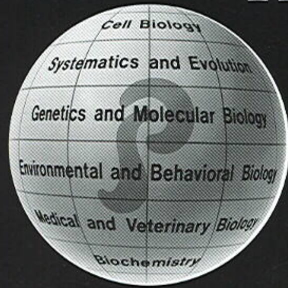
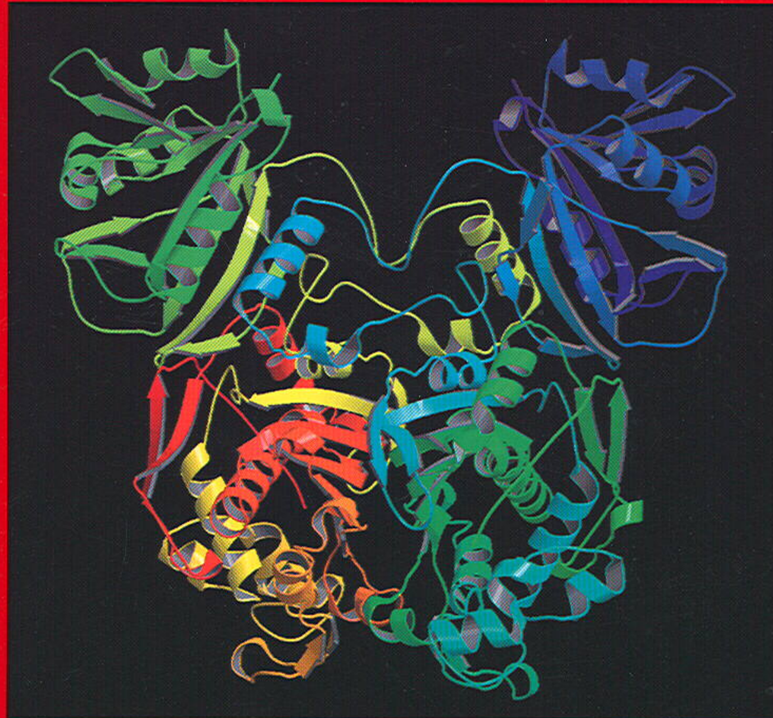
Supplement to Volume 50

The Journal of Eukaryotic Microbiology



Proceedings of the Eighth International Workshops on Opportunistic Protists International Conference on Anaerobic Protists Hilo, Hawaii July 23–29, 2003



Published by The Society of Protozoologists

Cover: The crystal structure of dihydrofolate reductase-thymidylate synthase (DHFR-TS) from *Cryptosporidium hominis*. From R. O'Neil, R. Lilien, B. R. Donald, R. Stroud, and A. Anderson. The crystal structure of dihydrofolate reductase-thymidylate synthase from *Cryptosporidium hominis* reveals a novel architecture for the bifunctional enzyme. *Jour. Eukaryotic Microbiology*, 50(6):555–556, 2003.

The Crystal Structure of Dihydrofolate Reductase-Thymidylate Synthase from *Cryptosporidium hominis* Reveals a Novel Architecture for the Bifunctional Enzyme

ROBERT H. O'NEIL,[a] RYAN H. LILIEN,[b] BRUCE R. DONALD,[b] ROBERT M. STROUD[c] and AMY C. ANDERSON[a]

[a]*Department of Chemistry, Dartmouth College, Burke Laboratories, Hanover, NH 03755;*
[b]*Department of Computer Science, Dartmouth College, Sudikoff Laboratory, Hanover, NH and*
[c]*Department of Biochemistry and Biophysics, University of California, San Francisco, CA*

Cryptosporidium hominis is an emerging pathogen that primarily affects immune-compromised patients, including those with AIDS. There is no effective cure at this point for the degenerative wasting disease that can follow infection. Dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a bifunctional enzyme in *C. hominis* and is crucial in the folate cycle for the production of dTMP, one of the four DNA bases. DHFR-TS is an excellent drug target since its inhibition leads to the death of the pathogen. In apicomplexan protozoa, including *C. hominis*, the gene for DHFR-TS can be partitioned into a DHFR domain, a linker domain and a TS domain. In human cells, DHFR and TS are separate, monofunctional enzymes.

