Published in final edited form as:

Biochem J. 2009 February 1; 417(3): 757–764. doi:10.1042/BJ20081247.

Disruption of the Crossover Helix Impairs Dihydrofolate Reductase Activity in the Bifunctional Enzyme TS-DHFR from *Cryptosporidium hominis*

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Abstract

In contrast to most species, including humans, having monofunctional forms of the folate biosynthetic enzymes thymidylate synthase (TS) and dihydrofolate reductase (DHFR), several pathogenic protozoal parasites, including *Cryptosporidium hominis*, contain a bifunctional form of the enzymes on a single polypeptide chain having both catalytic activities. The crystal structure of the bifunctional enzyme thymidylate synthase-dihydrofolate reductase (TS-DHFR) *Cryptosporidium hominis* reveals a dimer with a “crossover helix”, a swap domain between DHFR domains, unique in that this helical region from one monomer makes extensive contacts with the DHFR active site of the other monomer. In this study, we used site-directed mutagenesis to probe the role of this crossover helix in DHFR catalysis. Mutations were made to the crossover helix: an “alanine face” enzyme in which the residues on the face of the helix close to the DHFR active site of the other subunit are mutated to alanine, a “glycine face” enzyme in which the same residues are mutated to glycine, and an “all alanine” helix in which all residues of the helix were mutated to alanine. These mutant enzymes were studied using a rapid transient kinetic approach. The mutations cause a dramatic decrease in the DHFR activity. The DHFR catalytic activity of the alanine face mutant enzyme is 30 s⁻¹, the glycine face mutant enzyme is 17 s⁻¹, and the all alanine helix enzyme is 16 s⁻¹, all substantially impaired from the wild-type DHFR activity of 152 s⁻¹. It is clear that loss of helix interactions results in a marked decrease in DHFR activity, supporting a role for this swap domain in DHFR catalysis. The crossover helix provides a unique structural feature of *C. hominis* bifunctional TS-DHFR that could be exploited as a target for species-specific non-active site inhibitors.

Keywords

bifunctional; transient kinetics; dihydrofolate reductase; *Cryptosporidium hominis*; protozoal parasite; thymidylate synthase

INTRODUCTION

Protozoal parasites, such as *Cryptosporidium hominis*, *Leishmania major*, *Toxoplasma gondii* and *Plasmodium falciparum*, are unusual in that the thymidylate synthase (TS)² and dihydrofolate reductase (DHFR) enzymes exist on a single polypeptide chain to form the bifunctional enzyme TS-DHFR³ [1]. These are essential enzymes and have been established as drug targets. Thymidylate synthase catalyzes the conversion of 2'-deoxyuridine monophosphate (dUMP) and methylene tetrahydrofolate (CH₂H₄folate) to 2'-deoxythymidine monophosphate (dTMP) and dihydrofolate (H₂folate). DHFR then catalyzes the reduction of H₂folate by NADPH to form tetrahydrofolate (H₄folate), which is used for one carbon transfer reactions in many biochemical processes.

After the determination of the crystal structure of *C. hominis* TS-DHFR, it was suggested that there are two families of bifunctional TS-DHFR: a short linker family with an N-terminal tail, as in the kinetoplastids, which includes *L. major* and the trypanosomes; and a long linker family which contains a donated or crossover helix, as in the apicomplexan family, containing *C. hominis*, *P. falciparum*, and *T. gondii* [2]. The short linker family has a linker length of 2 residues (*L. major*) and an N-terminal tail of 22 residues, which stretches from the DHFR domain and wraps around the TS domain. However, in the apicomplexan TS-DHFR enzymes, there is no N-terminal tail in *C. hominis* and *T. gondii* and only a 5 amino acid tail in *P. falciparum*, and the linker region between the TS and DHFR domains is long (89 residues in *P. falciparum*, 72 residues in *T. gondii*, and 58 residues in *C. hominis*). This linker region begins in the DHFR domain of one monomer (A), crosses to the other monomer (B), forms the crossover helix that makes extensive contacts with the opposite DHFR domain, then crosses back to monomer A to form the TS domain (Figure 1).

