Divergent polyamine metabolism in the Apicomplexa

Tuesday Cook,¹ David Roos,² Mary Morada,¹ Guan Zhu,³ Janet S. Keithly,⁴ Jean E. Feagin,⁵ Gang Wu¹ and Nigel Yarlett^{1,6}

Correspondence

Nigel Yarlett
nyarlett@pace.edu

¹Haskins Laboratories, Pace University, New York, NY 10038, USA

²Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

³Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA

⁴Division of Infectious Diseases, David Axelrod Institute, Wadsworth Center, NYS Department of Health, Albany, NY 1220, USA

⁵Seattle Biomedical Research Institute, 307 Westlake Ave N., Seattle, WA 9810, USA

⁶Department of Chemistry and Physical Sciences, Pace University, New York, NY 10038, USA

The lead enzymes of polyamine biosynthesis, i.e. ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), were not detected in *Toxoplasma gondii* [the limit of detection for ODC and ADC was 5 pmol min⁻¹ (mg protein)⁻¹], indicating that *T. gondii* lacks a forward-directed polyamine biosynthetic pathway, and is therefore a polyamine auxotroph. The biochemical results were supported by results obtained from data-mining the *T. gondii* genome. However, it was possible to demonstrate the presence of a highly active backconversion pathway that formed spermidine from spermine, and putrescine from spermidine, via the combined action of spermidine/spermine N¹-acetyltransferase (SSAT) or spermidine N¹-acetyltransferase (SAT) and polyamine oxidase (PAO). With spermine as the substrate, *T. gondii* SSAT had a specific activity of 1.84 nmol min⁻¹ (mg protein)⁻¹, and an apparent K_m for spermine of 180 mM; with spermidine as the substrate, the SAT had a specific activity of 3.95 nmol min⁻¹ (mg protein)⁻¹, and a K_m for spermidine of 240 mM. *T. gondii* PAO had a specific activity of 10.6 nmol min⁻¹ (mg protein)⁻¹, and a K_m for acetylspermine of 36 mM. Furthermore, the results demonstrated that *T. gondii* SSAT was 50 % inhibited by 30 mM di(ethyl)nor-spermine. The parasite actively transported arginine and ornithine, which were converted via the arginine dihydrolase pathway to citrulline and carbamoyl phosphate, resulting in the formation of ATP via carbamate kinase. The lack of polyamine biosynthesis by *T. gondii* is contrasted with polyamine metabolism by other apicomplexans.

Received 24 August 2006

Revised 11 December 2006

Accepted 18 December 2006

INTRODUCTION

Polyamines are essential for the growth and function of all eukaryotic cells. To date, only two orders of the *Archaea*, the *Methanobacteriales* and the *Halobacteriales* (Hamana & Matsuzaki, 1992), have been found that lack these cationic molecules. The apparent universal distribution of polyamines, and the complexity of compensatory mechanisms that are invoked to maintain polyamine homeostasis, suggest that these molecules are critical to cell survival (Wallace *et al.*, 2003). The biosynthesis of polyamines

typically starts with the conversion of arginine to putrescine by one of two distinct pathways: arginase coupled with ornithine decarboxylase (ODC), or arginine decarboxylase (ADC) coupled with agmatine iminohydrolase. Putrescine can be further converted into spermidine and spermine by spermidine synthase and spermine synthase, respectively. When polyamines are available, spermine or spermidine can be retro-converted into spermidine and putrescine by the consecutive action of spermine/spermidine N¹-acetyltransferase (SSAT) and polyamine oxidase (PAO).

The phylum Apicomplexa comprises a large group of intracellular parasitic protists, including *Toxoplasma gondii*, *Plasmodium* spp., *Babesia* spp., *Cryptosporidium* spp. and *Eimeria* spp., all of which have a multistage life cycle that includes merozoites, meronts and gamonts, which are formed within the host cell membrane-bound parasitophorous vacuole. The merozoites and sporozoites are found

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ADC, arginine decarboxylase; DENSpm, di(ethyl)nor-spermine; DFMA, α -difluoromethylarginine; DFMO, α -difluoromethylornithine; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAT, spermidine N¹-acetyltransferase; SSAT, spermidine/spermine N¹-acetyltransferase.

