**Chart 1. Minimal kinetic mechanism and rate constants used to simulate C. hominis TS-DHFR.**

| U + E | UE | UE + M | UME | UME + I | UIE |
|-------|----|--------|------|---------|-----|
| $k_1$ | $k_{-1}$ | $k_2$ | $k_3$ | $k_{-3}$ | $k_4$ |

- U = dUMP
- E = TS active site
- M = CH$_2$H$_4$F
- Q = dTMP
- I = Intermediate

**Kinetic Characterization of C. hominis TS-DHFR**

| Rate Constants | Value |
|----------------|-------|
| $k_1$ | 2.3 μM$^{-1}$s$^{-1}$ |
| $k_2$ | 4 μM$^{-1}$s$^{-1}$ |
| $k_3$ | 20 s$^{-1}$ |
| $k_4$ | 0.1 s$^{-1}$ |
| $k_5$ | 200 s$^{-1}$ |
| $k_6$ | 2 μM$^{-1}$s$^{-1}$ |
| $k_7$ | 10.9 μM$^{-1}$s$^{-1}$ |
| $k_8$ | 40 s$^{-1}$ |
| $k_9$ | 5.7 μM$^{-1}$s$^{-1}$ |
| $k_{10}$ | 0.001 s$^{-1}$ |
| $k_{11}$ | 4.5 s$^{-1}$ |
| $k_{12}$ | 10 μM$^{-1}$s$^{-1}$ |
| $k_{13}$ | 95 s$^{-1}$ |
| $k_{14}$ | 2.6 μM$^{-1}$s$^{-1}$ |
Kinetic Characterization of C. hominis TS-DHFR

H.C. hominis /H11002 (45 –A/H9262 CH2H4folate (11 of H2folate, further discussed in subsequent sections. Even at 1 HPLC evidence for a TS intermediate and a large accumulation of CH2H4folate after a 1-min TS-DHFR reaction using the assay mixture (25 ml) containing buffer (50 mM Tris, pH 7.8, 1 mg/ml bovine serum albumin, 1 mM EDTA, 5 mM formaldehyde, 75 mM β-mercaptoethanol), 10.8 μM C. hominis TS-DHFR, and 28 μM CH2H4folate was incubated with 20 μM NADPH until the A260 stabilized, indicating enzymatic consumption of endogenous traces of H2folate. The reaction was then initiated with 100 μM dUMP (final concentration), and the decrease in NADPH absorbance at 340 nm was monitored in the absence of added H2folate. Experiments were performed using a PerkinElmer Life Sciences Lambda 2 UV-visible Spectrophotometer and running PECESS (PerkinElmer Computerized Spectroscopy Software), version 4.0.

RESULTS

Transient Kinetic Analysis to Assess TS, DHFR, and TS-DHFR Activity

The C. hominis TS and DHFR steady-state rates were found to be 2.4 and 2.3 s⁻¹, respectively, in a spectroscopic activity assay (340 nm). Because the specific activity of DHFR is typically significantly higher than that of TS, steady-state kinetics provided the first clue that C. hominis TS-DHFR is unusual. We followed up this initial steady-state experiment with a thorough transient kinetic analysis, including both single enzyme turnover and pre-steady-state burst conditions.

Single enzyme turnover experiments, which measure the rate of chemical conversion of substrate to product at the active site under conditions where enzyme concentration is sufficiently high that substrate binding is not rate-limiting, were performed using a rapid chemical quencher apparatus. To monitor the TS reaction, the bifunctional enzyme (78 μM) was preincubated with a saturating concentration of dUMP (500 μM) and then mixed with a limiting amount of radiolabeled CH2H4folate (11 μM). All reported concentrations are final, after mixing. C. hominis TS was found to be remarkably fast: 45 s⁻¹ (data not shown). The TS single enzyme turnover rate is typically significantly slower than that of DHFR, so the TS rate limits and is equivalent to that of TS-DHFR: it was confirmed that the rate of CH2H4folate consumption in the TS-DHFR reaction is also 45 s⁻¹ (Fig. 1A).

The C. hominis DHFR reaction was monitored by stopped-flow fluorescence and rapid chemical quench under similar conditions. Like with T. gondii but not L. major, DHFR appears to be in an “activated” state even in the absence of TS ligands: the DHFR rate for C. hominis was found to be 130 s⁻¹ (Supplemental Materials Fig. 1A).