We have determined the X-ray crystal structure of DHFR-TS from *C. hominis*. The structure reveals that the linker polypeptide between the DHFR and TS domains has important structural interactions with the opposite monomer of the homodimeric enzyme. A comparison of the structure of DHFR-TS from *C. hominis* and structures of DHFR-TS from *Plasmodium falciparum* [11] and *Leishmania major* [5] shows that there are significant structural differences between the apicomplexan and kinetoplastid forms of the enzyme. Additionally, the DHFR and TS domains of the *C. hominis* enzyme have important sequence and structural differences from the human forms of the enzymes. The species-specific differences are important for future inhibitor design targeting *C. hominis*.

MATERIALS AND METHODS

Cell extract from PA414 cells deficient in DHFR and over-expressing the *C. hominis* DHFR-TS protein, was applied to a methotrexate-agarose column (Sigma). After extensive washing, the protein was eluted with 2 mM dihydrofolate (Sigma) [10]. Fractions containing the *C. hominis* DHFR-TS protein were concentrated to 7 mg/ml.

The pure protein was incubated with 2 mM dUMP, 2 mM CB3717, 2 mM NADPH and 1 mM methotrexate. DHFR-TS was crystallized using vapor diffusion against a reservoir of 10% PEG 6K, 150 mM Li_2SO_4, 50 mM $(NH_4)_2SO_4$ and 100 mM Tris pH 8.0. Crystals grew within 2 weeks and were frozen using 25% ethylene glycol as a cryosolvent. Data were collected at Brookhaven National Laboratory to 2.8 Å resolution and were indexed and scaled with Denzo/Scalepack [9].

Molecular replacement attempts using CNS [3] and a model of TS from *Pneumocystis carinii* [2] successfully placed two full dimers of TS in the asymmetric unit and placed one dimer of TS across a symmetry axis. Five models of DHFR from *P. carinii* [4] were successfully placed in the asymmetric unit using the fixed models of TS. The two full dimers of DHFR-TS, the dimer across the symmetry axis and two symmetry-related dimers from adjacent asymmetric units form a non-crystallographic 5_1 axis. The residues of *C. hominis* DHFR-TS were substituted for the residues of *P. carinii* TS and DHFR, the ligands were added, the linker was built and water molecules were added to the complex. The entire model was refined to an R-factor = 22.7% and R_{free} = 24.7%.

Corresponding author: Amy C. Anderson. Telephone: 603-646-1154; Fax: 603-646-3946; Email: Amy.Anderson@Dartmouth.edu

RESULTS AND DISCUSSION

The crystal structure of DHFR-TS from *C. hominis* (ChDHFR-TS) reveals that the protein is a homodimer and forms the canonical TS dimer interface (Fig. 1). The two DHFR domains sit on the "shoulders" of the TS monomers. A two-fold non-crystallographic symmetry axis relates the two monomers of the protein. The ChDHFR-TS protein fold is unique. The ChDHFR-TS polypeptide forms the DHFR domain, crosses to the opposite monomer of the homodimeric enzyme, forms an 11-residue helix that interacts with the DHFR active site of the opposite monomer, and then crosses back and forms the TS domain. In general, the structures of the *C. hominis* DHFR and TS domains resemble other known structures of DHFR and TS, although there are some specific residue and loop differences that may prove to be important for species-specific drug design.

The residue differences between DHFR-TS from *C. hominis* and *C. parvum*, the bovine isolate of the parasite, are in regions that are seemingly insignificant to catalysis. Seven of the ten residue substitutions are in primarily surface-exposed residues. Two are in the second shell of residues away from the active site, in the interior of the protein and close to the linker domain that crosses between monomers. The last residue substitution is directly in the middle of the linker domain where the polypeptide crosses. The lack of significant structural differences or residue substitutions in catalytically important regions between the DHFR-TS proteins of *C. hominis* and *C. parvum* explains the similarities in IC_{50} values observed with several DHFR inhibitors [7].