Apart from structural differences, these enzymes also display unique kinetic behaviors in terms of how the DHFR catalytic activity may be modulated. Moreover, each protozoal species exhibits distinct modes of modulation. The catalytic activity of DHFR from *L. major* and *P. falciparum* is enhanced upon TS ligand binding, whereas *C. hominis* DHFR activity is unaffected by the presence of TS ligands at the TS active site (Table 1) [3-5]. Despite sharing a linker and crossover helix, *P. falciparum* and *C. hominis* clearly differ in terms of DHFR kinetics. A closer look at the *P. falciparum* structure shows that while the enzyme does form a crossover helix in the same general orientation as *C. hominis*, it does not contact the DHFR active site of the other monomer. However, the crossover helix in *C. hominis* DHFR makes extensive contacts with the catalytically important Helix B of the DHFR active site. This unique structural characteristic led us to hypothesize that although there is no domain-domain modulation of catalytic activity between the TS and DHFR domains of the same subunit, the crossover helix swap domain may be responsible for modulating catalysis for the *C. hominis* DHFR. The residues of this "crossover helix" were mutated in order to determine if these structural differences might account for some of the mechanistic differences between enzymes from different species.

Cryptosporidiosis, caused by *C. hominis* infection, is one of the major causative agents of the diarrheal diseases in AIDS patients [6-8]. There have been several outbreaks of *C. hominis* infections from contaminated water supplies in the past few years that have sickened thousands including a very recent episode in a New York water amusement park [9, 10]. There is currently no effective treatment for this disease, thus there is an urgent need for new drugs. Further understanding of the mechanistic and structural characteristics of the enzyme may reveal key features of catalytic function that could be exploited in the design of potential species-specific inhibitors.

MATERIALS AND METHODS

Chemicals and Reagents

All buffers and reagents were of the highest purity. NADPH and dUMP were purchased from Sigma. Concentration of NADPH was determined by using an extinction coefficient of $6220 \text{ M}^{-1}\text{cm}^{-1}$ at 340 nm. Tritium-labeled H_2 folate and CH_2H_4 folate were synthesized as previously described using tritiated folic acid as starting material [11, 12]. The $[3',5',7,9\text{-}^3\text{H}]$ -folic acid was purchased from Moravsek Biochemicals (Brea, CA).

Plasmids and Site-directed Mutagenesis

Full length *C. hominis* TS-DHFR was encoded in the pTrc99A-rHCp (the “genotype 1” TS-DHFR gene derived from a human parasite clone), kindly provided by Dr. Richard G. Nelson and Dr. Amy C. Anderson. Site directed mutagenesis was performed using the Stratagene QuikChange kit. The mutations for the alanine face mutant (K194A, D198A, L202A, D205A, I206A, and R210A) were all introduced using a single oligonucleotide that encoded the changes. The same residues were mutated to glycine in the glycine face mutant. In order to form the all alanine helix mutation, a second round of PCR was used to introduce the remaining alanine mutations at positions 195, 196, 197, 199, 200, 201, 203, 204, 207, and 208. DNA sequencing confirmed the presence of all the mutations. CD spectra of wild-type and all three mutant enzymes were globally the same suggesting overall folding of the proteins is maintained; however, because the expected change in percent helical content for the mutant enzymes were all within error of wild-type, we could not determine by CD whether the crossover helix is maintained as a helix. The poly-alanine sequence has a high propensity to form alpha helix [13], and therefore the alanine face and all-alanine mutant enzymes will presumably retain the alpha-helical structure. In addition, based on nnPREDICT, secondary structure prediction software, the alanine sequences are predicted to be helical, however this software would predict the glycine face enzyme would not maintain the helical structure [14]. Therefore, the glycine face mutant was made to determine whether simply the presence of a helix, and not specific interactions, is necessary for maximal catalytic activity.