To monitor the TS-DHFR reaction, the bifunctional enzyme (45-78 μM) was preincubated with saturating concentrations of dUMP (500 μM) and NADPH (500 μM) and then mixed with a limiting amount of radiolabeled CH2H4folate (11-13.5 μM). As mentioned above, the rate of CH2H4folate consumption was found to be 45 s⁻¹ (Fig. 1A). The TS-DHFR reaction also provided HPLC evidence for a TS intermediate and a large accumulation of H2folate, further discussed in subsequent sections. Even at 1 min, 13% of radioactivity was contained in a peak corresponding to H2folate. In a side-by-side experiment, using the same CH2H4folate substrate, 0-4% of radiolabel corresponded to H2folate after a 1-min TS-DHFR reaction using the T. gondii enzyme. No H2folate was present in the no enzyme control.

Stopped-flow and rapid chemical quench experiments were also performed under pre-steady-state burst conditions (substrate in slight excess over enzyme). Similar to the other bifunctional enzymes studied, a DHFR burst was visualized by stopped-flow fluorescence (Supplemental Materials Fig. 1B). Results of burst experiments aimed at elucidating the C. hominis TS mechanism are reported in the next section.

Further Analysis of C. hominis TS Activity

Determination of the Rate-limiting Step in the TS Reaction—Under pre-steady-state burst conditions, where C. hominis TS-DHFR (45 μM) was preincubated with excess [14C]dUMP (200 μM) prior to mixing with a large excess of CH2H4folate (1 mM), no burst was observed (Fig. 2A). The absence of a burst in dTMP formation is consistent with chemistry being overall rate-limiting at TS.

Presence of a TS Intermediate—C. hominis TS-DHFR is surprising in that the TS activity is very fast and there is a large accumulation of H2folate (Fig. 1B). Furthermore, HPLC analysis of TS and TS-DHFR rapid chemical quench samples yielded clear evidence of a TS intermediate (Fig. 3). The intermediate is present at very early time points; peaks at 22 ms, where it is fully separable from H2folate and represents 18% of radioactive material; and is no longer observable at 40 ms. H2folate is also present at very early time points, but peaks at 40-50 ms, where it represents 65% of tritiated material; leveling out by 200 ms (Fig. 1A). The intermediate peak, seen previously in T. gondii TS and TS-DHFR reactions (5) but not observed with L. major, was hypothesized to represent a breakdown product of the TS iminium ion intermediate, perhaps hydroxymethylene tetrahydrofolate (30).

L. major and T. gondii were also observed to exhibit a pre-steady-state burst for consumption of the cofactor, CH2H4folate (5, 8). The burst in CH2H4folate consumption is thought to correspond to the presence of a TS intermediate with a rate of formation that is faster than chemistry. Stopped-flow absorbance (340 nm; 25 or 40 μM TS-DHFR, 1 mM dUMP, 0.5 or 1 mM CH2H4folate) and fluorescence (excitation 287 nm, emission 340 nm; 7.5 μM TS-DHFR, 100 μM dUMP, 50 μM CH2H4folate) were used to look for a burst in CH2H4folate consumption, but no TS burst was observed with C. hominis TS-DHFR at any of the conditions tested (Fig. 2B). T. gondii TS-DHFR was used as a positive control (Supplemental Materials Fig. 2).

Characterization of the dUMP and TS Folate Binding Sites—C. hominis dUMP binding affinity was determined by stopped-flow analysis: koff was found to be 90 ± 10 s⁻¹ and kon was 2.3 s⁻¹ M⁻¹; Kd = koff/kon ~ 35 μM (Supplemental Materials Fig. 3A). This dUMP Kd is significantly weaker than that observed by the same methods with L. major and T. gondii TS-DHFR, 0.18 and 4.5 μM, respectively.