outside the host cell, and they invade new host cells (Entzeroth *et al.*, 1998; Coombs *et al.*, 1977). All apicomplexans examined to date, except for members of the *Cryptosporidium* genus, contain a non-photosynthetic plastid, termed the apicoplast, which was acquired by an ancestral apicomplexan from a member of the red or green algae, via a secondary endosymbiotic event (Fichera & Roos, 1997; Kohler *et al.*, 1997; Cai *et al.*, 2003). However, *Cryptosporidium parvum* possesses an unusual mitochondrial organelle (Keithly *et al.*, 1997; Riordan *et al.*, 2003; Slapeta & Keithly, 2004), as well as a number of unique molecular and biochemical features that differ from those found in other apicomplexans (Abrahamsen *et al.*, 2004; Thompson *et al.*, 2005).

Despite the importance of polyamines to cell growth and multiplication, polyamine metabolism has not been thoroughly characterized in the Apicomplexa. Published data have shown that *Plasmodium falciparum* and *Eimeria tenella* possess ODC, whereas *C. parvum* has a plant-like pathway that utilizes ADC (Keithly *et al.*, 1997). However, there have been no reports on polyamine metabolism in *T. gondii*. In this study, we investigated polyamine metabolism by *T. gondii*. Our results indicate that *T. gondii* lacks a forward-directed polyamine biosynthetic pathway. However, we were able to demonstrate a highly active polyamine retro-conversion pathway responsible for the conversion of spermine to spermidine and putrescine via SSAT and PAO. *T. gondii* SSAT was inhibited by di(ethyl)nor-spermine (DENSpm), which is a specific inhibitor of SSAT in other cells (Yarlett *et al.*, 2000). These observations are also supported by the bioinformatic analysis of apicomplexan genomic data that indicates that polyamine metabolism is highly divergent among apicomplexans.

METHODS

Organisms. *T. gondii* tachyzoites (strain RH) were cultivated in human foreskin fibroblast cells, as described (Furtado *et al.*, 1992). Parasites were harvested soon after emerging from host cells, passed through a 3 µm filter (Whatman no. 110612), centrifuged at 1000 g for 12 min, washed once with PBS, and pelleted with desktop centrifugation for 3 min. Oocysts of *C. parvum* (Iowa strain) were obtained from Bunch Grass Farms (Troy, Idaho, USA), and purified through discontinuous CsCl gradients at 1.4, 1.1 and 1.05 g ml⁻¹ by centrifugation for 1 h at 16 000 g at 4 °C. They were then rinsed three times in distilled water, resuspended in 2.5% (w/v) aqueous potassium dichromate, and stored at 4 °C for 2–10 days. On the day of use, 10⁹ oocysts were washed in distilled water, surface sterilized on ice for 10 min with 10% sodium hypochlorite, washed five times with distilled water, and resuspended in Hanks' balanced salt solution (HBSS). Excystation was performed by mixing equal volumes of excystation buffer [0.5% (w/v) trypsin and 1.5% (w/v) taurodeoxycholate in HBSS] and oocysts, and incubating for 1 h at 37 °C. To prepare free sporozoites from the chicken coccidium *E. tenella* (WIS), oocysts in PBS were broken by strong vortex with 1 mm glass beads in a 15 ml tube to release sporocysts. Excystation was then performed in a similar way to that for *C. parvum*, except that the incubation was conducted at 41 °C for 1.5 h. Mixed erythrocytic stage *P. falciparum* (strain C10; Hempelmann *et al.*, 1981) was supplied by Dr Jean Feagin.