Protein Expression and Purification

The proteins were overexpressed in BL21 *E. coli* and purified using previously described methods [3, 15]. The protein was further purified using a PD-10 column from Amersham Biosciences to remove residual H_2 folate. The concentration of purified *C. hominis* TS-DHFR was determined spectrophotometrically using an extinction coefficient of $80,722 \text{ M}^{-1}\text{cm}^{-1}$. The DHFR activity was determined by following the decrease in absorbance at 340 nm ($\Delta\epsilon = -12.8 \text{ mM}^{-1}\text{cm}^{-1}$) which corresponds to the conversion of NADPH and H_2 folate to NADP^+ and H_4 folate. The TS activity was determined by following the increase in absorbance at 340 nm ($\Delta\epsilon = 6.4 \text{ mM}^{-1}\text{cm}^{-1}$) as the substrates CH_2H_4 folate and dUMP are converted to H_2 folate and dTMP [16]. Mutant enzymes were purified similar to wild-type.

Rapid Chemical Quench Experiments

Rapid chemical quench experiments were performed using a Kintek RFQ-3 Rapid Chemical Quench Apparatus (Kintek Instruments, Austin, TX). Single turnover experiments were initiated by mixing 15 μL of enzyme solution (enzyme + 2x buffer: 1 mM EDTA, 50 mM MgCl_2 , 50 mM TRIS, pH 7.8) with 15 μL of the tritiated substrate (approximately 20,000 dpm). The DHFR single turnover reaction was monitored by addition of $[^3\text{H}]\text{-H}_2$ folate (10 μM) to enzyme (50 μM) and NADPH (500 μM); all concentrations in the text are after mixing. The TS-DHFR reaction was monitored by addition of $[^3\text{H}]\text{-CH}_2\text{H}_4$ folate (10 μM) to enzyme (50 μM), dUMP (500 μM), and NADPH (500 μM). The reactions were terminated by quenching with 67 μL of 0.78 N KOH to give a final concentration of 0.54 N KOH [5]. The quenching

solution also contained 10 % sodium-ascorbate and 200 mM 2-mercaptoethanol to prevent the degradation of the products. To confirm complete quenching of the enzymatic reactions, controls in which substrate was added to a premixed solution of enzyme and quench were performed for each experiment, showing stability of the CH₂H₄folate. In addition, a control in which enzyme is allowed to react with substrates for one minute was performed to show complete conversion to products and ensure the stability of the formed H₄folate. The rate constants were determined by fitting the data to either a single or double exponential equation using Kaleidagraph.

High Performance Liquid Chromatography (HPLC) Analysis

The tritiated products from the rapid chemical quench experiments were analyzed using reversed-phase HPLC connected to a radioactivity flow detector as described previously [17]. The isocratic separation was performed using a BDS-Hypersil C18 reverse phase column (250 × 6.4 mM) (Alltech) with a flow rate of 1 ml/min using 10 % methanol in 180 mM triethylammonium bicarbonate, pH 8.0. The elution times for the products were as follows: H₄folate, 9 min; H₂folate, 17 min; and CH₂H₄folate, 20 min.

Stopped-Flow Fluorescence Experiments

Stopped-flow experiments were performed using a Kintek SF-2001 apparatus (Kintek Instruments, Austin, TX). To determine the rate for the DHFR reaction, coenzyme fluorescence resonance energy transfer was measured at an excitation of 290 nm with an output filter at 450 nm. The signal measured at 450 nm would decrease as bound NADPH involved in the FRET is converted to NADP⁺ and released from the enzyme. In single turnover experiments, enzyme (50 μM) was incubated with 500 μM NADPH and then mixed with 10 μM H₂folate. The data was collected over a given time interval using software provided by Kintek. For burst experiments, 7.5 μM enzyme was incubated with 50 μM H₂folate and then mixed with 500 μM NADPH. In order to determine if ligands bound at the TS site would result in an activated DHFR rate, as is seen with other TS-DHFR enzymes from other species, DHFR burst experiments were also examined in the presence of TS ligands. For these experiments, enzyme was incubated in the presence of 100 μM FdUMP (a TS inhibitor), 200 μM CH₂H₄folate, and 1mM NADPH, and the reaction initiated by rapidly mixing with 200 μM H₂folate. The data was collected over a given time range. 4-7 runs were collected and averaged. The data were fit to either a single exponential or burst equation to obtain the rate constants.

