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Short sequence-paper

Cloning and expression of the dihydroorotate dehydrogenase from *Toxoplasma gondii*☆

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Abstract

A full-length dihydroorotate dehydrogenase (DHODase) sequence was cloned from a *Toxoplasma gondii* tachyzoite cDNA library. The sequence was most similar to family 2 DHODases, and had a calculated molecular mass of 65.1 kDa. The full-length and two N-terminally truncated *T. gondii* DHODase sequences were expressed as recombinant proteins. One of the truncated sequences complemented a DHODase-deficient bacterial host.

Keywords: Dihydroorotate dehydrogenase; *Toxoplasma gondii*; Pyrimidine biosynthesis

Toxoplasma gondii is a protozoan parasite responsible for life-threatening disease in immunocompromised individuals [1] and in the fetus [2]. Recently, Fox and Bzik [3] demonstrated that de novo pyrimidine biosynthesis is necessary for the virulence of this parasite in mice. Mutants of *T. gondii* with disruptions in carbamoyl phosphate synthetase II, the first enzyme of the pyrimidine biosynthetic pathway, are able to invade normally, but are unable to replicate [3]. Moreover, pyrimidine starvation is one of the conditions that cause stage conversion from the tachyzoite, the form present in active infections, to the bradyzoite, present in latent infections [4]. These findings, together with the observation that the parasite has a limited capacity to salvage pyrimidines [5], place a new importance on the enzymes of de novo pyrimidine biosynthetic pathway in *T. gondii* [6]. The presence of the pathway in *T. gondii* has been known since 1981 [7]. In a seminal work, Asai et al. [8] measured specific activities of all six pyrimidine biosynthetic enzymes in *T. gondii* extracts. We recently cloned and characterized the *T. gondii* aspartate transcarbamoylase, the enzyme catalyzing the second step of the pathway [9].

Dihydroorotate dehydrogenase (DHODase) catalyzes the fourth, and only redox step of pyrimidine biosynthesis. In humans, this enzyme is the target of leflunomide, an immunosuppressive drug used in treatment of rheumatoid arthritis [10], and brequinar sodium, an antiproliferative drug [11]. The enzyme from *Plasmodium falciparum*, the parasite causing malaria, was recently evaluated as a possible drug target [12,13].

DHODases are classified into two families; family 1 enzymes are soluble, cytosolic proteins, and are found in some bacteria and some lower eukaryotes, while family 2 DHODases are membrane associated [14]. The mammalian DHODase is bound to the inner mitochondrial membrane, and transfers electrons via ubiquinone to the respiratory chain [15]. Asai et al. [8] locate the *T. gondii* DHODase activity in the particulate fraction of cell extracts, and show that it is inhibited by respiratory chain inhibitors.

To clone the DHODase, we screened 5×10^6 plaque forming units of a *T. gondii* RH tachyzoite cDNA library (catalog #1896, AIDS Research and Reference Reagent Program, NIAID, NIH) using the polymerase chain reaction (PCR) method of Israel [16] with primers, sense, 5′TCTTCTCCCAACACACCGGGT3′; and antisense, 5′CCGGCCGCTGAGACCGCCAGT3′. We isolated and sequenced five overlapping clones containing DHODase

Abbreviations: DHODase, dihydroorotate dehydrogenase; PCR, polymerase chain reaction

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under the accession number AF271664.

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sequences with inserts of ~ 2.5, 2.3, 1.5, 1.4, and 1.2 kb. A full-length *T. gondii* DHODase sequence (1770 bp) was constructed from two of these clones by digestion at an internal *Cla*I restriction site, purification of appropriate fragments, and ligation. The junction region containing the *Cla*I site of the new construct was verified by sequencing.