It has been shown previously that increased affinity for folate substrate can alter the TS mechanism (20), so we sought to determine the relative affinities for folates of the C. hominis and T. gondii TS active sites. As it is not possible to directly measure the rate of CH2H4folate binding by stopped-flow absorbance or fluorescence, we took advantage of a fluorescence resonance energy transfer peak produced upon binding of a folate analog, PDDF, to enzyme. The kobs was obtained by titrating 100 nM C. hominis or T. gondii TS-DHFR with increasing amounts of PDDF and plotting kobs (s⁻¹) versus concentration of PDDF added: kobs = kobs[PTHF] + koff (Supplemental Materials Fig. 3B). Interestingly, in the case of C. hominis but not T. gondii, the data used to calculate koff were biphasic, fitting a double exponential equation with a fast PDDF concentration-dependent phase (kobs) followed by a slower PDDF concentration-independent phase of 5.7 s⁻¹ (Fig. 4). The slow PDDF concentration-independent phase is likely to represent a conformational change following PDDF binding. The koff was measured independently in a competition experiment (Supplemental Materials Fig. 3C): koff was found to be 2.3 s⁻¹ and koff was 48.6 s⁻¹ M⁻¹; Kd = koff/kon = 47.3 nM for folate cofactor binding. For T. gondii TS-DHFR, koff was found to be 1.1 s⁻¹ and koff was 126.1 s⁻¹ M⁻¹, so the Kd for PDDF was 8.5 nM (data not shown).
KinTekSim (Chart 1). The minimal kinetic mechanism that approximates experimental data obtained with *C. hominis* TS-DHFR differs from the *T. gondii* mechanism (5) in that it requires fast conversion of the TS intermediate to H$_2$folate, followed by a slow conformational change (predicted by the PDDF binding studies), with subsequent fast product release at TS. The KinTekSim model of the TS-DHFR single enzyme turnover reaction superimposed on experimental data is presented in Fig. 1C. H$_2$folate and the TS intermediate are plotted separately in the inset.

**Substrate Channeling**

Special attention was paid to the early TS-DHFR time points because a lag in H$_4$folate production and a large build-up of H$_2$folate would be evidence against substrate channeling. There is clear evidence for H$_2$folate channeling by *L. major* TS-DHFR (7, 8, 21). H$_2$folate is formed from the earliest time points with the *L. major* wild-type bifunctional TS-DHFR enzyme (no lag in product formation at DHFR) and only a modest amount of H$_2$folate accumulates, peaking at 14% of tritiated
Material (8). Similar behavior is observed with *T. gondii* TS-DHFR (5). Conversely, when *E. coli* monofunctional TS and DHFR are combined as a non-channeling control, H$_2$folate concentration rises more rapidly than H$_4$folate and there is almost no H$_4$folate present at the earliest time points, suggesting a lag in product formation at DHFR; H$_2$folate peaks at 44–60% of tritiated material (8). In contrast to the other bifunctional enzymes studied, *C. hominis* generates a large accumulation of H$_2$folate: comprising up to 65% of radiolabeled material (Fig. 1A), and there is a clear lag in production of H$_4$folate (Fig. 1B). Although the minimal kinetic mechanism developed for *C. hominis* TS-DHFR predicts a shorter but more complete lag (Fig. 1C), addition of a channeling step to the mechanism results in a significantly worse fit at early time points.

Substrate channeling was also assessed by a steady-state spectroscopic method previously used to visualize the presence or absence of a lag in NADPH$^+$ production via the DHFR-catalyzed oxidation of H$_2$folate formed at TS (9). Trujillo *et al.* (10) report a lag in the production of NADPH$^+$ in a coupled assay using monofunctional TS and DHFR, or a mixture of the *T. gondii* TS domain with the *T. gondii* DHFR domain. In the case of the *T. gondii* bifunctional TS-DHFR enzyme, no lag was observed suggesting that H$_2$folate is directly channeled from TS to DHFR. Spectroscopic signal change resulting from NADPH consumption is monitored at 340 nm. The equation for NADPH$^+$ formation via the DHFR catalyzed oxidation of H$_2$folate produced at TS is:

$$[\text{NADPH}^+] = v_1 t + (v_1/v_2)K_m e^{-v_2/t},$$

where $v_1$ is the rate of TS (μM/min) under coupled assay conditions, and $v_2$ is the DHFR rate (μM/min) using near-saturating substrate concentrations. When the NADPH$^+$ concentration is plotted versus time, the steady-state concentration of H$_2$folate corresponds to $v_1 K_m/v_2$ and the predicted lag time prior to steady-state H$_2$folate accumulation is $K_m/v_2$. In the case of *C. hominis*, conditions corresponding to a predicted lag of 30 s in the absence of substrate channeling (10.8 nM enzyme), produced such a lag (Fig. 5).