RESULTS

Expression and Steady-State Activity of Helix Mutants

All of the helix mutants were purified using the previously published protocol and all enzymes were ~95% pure as measured by SDS-PAGE gel electrophoresis. The DHFR steady state rates are $2.7 \pm 0.1 \text{ s}^{-1}$, $1.7 \pm 0.1 \text{ s}^{-1}$, $1.9 \pm 0.4 \text{ s}^{-1}$, and $1.1 \pm 0.4 \text{ s}^{-1}$, for wild-type, alanine face, glycine face, and all alanine mutant enzymes, respectively. The TS steady state rates are $3.5 \pm 0.4 \text{ s}^{-1}$, $3.0 \pm 0.2 \text{ s}^{-1}$, $1.6 \pm 0.4 \text{ s}^{-1}$, and $1.7 \pm 0.1 \text{ s}^{-1}$ for wild-type, alanine face, glycine face, and all alanine mutant enzymes respectively. All rates were determined using a spectrophotometric assay.

Single Turnover of the DHFR Reaction

Single turnover experiments were conducted to directly assess the effects of the mutations on the rate of catalysis at the DHFR site. Experiments were performed using stopped-flow fluorescence (Figure 2). Bifunctional TS-DHFR from wild type or mutant enzymes was preincubated with saturating amounts of NADPH and then mixed with limiting amounts of H₂folate. The alanine face mutant catalytic rate is $30 \pm 1 \text{ s}^{-1}$, the glycine face catalytic rate is

$17 \pm 1 \text{ s}^{-1}$ and the all alanine catalytic rate is $16 \pm 1 \text{ s}^{-1}$, compared to a catalytic rate of $152 \pm 7 \text{ s}^{-1}$ for the wild-type enzyme (Table 2). Similar experiments were performed using rapid chemical quench. Bifunctional TS-DHFR was preincubated with a saturating amount of unlabeled NADPH and then rapidly mixed with a limiting amount of radiolabeled H_2 folate. Similar rates were obtained as compared to the stopped-flow results. Both methods confirm a reduced catalytic rate for all mutant enzymes. Doubling the enzyme concentration did not change the catalytic rate, demonstrating that binding was not rate limiting in these assays.

Pre-steady state burst experiments of the DHFR reaction

The DHFR reaction was also studied under pre-steady state burst conditions (substrate in slight excess over enzyme), using stopped-flow fluorescence. Bifunctional TS-DHFR was incubated with H_2 folate and then mixed with saturating NADPH. A burst is observed for all enzymes (Figure 3), suggesting that chemistry is not the rate limiting step, but rather a later step, perhaps product release, limits the catalytic cycle. DHFR burst reactions were also performed in the presence of TS ligands in order to determine if the DHFR rate would be activated in the presence of TS ligands. The DHFR rate did not change in the presence of ligands for any of the mutant enzymes, indicating there is no enhancement in the DHFR catalytic rate even if the TS active site has ligands bound.