Alignments of the full-length *T. gondii* DHODase sequence with a predicted DHODase sequence fragment

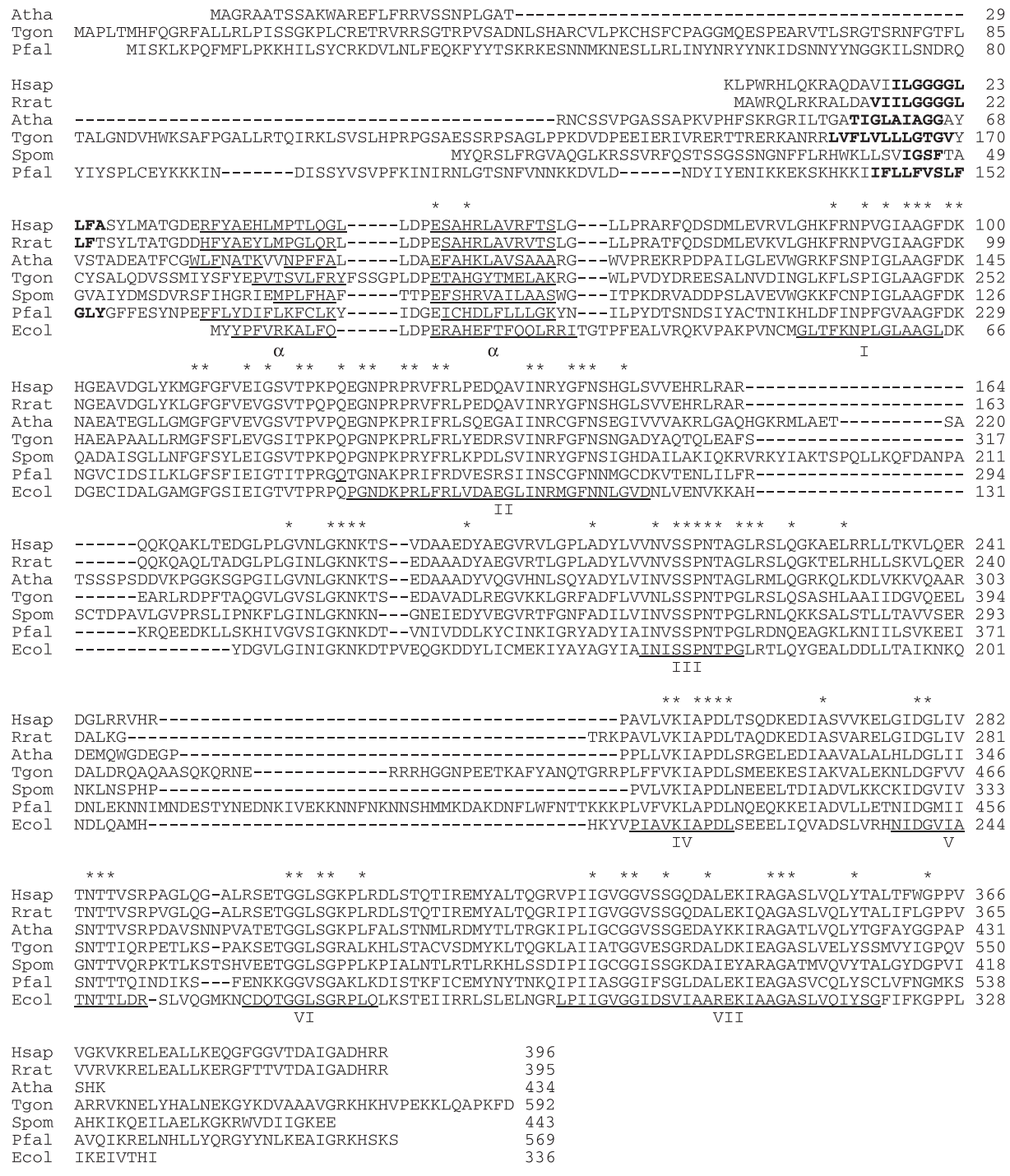

Fig. 1. Alignment of the *T. gondii* DHODase predicted amino acid sequence with other family 2 DHODase. Seven conserved DHODase regions are underlined in the *E. coli* sequence (I–VII). The locations of two alpha helices observed in crystal structures of the human DHODase are underlined and marked (α). The corresponding sequences predicted to form alpha helices in the other DHODases are also underlined. N-terminal sequences predicted to be transmembrane segments are shown in bold. Asterisks indicate conserved residues. Abbreviations and GenBank accession numbers are: *A. thaliana*, Athal, X62909; *E. coli*, Ecol, X02826; *H. sapiens*, Hsap, M94065; *P. falciparum*, Pfal, L15446; *R. rattus*, Rrat, X80778; *S. pombe*, Spom, X65114; *T. gondii*, Tgon, AF271664.