**Domain-Domain Communication: Ligand Binding Effects**

It was observed with *L. major* TS-DHFR that ligand binding at one active site affects activity at the active site of the other enzyme, evidence of communication between the TS and DHFR domains (7). The effects of ligand binding in apicomplexa were evaluated with *C. hominis* TS-DHFR and with the *T. gondii* enzyme for the sake of comparison.

Effects of TS ligands on DHFR burst and single enzyme turnover reactions were assessed by stopped-flow fluorescence (excitation 290 nm, emission 450 nm). In the case of *L. major,*
formation of the dUMP-CH$_2$H$_4$folate-enzyme ternary complex at TS accelerates the DHFR rate 20-fold (7). With C. hominis and T. gondii, the DHFR burst rate was fast even in the absence of TS ligands (7.5 μM TS-DHFR, 50 μM H$_2$folate), and no acceleration or amplitude changes were observed in the presence of 100 μM FdUMP or 100 μM FdUMP + 50 μM CH$_2$H$_4$folate. Instead there was slight decrease in the DHFR rate, likely because of direct inhibition of the DHFR active site by CH$_2$H$_4$folate. It was confirmed spectroscopically that addition of FdUMP + CH$_2$H$_4$folate annulled TS activity, establishing formation of a non-productive ternary complex (unable to undergo catalysis). 74% of C. hominis and 79% of T. gondii DHFR activity was observed in the presence of the ternary complex; 70% of T. gondii DHFR activity was observed in the presence of 50 μM CH$_2$H$_4$folate alone.

The TS dimer is a half-site reactivity enzyme (at any given time, only one-half of the TS dimer is catalytically active) and conformational changes upon ligand binding lead to asymmetric binding sites with differential substrate affinities at each half of the dimer (22–24). We sought to determine whether coordination of the two TS active sites to provide half-site reactivity impacts DHFR activity: DHFR single enzyme turnover rates were determined under conditions where one or both TS active sites are predicted to be ligand-bound. Experiments were carried out with 50 μM TS-DHFR, 10 μM H$_2$folate, and two sets of TS-specific ligands: FdUMP (500 μM) + CH$_2$H$_4$folate (50 μM) to provide covalent ligation of the TS active site and dUMP (25 or 500 μM) + PDDF (25 μM) to provide non-covalent ligation of the TS folate-binding site (PDDF lacks an N$_5$-methyl moiety, prohibiting covalent bonding to dUMP). C. hominis and T. gondii DHFR rates were unchanged by half- or full site TS occupancy with covalent or non-covalent ligands.

Effects of DHFR ligands on TS burst and single enzyme turnover reactions were assessed by stopped-flow fluorescence (excitation 287 nm, emission 340 nm) and rapid chemical quench, respectively, under conditions where ligand-binding effects were observed with L. major. With L. major, an additional conformational change at TS occurs in the presence of...
the DHFR ligand, NADPH; and addition of the DHFR folate inhibitor, methotrexate, results in a 4-fold slower TS single turnover rate (7). Combination of T. gondii TS-DHFR (7.5 μM) and FDUMP (100 μM) resulted in a single exponential TS burst of 2.5 s⁻¹ in the presence or absence of NADPH (500 μM) ± methotrexate (8 μM). A single enzyme turnover experiment (41 μM T. gondii TS-DHFR, 500 μM dUMP; 14 μM CH₂H₄folate) revealed 80% of T. gondii TS single enzyme turnover activity in the presence of methotrexate (45 μM). The corresponding C. hominis experiments were performed using trimethoprim, a more specific DHFR inhibitor under burst conditions; the TS profile was similar in the presence or absence of NADPH ± trimethoprim (in this case, no burst). C. hominis exhibited 100% of TS single turnover activity in the presence of trimethoprim (45 μM TS-DHFR; 50 μM trimethoprim).