Enzyme assays. Parasites were resuspended in 0.1 M KH₂PO₄/K₂HPO₄ buffer, pH 7.4, and extracts were prepared by 30 strokes in a Potter-Elvehjem at 4 °C. ADC was assayed in incubations containing 7 µCi (259 kBq) [1-¹⁴C]arginine (327 mCi mmol⁻¹; 12.10 GBq mmol⁻¹), 10 mM Tris/HCl, pH 6.0–8.0, 60 µM pyridoxal phosphate, 1 mM 2-mercaptoethanol, and varying amounts of arginine (0.06–2.0 mM), for 30 min at 37 °C. The ¹⁴CO₂ released in 30 min was trapped on filter paper soaked with 1 M benzethonium hydroxide, and measured by scintillation (Smith, 1983). ODC was assayed as described for ADC, except that 1 µCi (37 kBq) [1-¹⁴C]ornithine (51.3 mCi mol⁻¹; 1.90 GBq mmol⁻¹) was used instead of [1-¹⁴C]arginine. SSAT and spermidine N¹-acetyltransferase (SAT) were assayed in incubations containing 0.5 µCi (18.5 kBq) [1-¹⁴C]acetyl coenzyme A (60 mCi mmol⁻¹; 2.22 GBq mmol⁻¹), and supplemented with 60 µM acetyl coenzyme A, 0.1 mM Bicine, pH 7.0, and varying amounts of spermine (0.02–0.60 mM) or spermidine (0.02–0.50 mM), for 20 min. The reaction was stopped by the addition of 0.2 M hydroxylamine, and placing on ice. Labelled samples were placed in a boiling water bath for 3 min, and they were measured as for ODC and ADC (Keithly *et al.*, 1997). PAO activity was determined by measuring the substrate-dependent formation of hydrogen peroxide in 10 mM glycine (pH 8.0) containing 10 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (BioChemika), 1 mM N¹-acetylspermine, 5 units horseradish peroxidase, and 50–100 µg protein. The peroxide released was detected by measuring the change in absorption of ABTS at 420 nm. Arginine deiminase was determined by measuring the colorimetric formation of citrulline at 37 °C. The assay contained 1 mM L-arginine, 40 mM MES, pH 6.0, and 0.07 mg protein, in a final volume of 1.0 ml. After 1 h, the reaction was stopped by the addition of 0.075 ml 100% (w/v) TCA, and the amount of citrulline formed was determined using diacetyl monoxime, as described by Boyde & Rahmatullah (1980). Catabolic ornithine carbamoyl transferase was determined by measuring ¹⁴CO₂ release from L-[¹⁴C-carbamoyl]citrulline. The reaction mixture contained 40 mM Tricine, pH 8.0, 0.1 mM L-citrulline, 17.2 mM L-[¹⁴C-carbamoyl]citrulline (57.7 mCi mmol⁻¹; 2.13 GBq mmol⁻¹) (DuPont NEN Life Science), and 0.07 mg protein, in a final volume of 1 ml. After incubation at 37 °C for 1 h, the reaction was stopped with 1 ml 40% TCA, and incubated for a further 30 min. CO₂ was trapped using filter paper soaked in benzethonium hydroxide. Carbamate kinase was determined in incubations containing 1 mM ADP, 20 mM MgSO₄, 0.15 mM luciferin, 1 mg firefly lantern extract, and 1 mM carbamoyl phosphate, in 50 mM potassium phosphate buffer, pH 7.6. ATP formation was determined by monitoring the luminescence using a photomultiplier tube. Proteins were determined using the method of Lowry *et al.* (1951).

Enzyme inhibition studies. Extracts were incubated with 30 µM α-difluoromethylornithine (DFMO) or α-difluoromethylarginine (DFMA) for 30 min prior to determination of enzyme activity by addition of 1 µCi (37 kBq) [1-¹⁴C]ornithine plus 2 mM ornithine, or 7 µCi (259 kBq) [1-¹⁴C]arginine plus 2 mM arginine, as described for ODC and ADC activity (Yarlett *et al.*, 1992). The inhibitory effect of DENSpm on SSAT was evaluated by measuring the enzyme activity in incubations containing 0.1 mM bicine (pH 7.0), 60 µM acetyl-CoA, 25 µM spermine and varying concentrations of DENSpm (10–100 µM). The assay was repeated with 75, 150 and 300 µM spermine and the amount of N¹-acetylspermine produced after 30 min was determined as described previously (Yarlett *et al.*, 2000).