Single Turnover of the TS-DHFR reaction

The full bifunctional TS-DHFR reaction was examined using rapid chemical quench. In this experiment, the formation of H_2 folate from CH_2H_4 folate can be measured at the TS site and subsequent conversion of the H_2 folate to H_4 folate can be measured at the DHFR site. The bifunctional TS-DHFR was pre-incubated with saturating amounts of unlabeled dUMP and NADPH, and then rapidly mixed with a limiting amount of radiolabeled CH_2H_4 folate. The time course for the disappearance of CH_2H_4 folate, the appearance and disappearance of H_2 folate, and the formation of H_4 folate is shown in Figure 4. The buildup of H_2 folate in all enzymes is consistent with previously determined characteristics of *C. hominis* TS-DHFR. The accumulation of H_2 folate is seen to a greater extent in the all alanine helix than in wild-type or face enzymes ($6.2 \mu\text{M}$ compared to $4.1 \mu\text{M}$ for wild-type enzyme) (Table 3). The H_2 folate also persists for longer times in all of the mutant enzymes than in the wild-type enzyme (Table 3). Interestingly, the H_2 folate buildup lasts for much longer times ($\sim 0.7 \text{ s}$) in the glycine face enzyme than either of the alanine mutants. The end result, the rate of H_4 folate formation, is decreased in all of the mutants: wild-type, $10.1 \pm 0.9 \text{ s}^{-1}$; alanine face, $6.7 \pm 0.8 \text{ s}^{-1}$; all alanine, $4.0 \pm 0.4 \text{ s}^{-1}$, and glycine face, $1.0 \pm 0.3 \text{ s}^{-1}$.

The rates of catalysis for the TS reaction were derived from the full TS-DHFR reaction time courses. Rates were determined from $\text{CH}_2\text{H}_4\text{F}$ consumption. The rates for the alanine face and the all alanine helix mutant enzymes were not significantly different than that of wild-type (all within error of 15 s^{-1}). However, in the case of the glycine face enzyme, the rate for the disappearance of CH_2H_4 folate is much slower than the other enzymes with a rate of $2.7 \pm 0.5 \text{ s}^{-1}$.

DISCUSSION

Based on currently known structures and sequence alignments, the crossover helix appears to be present only in TS-DHFR of the apicomplexan family - including *C. hominis*, *P. falciparum*, and *T. gondii* [2]. The linker and crossover helix in *C. hominis* brings the two DHFR domains in much closer proximity than bifunctional TS-DHFR enzymes with very short linker regions, such as *L. major*. Based on the structural differences between the families, we tested the role of the crossover helix using a site-directed mutagenesis approach. Our kinetic

characterization of *C. hominis* helix mutations has provided new insight into the role of the crossover helix.

The alanine face, glycine face, and all alanine helix enzymes have lower DHFR catalytic rates than wild-type enzyme suggesting that the interactions between the crossover helix and the residues of the opposite DHFR domain are important for maximal DHFR catalytic activity. The crossover helix packs against Helix B in the opposite DHFR domain. Residues on Helix B opposite from the crossover helix form a portion of the DHFR active site and contain many highly conserved residues including Phe36, which is universally conserved and a key residue in the catalytic mechanism of DHFR, specifically the conformational change and hydride transfer [18, 19]. Helix B and the crossover helix form at least seven tight interactions, some of which are as follows: Asp198 makes a salt bridge to Asn42, Leu202 is involved in hydrophobic interactions with Ile39, Asp205 makes a salt bridge with Lys38, and I206 makes hydrophobic interactions with the aliphatic portion of the Lys34 side chain and also the side chain of Phe35. Therefore, mutating residues on the crossover helix may cause slight shifts in the helix of the active site. As shown in *E. coli*, the DHFR domain proceeds through a series of conformational changes along the reaction pathway, some of which are distal to the active site, and it is therefore possible that small perturbations in these conformational changes, caused by mutations altering Helix B-crossover helix packing, could affect catalysis [18, 20]. The additional loss in activity for the all alanine and glycine face mutant enzymes can be explained by the loss of additional interactions. Although they do not involve Helix B, several of these interactions are between the two DHFR domains. Residues on an orthogonal face of the crossover helix interact with a β -sheet of the DHFR domain. There are also interactions between the crossover helix and residues located in the flexible tethers of the crossover domain. It is likely that these interactions are necessary for a maximal DHFR catalytic activity, possibly by positioning the crossover helix in an optimal orientation. The returning tether makes several interactions and hydrogen bonds with the TS domain. The loss of these interactions could explain the reduction in TS steady state activity for the all alanine mutant enzyme. Cumulatively, these lost interactions result in an additional 2-fold loss in activity compared to the alanine face mutant enzyme. In fact, initial experiments mutating residues L203 and F207 on the orthogonal face of the crossover helix cause a loss of activity to 30 s⁻¹. The argument could be made that a stretch of alanine mutations introduced anywhere in the protein could cause this reduction in rate, however when the analogous mutations are made in the *P. falciparum* enzyme, there is *no* reduction in activity [4]. The crossover helix appears to be necessary to retain the productive conformation of the active site helix and to allow for proper coordinated movement, and thus maximal activity. In the alanine face and all alanine mutant enzymes, the crossover helix would presumably still be present; however, in the case of the glycine face mutant, we would predict that the crossover helix is no longer maintained as a helix. The glycine face enzyme results in a similar DHFR rate to the all alanine mutant enzyme, but surprisingly, substantially alters the TS rate. Because the linker, upon returning to its own domain, makes many contacts to the TS domain, this entire region could be disrupted by the lack of a structurally stable helix. Although we do not observe that ligands binding to TS enhance DHFR activity, there may be a reciprocal modulation of TS activity by DHFR mediated through proper positioning of the crossover helix and linker region. Based on the mutant enzymes made in this study, it appears that the specific interactions of the crossover helix are necessary for a fully active DHFR domain, while simply the presence of a stable helix is important for full TS activity.