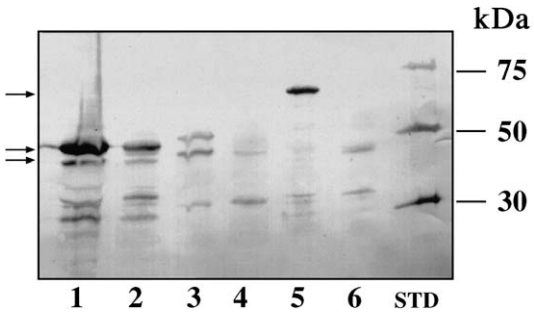

Fig. 2. Western blot showing expression of *T. gondii* DHODase recombinant proteins in *E. coli*. Full-length and N-terminally truncated sequences cloned into expression vectors were transformed into *E. coli* host cells and grown on minimal media under inducing conditions. *E. coli* strain pyr D^- (American Type Culture Collection, ATCC12632) was used to express TgDHOD-MAPL and TgDHOD-MIYS recombinant proteins, while strain BL21CodonPlus(DE3)RP (Stratagene) was used to express TgDHOD-ALQD. Cells were sonicated in the presence of detergent (50 mM sodium phosphate, 300 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine), and the extracts were centrifuged. Supernatants and washed pellets were fractionated by denaturing gradient polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Recombinant proteins were visualized by antibodies recognizing histidine tags (Qiagen). The expected molecular masses of the proteins were: TgDHOD-MIYS, 46.3 kDa (Lane 1, pellet and Lane 2, supernatant); TgDHOD-ALQD, 48.1 kDa (Lane 3, pellet and Lane 4, supernatant); and TgDHOD-MAPL, 66.2 kDa (Lane 5, pellet and Lane 6, supernatant). Inclusion of flavins in the growth media did not affect band intensities. No bands were visible in host cell strains transformed with parent vectors grown under inducing conditions.

from a *T. gondii* tachyzoite library (398 bp, GenBank BG660232) showed differences at four nucleotide positions, with an identical deduced amino acid sequence. A DHODase fragment from a *T. gondii* bradyzoite library (535 bp, GenBank AA520414, Ref. [17]) differed at one nucleotide position, and was missing nucleotides at two positions resulting in frame shifts.

A possible translation initiation site in the full-length sequence was identified, and agreed with a *T. gondii* translation initiation consensus sequence described by Seeber [18]. The deduced amino acid sequence had a predicted molecular mass of 65,064 Da. An alignment [19] showed that it contained the seven DHODase conserved regions predicted by the BLOCKS program [20] (Fig. 1). Pairwise alignments using the ALIGN program (http://www2.igh.cnrs.fr/) showed that the *T. gondii* sequence shared highest percent with family 2 type DHODases (*Homo sapiens*, 33.6%; *Arabidopsis thaliana*, 33.4%; *Schizosaccharomyces pombe*, 31.6%; *P. falciparum*, 31.7%; *Escherichia coli*, 24.4%), and was least similar to family 1 type DHODases (*Lactococcus lactis* A, 17.0%; *L. lactis* B, 16.2%). Additional sequences were observed between conserved regions III and IV in both *T. gondii* (31 residues) and *P. falciparum* (44 residues) sequences (Fig. 1).