Uptake and interconversion of [¹⁴C]spermine, [¹⁴C]arginine and [¹⁴C]ornithine. *T. gondii* tachyzoites (10⁴) were incubated in HBSS, pH 7.4, containing 0.09 µM (0.5 µCi; 18.5 kBq) [5,8-¹⁴C]spermine mixed with 2 mM spermine, 6 µM (2 µCi; 74 kBq) L-[U-¹⁴C]arginine mixed with 2 mM arginine, or 0.64 µM (20 µCi; 740 kBq) L-[2,3-³H]ornithine mixed with 2 mM ornithine, for 15 min

at 37 °C. In some experiments, the incubation medium also contained 5 mM DFMO or 5 mM DFMA. Cells were sedimented by centrifugation at 14 000 g for 1 min, and the supernatant was removed. The cell pellet was resuspended in ice-cold 4% TCA and vortexed, and the precipitated protein was removed by centrifugation at 14 000 g for 5 min. Amino acids and polyamines in the protein-free supernatant were separated by HPLC, and detected by dual analysis using radiometric and fluorescence detection, as described below.

HPLC. Polyamines were separated by reverse-phase HPLC, using a series LC 410 pump (Perkin-Elmer) coupled to a C-18 10 µm column (4.5 × 250 mm), at a flow rate of 1 ml min⁻¹. The method employed a 70 min discontinuous gradient starting with 85% (v/v) buffer A: 2.5 g lithium citrate l⁻¹, pH 2.65, containing 0.22 g octane sulfonic acid l⁻¹ and 15% (v/v) acetonitrile. Separation of polyamines used a gradient change in the buffer, as described (Yarlett & Bacchi, 1988). Standards and samples were derivatized prior to injection by mixing one part standard or sample with two parts 0.8 g o-phthalaldehyde l⁻¹ (dissolved in 3 ml methanol and 30.9 g boric acid l⁻¹ containing 24 g KOH l⁻¹ and 1 ml 2-mercaptoethanol, pH 10.4). The derivatized compounds were subjected to dual analysis using a fluorescence monitor ($\lambda_{\text{excitation}}$ 320 nm, $\lambda_{\text{emission}}$ 455 nm) coupled to a flow-through model 1B Radiometric detector (IN/US Systems) that mixed three parts scintillant (INFLOW ES) to one part sample. Areas under the peaks were determined using β -RAM computer software (IN/US Systems), version 1.62.

Bioinformatic analysis. To validate our biochemical observations, we also data-mined the complete or nearly complete genome-sequencing data for *T. gondii* (<http://ToxoDB.org/toxo/home.jsp>, release 3.0; and www.tigr.org/tdb/e2k1/tga1), *P. falciparum* (<http://PlasmoDB.org/plasmo/home.jsp>, release 4.4), *C. parvum* (<http://CryptoDB.org/cryptodb>, release 3.2), and *E. tenella* (www.sanger.ac.uk/Projects/E_tenella). Because the divergence in apicomplexan polyamine metabolism is mainly found in the forward direction, we focused on the homology search of ODC and ADC genes that represent two distinct pathways to convert arginine to putrescine. The annotated ODC and ADC protein sequences from all major taxonomic groups were used as queries to perform a BLAST search of DNA and protein databases (where available). SSAT and PAO were searched using conserved sequences for these proteins in the lower eukaryotes. Hits from the apicomplexan databases were retrieved, and used as queries to search homologues in all non-redundant protein databases at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/blast) to verify their true identities.

RESULTS

Enzyme analysis

T. gondii was found to be incapable of converting arginine to putrescine, whereas *P. falciparum*, *E. tenella* and *C. parvum* use different pathways for this process. Extracts of *T. gondii* had no detectable ODC and ADC activity (Table 1). The addition of pyridoxal phosphate (0.06 mM) or Mg²⁺ (0.1 mM) at concentrations shown to be necessary for these enzymes in other cells did not result in detectable enzyme activity when assayed over a pH range of 4.5–8.5. Positive controls included lysates of *Trichomonas vaginalis* and *Saccharomyces cerevisiae* expressing an *Escherichia coli* ADC. These were used at 1/10 of the protein content for *T. gondii* (not shown). The activity of these enzymes was examined in extracts from other apicomplexans. In agreement with previous observations, ODC was detected in extracts of *P. falciparum* and *E. tenella*, while ADC activity was detected in extracts of *C. parvum* (Table 1). The ADC and ODC detected in the respective apicomplexans were specifically inhibited by fluorinated analogues of the parent amino acid. The ADC activity in *C. parvum* was 50% inhibited by 30 µM DFMA, but was unaffected by the same concentration of DFMO (Table 1). In contrast, the ODC activity observed in *P. falciparum* and *E. tenella* was 84 and 56% inhibited by 30 µM DFMO, respectively, and 58 and 12% inhibited by 30 µM DFMA, respectively (Table 1).