Interestingly, *L. major*, which has a very short linker (~2 amino acids, and therefore no crossover helix), has a very low DHFR activity of 14 s⁻¹. However, when ligands are bound at the TS site, the DHFR activity is enhanced almost 10-fold to a rate of 120 s⁻¹. This enhanced rate is comparable to the activity of *C. hominis* DHFR. Interestingly, the *C. hominis* all alanine mutant enzyme has an activity (16 s⁻¹) equivalent to that of *L. major* in the unliganded, unenhanced state (14 s⁻¹). The bifunctional TS-DHFR enzyme from *P. falciparum* is an

interesting combination of *C. hominis* and *L. major* both structurally and catalytically. Structurally, *P. falciparum* has a long linker containing a crossover helix between the TS and DHFR domains (similar to *C. hominis*), but also has an N-terminal tail similar to *L. major*. Unlike *C. hominis*, *P. falciparum* DHFR activity increases 2-fold when TS ligands are bound, to reach an enhanced activity of 130 s^{-1} , similar to the inherent rate of *C. hominis* DHFR. *P. falciparum* does have a crossover helix, however upon mutation of the helix face residues to alanine, there is no reduction in DHFR activity in contrast to that observed for *C. hominis* [4], as expected since the helix does not contact the DHFR active site, but rather has electrostatic residues which make contacts with several lysine residues scattered throughout the DHFR domain. It appears the crossover helix plays a different role in *P. falciparum* than in *C. hominis* offering further evidence that these bifunctional enzymes have developed different modes of modulating or enhancing activity. TS-DHFR from *T. gondii* also shows an enhanced DHFR activity whether or not ligands are bound at TS, similar to *C. hominis* TS-DHFR. This parasite is also a member of the apicomplexan family; and, therefore has a long linker and crossover helices, based on secondary structure prediction. Based on the information in this study, we would predict that the crossover helix in *T. gondii* is much like *C. hominis*, and makes extensive contacts with the DHFR active site of the opposite monomer. Crystallography and mutational studies are currently underway to validate this hypothesis.