The *T. gondii* DHODase sequence had a predicted isoelectric point of 9.55 (http://www.expasy.ch/tools/pi_tool.html). High isoelectric points are predicted for other family 2 DHODases, and are consistent with their observed mitochondrial locations [21,22]. The N-terminal extensions found in family 2 enzymes, which are absent in family 1 enzymes, play a role in cellular localization. The *Rattus rattus* DHODase N-terminus contains a mitochondrial targeting sequence (residues 2–10) and a hydrophobic membrane anchor (residues 11–18) that arrests the protein in the inner mitochondrial membrane [23]. Potential membrane-associated N-terminal sequences predicted by the DAS program [24] are shown in Fig. 1. Interestingly, the N-terminal extensions of the *T. gondii* and *P. falciparum* enzymes are 147 and 129 residues longer, respectively, than that of the rat enzyme (Fig. 1).

Crystal structures of a truncated human enzyme (starting at the sequence MATG) demonstrate that residues 30–63 form a small domain containing two alpha helices involved in binding a brequinar analog and a leflunomide derivative [25]. This domain forms the entrance to a hydrophobic tunnel that leads to the ubiquinone reduction site [25]. Secondary structure predictions using PredictProtein [26] of the corresponding sequences in the *T. gondii* DHODase and the other family 2 DHODases predict the presence of alpha helices that align with the observed helices in the human enzyme (Fig. 1).

We cloned the full-length DHODase sequence (MAPL-PKFD, starting at amino acid residue 1), and a truncated sequence (MIYS-PKFD, starting at residue 181), into the vector pBAce, under the control of the phoA promoter [27]. These two constructs contained the amino acid sequence $DRGH_6$ at the C-terminal end of the DHODase coding sequence. A third truncated sequence (ALQD-PKFD, starting at residue 174) was cloned into the vector P343, under the control of the *tet* promoter [28]. This construct contained an added methionine at the N-terminus, and the sequence PGD_4KH_8SGS at the C-terminus of the coding sequence.

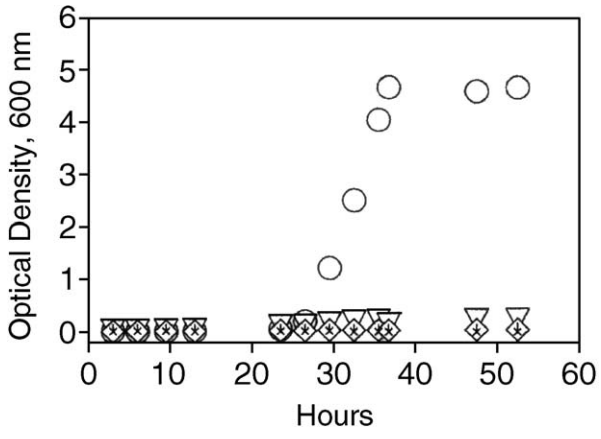

Fig. 3. A truncated recombinant *T. gondii* DHODase complements a DHODase-deficient *E. coli* strain. DHODase-deficient *E. coli* cells (ATCC12632) were transformed with parent vectors and with constructs expressing full-length and truncated *T. gondii* DHODase. Transformed cells were grown under inducing conditions in minimal media lacking uracil, and growth was monitored by optical density measurements at 600 nm. Cells were transformed with (○) TgDHOD-MIYS; (▽) TgDHOD-ALQD; (◇) TgDHOD-MAPL; (×) P343; and (|) pBAce.

The full-length recombinant protein was present in the insoluble fraction after sonication in the presence of detergent (Fig. 2). It did not complement the DHODase deficient pyr D^- strain when grown in minimal media under inducing conditions (Fig. 3) [9,27]. Pyr D^- cells expressing TgDHOD-ALQD recombinant protein grew poorly (Fig. 3), and the protein was primarily present in the pellet after sonication and centrifugation. The shortest recombinant protein was partially solubilized by the detergent (Fig. 2), and complemented the DHODase-deficient strain, and thus appeared to be catalytically active (Fig. 3). Purification of this recombinant enzyme will allow us to begin characterization of the *T. gondii* DHODase.

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