Genomic data support the notion that the forward-converting pathway is divergent among apicomplexans

Our biochemical observations agree with the genomic data. By data-mining various apicomplexan genome sequences, we failed to detect any ODC and ADC homologues encoded by the *T. gondii* genome (ToxoDB, release 3.0, with 10 × coverage), but we identified ODC genes from *P. falciparum* which has a well characterized bifunctional ODC/S-adenosyl-L-methionine decarboxylase (Wrenger *et al.*, 2001) and *E. tenella* which has ODC activity (Keithly *et al.*, 1997; The Sanger Institute, with > 8 × coverage). No ADC homologues were detected from the *T. gondii*, *P. falciparum*

Table 1. Effect of DFMO and DFMA on ADC and ODC activity in the apicomplexa

ODC and ADC activity was determined as described in Methods. Parasite extracts were incubated for 30 min with 30 µM DFMA or DFMO prior to the addition of L-[1-¹⁴C]ornithine or L-[1-¹⁴C]arginine, as described in Methods. One unit of enzyme activity is defined as 1 µmol product formed min⁻¹ (mg protein)⁻¹. ND, Not detected; limit of detection, 5 pmol min⁻¹ (mg protein)⁻¹.

Organism	ODC (mU)	ADC (mU)	DFMO (% inhibition)	DFMA (% inhibition)
<i>T. gondii</i>	ND	ND	ND	ND
<i>P. falciparum</i>	0.309	ND	84	58
<i>C. parvum</i>	ND	0.005	0	50
<i>E. tenella</i>	0.28	ND	56	12

and *E. tenella* genomes, except for the lysine decarboxylase (LDC) homologues that typically display significant sequence similarity with ADC and ODC. No ODC homologue was present in the *C. parvum* genome, which agrees with the absence of ODC activity in this parasite. Despite the consistent enzymic detection of ADC in *C. parvum* (Keithly *et al.*, 1997; this study), we failed to find any ADC or even LDC homologues in the *C. parvum* genome. It is possible that the ADC gene in *C. parvum* is highly divergent from known ADC genes that have so far been found in bacteria and plants only, or that this parasite may possess another uncharacterized pathway that displays activity similar to ADC. The *T. gondii* SSAT gene identified with sequence 12 290 from the *T. gondii* genome database. BLAST search of this sequence indicated the highest homology with the SSAT gene from *C. parvum* (Yarlett *et al.*, 2007). A putative PAO gene was also identified in the *T. gondii* genome database, and it had the contiguous sequence chr 0-995297-63. The *T. gondii* PAO gene is highly divergent, but it encodes a protein of similar molecular size (508 aa residues) to that observed for PAO from other organisms.

T. gondii possesses highly active back-converting activity to synthesize putrescine

Although neither ODC nor ADC activity was detected in *T. gondii*, SSAT and SAT activities were clearly detected in extracts of *T. gondii* by measuring the acetylation of exogenously supplied spermine and spermidine, respectively (Table 2). The enzymes had a pH optimum of 8.0 for both substrates, and exhibited Michaelis–Menten kinetics. Hanes–Woelf analysis of the SSAT activity for spermine resulted in a linear plot with maximal activity at a saturating spermine concentration of 1.84 $\mu\text{M min}^{-1}$ (mg protein) $^{-1}$, and an apparent K_M for spermine of 180 μM (Table 2). The maximal activity with spermidine was 3.95 $\mu\text{M min}^{-1}$ (mg protein) $^{-1}$, and an apparent K_M for spermidine of 240 μM (Table 2). *T. gondii* SSAT was competitively inhibited by DENSp_m, with a calculated K_i of 30 μM . These results indicate that *T. gondii* may rely on polyamine uptake and retro-conversion to satisfy its polyamine requirements. PAO, the enzyme transforming *N*¹-acetylspermine to spermidine, and *N*¹-acetylspermidine to putrescine, was

Table 2. Activities of polyamine metabolizing enzymes in representative members of the Apicomplexa

Enzyme analysis was performed as described in Methods. One unit of enzyme activity is defined as the amount of enzyme required to generate 1 $\mu\text{mol product min}^{-1}$ (mg protein) $^{-1}$; K_m values are expressed in μM .