The structure of ChDHFR-TS is similar in many respects to the structure of DHFR-TS from *Plasmodium falciparum* (PfDHFR-TS) [11], but both of the apicomplexan DHFR-TS proteins differ significantly from the structure of DHFR-TS from the kinetoplastid, *Leishmania major* (LmDHFR-TS) [5]. Both ChDHFR-TS and PfDHFR-TS have long linker regions between the DHFR and TS domains (58 and 89 residues, respectively). Both structures have helices, donated from the opposite monomer, located at the active site of DHFR. In both cases, the polypeptide chain then returns to form the TS domain. In the PfDHFR-TS structure, however, the electron density for the linker region was not clear and only the helix and connection back to TS were modeled. In ChDHFR-TS, this entire region was visible in the electron density and was modeled.

In stark contrast, however, the LmDFHR-TS protein has a very short (two-residue) linker between the DHFR and TS domains. The taut tether restricts the orientation of DHFR relative to TS. As a result, the LmDHFR domain is rotated approximately 180 degrees relative to the ChDHFR domain. The DHFR active site in ChDHFR-TS points toward the cavity between the two DHFR monomers and the DHFR active site in LmDHFR-TS points in the opposite direction, toward the outside of the molecule. Therefore, in LmDHFR-TS, the DHFR and TS active sites are on the same side of the monomer whereas in ChDHFR-TS, they are on opposite sides of the monomer. Given the structural differences between the apicomplexan and *Leishmania* forms of DHFR-TS, it is important, especially when modeling any compounds that are located outside the active site, to use a protein template that correlates with the species of interest.

In the DHFR active site, dihydrofolate and NADPH are bound using several of the canonical interactions seen in other structures of

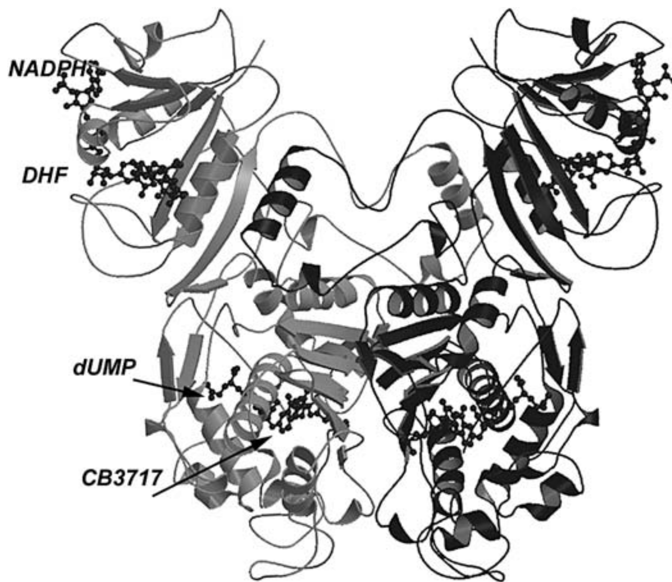


Fig. **1**. The crystal structure of DHFR-TS from *Cryptosporidium hominis*. The bifunctional enzyme is bound to dihydrofolate (DHF) and NADPH in the DHFR active site and to dUMP and CB3717 in the TS active site. The two monomers are shown in two shades of grey.

DHFR [4,5,8]. The molecule of dihydrofolate is bound in the same orientation seen in other structures of DHFR bound to dihydrofolate [8]. However, several key residue differences between ChDHFR and human DHFR (hDHFR) will be important in the design of species-selective drugs. ChDHFR has a cysteine residue in the active site of DHFR, Cys 113, pointed away from the molecule of dihydrofolate where hDHFR has a valine that makes more significant van der Waals' interactions with the substrate. In several instances, ChDHFR positions a smaller residue where hDHFR uses a larger residue: Val 9 in ChDHFR replaces Ile 7 in hDHFR, Phe 111 (ChDHFR) replaces Trp 113 (hDHFR), Leu 33 (ChDHFR) replaces Phe 31 (hDHFR) and Lys 34 (ChDHFR) replaces Arg 32 (hDHFR). Finally, hDHFR has a four-residue loop at the exit from the active site and ChDHFR is missing the loop altogether.