It appears that for all species studied, there is a maximal DHFR catalytic rate. An optimized DHFR catalytic rate may be essential in order to help the parasites compete for host folates. It is important to note that folate dependent enzymes are present in much greater concentrations than folates in the cell (making the concentration of free folates in the cell negligible) and therefore these single enzyme turnover rates are also likely relevant when studying these enzymes [21, 22]. Rather than TS to DHFR modulation of catalytic activity as seen in *L. major* or *P. falciparum*, our data suggests an optimal rate is affected by a swap domain between subunits. In addition to influencing the DHFR activity of the monomer to which it crosses, another potential role for the crossover helix is to provide a structural motif which is used for interdomain communication, first proposed by O'Neil, et al [2]. The crossover helix is connected to its own domain through two linkers. The subdomains of the DHFR active site (including Helix B) move during the catalytic cycle, resulting in as great as a one angstrom shift in position at the active site [23]. Because Helix B and the crossover helix are so closely associated, movements of Helix B during the catalytic cycle may be transferred to the crossover helix and this "information" can then be relayed to its own active site through the flexible linker. The information may be used to activate or coordinate activity with the other DHFR active site. This would be a unique type of domain-domain communication among the characterized bifunctional TS-DHFR enzymes.

The mutational analysis and mechanistic information we have gained in this study opens an exciting new avenue of investigation that may be exploited for inhibitor design. Ligands targeted to bind near or adjacent to the crossover helix may also interfere with domain-domain communication and thereby inhibit enzyme activity. A unique pocket is formed by the crossover helix and the tethers that can be targeted for non-active site inhibitors using a molecular docking strategy coupled with virtual library screening [24]. A non-active site TS-DHFR inhibitor would be advantageous because this site would be specific for the bifunctional enzyme and therefore not inhibit the monofunctional (host) enzymes. The successful use of non-active site inhibitors as a component of combination therapy is well established in the treatment of HIV infections [25]. A potential non-active site inhibitor of *C. hominis* could work in two ways: 1) by interfering directly with the crossover helix and Helix B or 2) by binding to a site adjacent to the crossover helix. These non-active site inhibitors could be used in conjunction with potential *C. hominis* DHFR active site inhibitors as combination therapy [26, 27], theoretically decreasing the onset of resistance mutations. Development of effective

species-specific inhibitors for *C. hominis* is essential as there are currently no effective treatments for cryptosporidiosis.

Acknowledgments

This work was supported by National Institutes of Health Grant AI 44630 to K.S.A. and NIH Grant 5T32-AI07404 for predoctoral support for W.E.M.

The abbreviations used are

TS, thymidylate synthase; DHFR, dihydrofolate reductase; dTMP, 2'-deoxythymidine monophosphate; dUMP, 2'-deoxyuridine monophosphate; CH₂H₄folate, methylene tetrahydrofolate; H₂folate, dihydrofolate; H₄folate, tetrahydrofolate.

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Figure 1. *Cryptosporidium hominis* TS-DHFR structure (PDB ID: 1QZF)

(A) Dimer structure of TS-DHFR. TS and DHFR domains are labeled. Crossover helix and Helix B are also labeled in the DHFR domains. The DHFR ligands, NADP⁺ and H₂F are shown in sticks. (B) Close-up of the crossover helix region. Residues on the crossover helix (light grey) are displayed as well as residues on the active site helix (dark grey). (C) Space filling representation highlighting the close interactions of the crossover helix (light grey) and helix B (dark grey) residues. DHFR active site ligands are shown in sticks.

Figure 2. DHFR catalysis as measured by coenzyme fluorescence energy transfer

Fluorescence excitation was at 290 nm and emission was at 450 nm. In all panels, 50 μM enzyme was incubated with 500 μM NADPH and then rapidly mixed with 10 μM H_2folate . The traces show the change in fluorescence signal after mixing as a function of time. **(A)** A representative stopped-flow trace of wild-type TS-DHFR. Data were fit to a single-exponential with a rate of $152 \pm 7 \text{ s}^{-1}$. **(B)** A representative stopped-flow trace for the alanine face TS-DHFR. Data were fit to a single exponential with a rate of $30 \pm 1 \text{ s}^{-1}$. **(C)** A representative stopped-flow trace of the all-alanine TS-DHFR. Data were fit to a single exponential with a rate of $16 \pm 1 \text{ s}^{-1}$. **(D)** A representative stopped-flow trace for the glycine face TS-DHFR. Data were fit to a single exponential with a rate of $17 \pm 1 \text{ s}^{-1}$.