**DISCUSSION**

Our kinetic characterization of the recently crystallized C. hominis TS-DHFR enzyme yielded surprising results in all three areas of focus: determination of the enzyme mechanism, substrate channeling, and domain-domain communication. Interestingly, C. hominis TS-DHFR exhibited significant mechanistic differences from enzymes of both the kinetoplastid protozoa, L. major, and the apicomplexan protozoa, T. gondii. In fact, all of the mechanistic features found to be shared across the two bifunctional TS-DHFR classes are also shared by monofunctional TS and DHFR, suggesting that these are fundamental characteristics that preceded the gene-fusion event. In all cases, DHFR catalytic activity is faster than TS, and chemistry is overall rate-limiting at TS (no burst in dTMP formation), whereas a step after chemistry, such as product formation, is rate-limiting at DHFR (a burst in NADPH consumption is observed).

Remarkably, the most unusual feature of the C. hominis mechanism involves the TS domain, which is more highly conserved than DHFR. At 45 s⁻¹, C. hominis TS is 10–40-fold faster than other TS enzymes characterized. In delineating the C. hominis TS-DHFR kinetic reaction mechanism, it was necessary to include the slow conformational change observed following binding of the folate analog, PDDF. Additionally, while there is HPLC evidence for a C. hominis TS intermediate, no pre-steady-state burst in consumption of the cofactor, CH₂H₄folate, is observed. The HPLC traces indicate that, like with T. gondii, a TS intermediate forms early in the time course and is consumed. With T. gondii and L. major, however, a pre-steady-state burst in consumption of the cofactor, CH₂H₄folate, is observed. The burst is also thought to indicate a TS intermediate, possibly the iminium form of CH₂H₄folate, for which the rate of formation is faster than chemistry. Modeling indicates that the fast C. hominis TS activity would make a pre-steady-state burst in consumption of CH₂H₄folate very difficult to detect.

In C. hominis TS-DHFR, the essential TS catalytic residues from the well characterized L. casei enzyme are conserved (25); however, differences in two additional conserved residues near the folate-binding domain could help explain the unusual TS activity of C. hominis. In place of a glycine found in human, Pneumocystis carinii, L. major, T. gondii, and P. falciparum TS, C. hominis has a serine (Ser-290). This serine provides an additional hydrogen bond to the glutamate tail of the folate substrate. In place of a phenylalanine found in all of the species listed above, C. hominis has an alanine (Ala-287) (4). Unlike alanine, bulky phenylalanine impedes the charged glutamate tail. It was previously observed with folypolyglutamates, which also increase folate substrate affinity for TS via the glutamate tail, that the TS mechanism is altered (20). C. hominis TS does have a higher affinity for the folate analog PDDF than does L. major TS (with a faster on rate and similar off rate) (8), but T. gondii TS has an even higher affinity for PDDF, so affinity alone fails to explain the unusual TS behavior of C. hominis. On the other hand, only with the C. hominis enzyme does addition of PDDF result in biphasic behavior, with a fast PDDF concentration-dependent phase followed by a slower PDDF concentration-independent phase thought to represent a conformational change following binding.

Perhaps the most important finding of this research is that C. hominis TS-DHFR is the first bifunctional TS-DHFR enzyme studied for which there is clear mechanistic evidence against H₂folate substrate channeling. With L. major TS-DHFR there are multiple lines of structural and mechanistic evidence to support the possibility of channeling of H₂folate from the TS active site, where it is produced, to the DHFR active site, where it is reduced to H₄folate (1, 7, 8). With the newly crystallized apicomplexan proteins, however, there is no obvious structural explanation for substrate channeling, as solvent-exposed basic residues do not follow a clear path. Furthermore, the TS and DHFR active sites are found on opposite faces of each apicomplexa TS-DHFR monomer, meaning that H₂folate would have to turn a corner to transit from TS to DHFR or, as suggested by Yuvaniyama et al. (2) follow a longer but straighter path from the TS active site of one monomer to the DHFR active site of the opposite half of the dimer. The orientation of the TS and DHFR active sites in the apicomplexa proteins also make channeling via conformational change less likely (6).

Consistent with the structural predictions, C. hominis exhibits an even more significant accumulation of H₂folate and lag in H₂folate formation than was observed with the combination of E. coli monofunctional TS + DHFR, used previously as a non-channeling control (8). C. hominis TS-DHFR also exhibits a lag in NADPH formation at DHFR following H₂folate formation at TS in a steady-state channeling assay. It is not clear why the accumulated H₂folate is not fully consumed over the course of 1 min, although one possibility may be that it is bound non-productively.