At the ChTS active site, dUMP, the substrate, and CB3717, a cofactor mimic, are bound using many of the same residues seen in other structures of TS [1,6]. However, ChTS interactions with the glutamate tail of CB3717 differ from most structures of TS bound to this analog. ChTS positions Ala 287 (Phe 80 in hTS) and Ser 290 (Gly 83 in hTS) near the glutamate tail. The Ala 287 substitution removes the steric bulk of the Phe residue in hTS and the Ser 290 substitution creates an additional hydrogen bond with the glutamate tail.

In conclusion, the crystal structure of ChDHFR-TS reveals a new fold for DHFR-TS in which each polypeptide chain of the homodimeric enzyme is associated with both monomers. There are few significant residue substitutions between DHFR-TS of *C. hominis* and *C. parvum*, allowing the successes of drug design for DHFR-TS from *C. hominis* to extend to *C. parvum*. Finally, there are important residue substitutions between *C. hominis* and human DHFR and TS that can be used in the design of species-selective inhibitors.

ACKNOWLEDGMENTS

Rick Nelson and John Vasquez (SFGH) provided pure ChDHFR-TS. Chris Bailey-Kellogg assisted with computer programming expertise to interpret the results of the cross rotation function during molecular replacement. This work was supported by NIH GM067542 to ACA.

LITERATURE CITED

1. Anderson, A., Perry, K., Freymann, D. & Stroud, R. 2000. The crystal structure of thymidylate synthase from *Pneumocystis carinii* reveals a fungal insert important for drug design. *J. Mol. Biol.,* **297**:645–657.
2. Anderson, A., O'Neil, R., Surti, T. & Stroud, R. 2001. Approaches to solving the rigid receptor problem by identifying a minimal set of flexible residues during ligand docking. *Chem. Biol.,* **8**:445–457.
3. Brunger, A. 1998. Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Cryst. D,* **54**:905–921.
4. Cody, V., Galitsky, N., Rak, D., Luft, J., Pangborn, W. & Queener, S. 1999. Ligand-induced conformational changes in the crystal structures of *Pneumocystis carinii* dihydrofolate reductase complexes with folate and NADP+. *Biochemistry,* **38**:4303–4312.
5. Knighton, D., Kan, C., Howland, E., Janson, C., Hostomska, Z., Welsh, K. & Matthews, D. 1994. Structure of and kinetic channeling in bifunctional dihydrofolate reductase-thymidylate synthase. *Nat. Struct. Biol.,* **1**:186–194.
6. Montfort, W., Perry, K., Fauman, E., Finer-Moore, J., Maley, G., Hardy, L., Maley, F. & Stroud, R. 1990. Structure, multiple site binding, and segmental accommodation in thymidylate synthase on binding dUMP and an anti-folate. *Biochemistry,* **29**:6964–6977.
7. Nelson, R. & Rosowsky, A. 2001. Dicyclic and tricyclic diaminopyrimidine derivatives as potent inhibitors of *Cryptosporidium parvum* dihydrofolate reductase: structure-activity and structure-selectivity correlations. *Antimicrob. Agents Chemother.,* **45**:3293–3303.
8. Oefner, C., D'Arcy, A. & Winkler, F. 1988. Crystal structure of human dihydrofolate reductase complexed with folate. *Eur. J. Biochem.,* **174**: 377–385.
9. Otwinowski, Z. 1993. DENZO 1-56-62. *In*: SERC Daresbury Laboratory; Warrington, U.K.
10. Vasquez, J., Gooze, L., Kim, K., Gut, J., Petersen, C. & Nelson, R. 1996. Potential antifolate resistance determinants and genotypic variation in the bifunctional dihydrofolate reductase-thymidylate synthase gene from human and bovine isolates of *Cryptosporidium parvum*. *Mol. Biochem. Parasitol.,* **79**:153–165.
11. Yuvaniyama, J., Chitnumsub, P., Kamchonwongpaisan, S., Vanichtanankul, J., Sirawaraporn, W., Taylor, P., Walkinshaw, M. & Yuthavong, Y. 2003. Insights into antifolate resistance from malarial DHFR-TS structures. *Nat. Struct. Biol.,* **10**:357–365.