Figure 3. *C. hominis* DHFR stopped-flow pre-steady state burst experiments

Fluorescence excitation was at 290 nm and emission was at 450 nm. In all panels, 7.5 μM enzyme was incubated with 50 μM H_2folate and then rapidly mixed with 500 μM NADPH. The change in fluorescence was measured over a given time range. **(A)** A representative stopped-flow trace for wild-type TS-DHFR. Data were fit to the burst equation with the fast rate of $60 \pm 2 \text{ s}^{-1}$ and the slower rate consistent with the steady state rate. **(B)** A representative stopped-flow trace for alanine face TS-DHFR. Data were fit to the burst equation with a faster rate of $22 \pm 1 \text{ s}^{-1}$ and a slower phase similar to the steady state rate. **(C)** A representative stopped-flow trace for the all alanine TS-DHFR. Data were fit to a burst equation with a faster rate of $9.5 \pm 0.1 \text{ s}^{-1}$. **(D)** A representative stopped-flow trace for the glycine face TS-DHFR. Data were fit to the burst equation with a faster rate of $8.9 \pm 0.3 \text{ s}^{-1}$.

Figure 4. *C. hominis* TS-DHFR single enzyme turnover reaction time course

Full TS-DHFR reaction: ● CH₂H₄folate, ■ H₂folate, ◆ H₄folate. In all panels, 50 μM enzyme is incubated with 500 μM dUMP and 500 μM NADPH, followed by rapid mixing with 10 μM H₂folate. (A) Wild-type enzyme: H₄folate production is $10.1 \pm 0.9 \text{ s}^{-1}$. (B) Alanine face enzyme: H₄folate production is $6.7 \pm 0.8 \text{ s}^{-1}$. (C) All alanine enzyme: H₄folate production is $4.0 \pm 0.4 \text{ s}^{-1}$. (D) Glycine face enzyme: H₄folate production is $1.0 \pm 0.3 \text{ s}^{-1}$ (note the change in the time scale for this panel)

Table 1
Structural and Kinetic Comparison of TS-DHFR from *L. major* [5], *P. falciparum* [4], and *C. hominis* [3].

Species	N-terminal Tail? (aa Length)	Linker Region (aa Length)	Crossover Helix?	DHFR activity in absence of TS ligands	DHFR activity in presence of TS ligands	DHFR Rate Enhancement
<i>Leishmania major</i>	Yes (22)	Short (2)	No	14 s ⁻¹	120 s ⁻¹	~ 10 fold
<i>Plasmodium falciparum</i>	Yes (5)	Long (89)	Yes	61 s ⁻¹	135 s ⁻¹	~ 2 fold
<i>Cryptosporidium hominis</i>	No	Long (58)	Yes	152 s ⁻¹	152 s ⁻¹	none

Table 2

Rate constants for the DHFR Single Turnover Reaction.

	DHFR Reaction Rate (s ⁻¹)
Wild-type	152 ± 7
Alanine Face	30 ± 1
Glycine Face	17 ± 1
All alanine	16 ± 1

Table 3

Data from the TS-DHFR single turnover reactions.

	Maximum Accumulation of H ₂ F (μ M)	Persistence of H ₂ F peak (amount of H ₂ F at 0.2 s in μ M)	TS-DHFR Reaction Rate (s ⁻¹)
Wild-type	4.1	1.5	10.1 $\pm$ 0.9
Alanine Face	5.1	2.6	6.7 $\pm$ 0.8
Glycine Face	3.8	3.6	1.0 $\pm$ 0.3
All alanine	6.2	3.9	4.0 $\pm$ 0.4