Just as the recent crystal structures provide evidence for two structural families (short and long linker), our data suggest two corresponding mechanistic families of TS-DHFR enzymes: those that do or do not exhibit domain-domain communication via ligand binding at TS affecting DHFR activity and vice versa. TS-DHFR from the apicomplexa parasites C. hominis and T. gondii are similar in that they exhibit maximal DHFR activity even when TS is in an unliganded state (130 and 180 s⁻¹, respectively), with no further acceleration upon complex formation at TS. This differs from L. major in which the DHFR rate is relatively slow at 6.4 s⁻¹, but is accelerated to 120 s⁻¹ following ternary complex formation at TS (7). Indeed, whereas there are multiple lines of evidence for domain-domain communication in L. major TS-DHFR, no such ligand binding effects were found with the apicomplexa proteins studied.

The structural variations may account for these mechanistic distinctions between parasite classes. The L. major TS-DHFR structure is different from those of the apicomplexan proteins in that there is virtually no junctional region between TS and DHFR, but there is a 22-amino acid N-terminal DHFR tail that wraps the TS domain. Attempts to fully or partially remove the N-terminal tail did not yield active protein and similarly, attempts to delete the L. major DHFR domain did not yield active TS, suggesting that the N-terminal tail is crucial for protein folding and/or stability.³ Whereas the short linker between L. major TS and DHFR limits the range of DHFR orientations...
relative to TS (3), we hypothesize that this N-terminal tail may serve as a sensor or brake, facilitating communication between domains and modulating activity.

By contrast, the N-terminal tail is essentially absent in the apicomplexan proteins. *P. falciparum* does have a short N-terminal extension, but it points upward away from TS. A recent study confirms that the 6-amino acid *P. falciparum* N-terminal extension is crucial to DHFR but not to TS activity (26). What the apicomplexan proteins do possess is extensive linker regions, ranging in length from 58 to 89 amino acids. In a corset-like fashion, crossover or donated helices make extensive contacts with the DHFR domain of the opposite TS-DHFR monomer. Like the *L. major* N-terminal tail, there is evidence that both the *P. falciparum* junctional region and DHFR domain are required for TS activity (2, 27). The *C. hominis* crossover helix makes extensive van der Waals and hydrogen bonding interactions with the DHFR domain from the other half of the dimeric enzyme. It is possible that the crossover helices serve to hold the dimeric, bifunctional apicomplexa TS-DHFR proteins together in a stable, active conformation.

Without evidence for domain-domain communication from ligand binding experiments and, at least in the case of *C. hominis* TS-DHFR, evidence against substrate channeling, why might the apicomplexa proteins exist as bifunctional enzymes? One remaining potential advantage of bifunctionality is for coupled translational autoregulation. In translational regulation, mRNA binding to protein results in translational repression (28). Indeed, Zhang et al. (31) report the existence of translational autoregulation in *P. falciparum* TS-DHFR: mRNA coding for TS and the junctional region binds to the DHFR domain of the protein, blocking translation. The possibility of coupled translational autoregulation by *C. hominis* TS-DHFR will be a topic of future investigation.

The recent availability of crystal structures from *P. falciparum* and *C. hominis* TS-DHFR combined with this detailed mechanistic analysis of the *C. hominis* enzyme greatly enhances our understanding of bifunctional TS-DHFR. Points learned from this research will serve to guide further study. Whereas it is typically more difficult to achieve species specificity with inhibitors directed at the TS versus the DHFR active site, unique mechanistic features of *C. hominis* TS suggest the possibility for a specific inhibitor directed at this active site. Previous efforts to find a non-active site inhibitor specific for a bifunctional enzyme were targeted at the putative channeling region of *L. major* TS-DHFR (29). Mechanistic evidence against substrate channeling by *C. hominis* TS-DHFR, as well as structural differences, indicate that novel non-active site targets should be explored in this enzyme. Growing rates of infection combined with emerging resistance, ineffective therapies, and unacceptable side effects prove that there is a real and urgent need for new therapies and novel therapeutic targets for the treatment of disease caused by protozoal parasites. We anticipate that detailed structure-function studies, such as described here, will be key to the rational development of novel drug targets and the design of new therapeutics specific for apicomplexan bifunctional TS-DHFR.

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