Organism	SSAT		SAT		PAO	
	K_m	mU	K_m	mU	K_m	mU
<i>T. gondii</i>	180	1.84	240	3.95	36	10.6
<i>C. parvum</i>	50	0.13	130	0.017	28	2.4

detected in extracts of *T. gondii* tachyzoites. As described for other cells, the SSAT is the rate-limiting step in the reaction.

Spermidine is the major product of spermine by T. gondii

Incubations of *T. gondii* tachyzoites (10^4) with a mixture of 2.02 mM [5,8-¹⁴C]spermine (0.25 $\mu\text{Ci mmol}^{-1}$; 9 kBq mmol^{-1}) for 30 min, and HPLC analysis of the products, revealed that spermine was readily transported by the parasite (retention time, 28 min), and that spermidine was the major product (retention time, 32 min) of spermine metabolism by *T. gondii* tachyzoites (Fig. 1). A minor signal corresponding to *N*¹-acetylspermine was also observed at a retention time of 5 min (Fig. 1).

Fig. 1. HPLC analysis of *T. gondii* tachyzoites after incubation with [¹⁴C]spermine. (a) *T. gondii* tachyzoites incubated with 2 mM (1 $\mu\text{Ci } \mu\text{mol}^{-1}$; 37 kBq μmol^{-1}) [5,8-¹⁴C]spermine for 30 min, as described in Methods, took up 1.77 μmol spermine per 10^4 tachyzoites (28 min), and converted it to 0.29 μmol *N*¹-acetylspermine per 10^4 tachyzoites (5 min) and 1.33 μmol spermidine per 10^4 tachyzoites (32 min), confirming the enzymic analysis with *T. gondii* tachyzoite extracts, and the data-mining results. (b) Standards: 2 mM (2 $\mu\text{Ci } \mu\text{mol}^{-1}$; 74 kBq μmol^{-1}) [1,4-¹⁴C]putrescine (39 min), 2 mM (0.30 Ci μmol^{-1} ; 11.1 GBq μmol^{-1}) [1,7-³H]spermidine (32 min), 2 mM (1 $\mu\text{Ci } \mu\text{mol}^{-1}$; 37 kBq μmol^{-1}) [5,8-¹⁴C]spermine (28 min).

Table 3. Polyamine content of *T. gondii*

Values [nmol polyamine (mg protein)⁻¹] were determined using whole-cell homogenates. Analysis was performed using control homogenates with no addition, homogenates incubated with 10 mM ornithine, and homogenates incubated with 10 mM arginine. Values are means $\pm$ SD; the number of experiments is in parentheses.

Treatment	Putrescine	Spermidine	Spermine	Total
Control	137 $\pm$ 24 (10)	191 $\pm$ 101 (10)	256 $\pm$ 90 (10)	369–799 (10)
Ornithine	119 $\pm$ 24 (3)	251 $\pm$ 147 (3)	178 $\pm$ 53 (3)	324–772 (3)
Arginine	142 $\pm$ 27 (3)	125 $\pm$ 58 (3)	262 $\pm$ 104 (3)	340–718 (3)

Citrulline, ornithine and carbamoyl phosphate are the major products in arginine metabolism in *T. gondii*

The enzymes of the arginine dihydrolase pathway were detected in extracts of *T. gondii*. In common with this pathway in other cells, the activities of arginine deiminase and ornithine carbamoyl transferase were approximately equal, whereas that for carbamate kinase was significantly higher: 3.0 ± 0.16 , 2.1 ± 0.09 and 980 ± 22.0 μ mol product min⁻¹ (mg protein)⁻¹ (mean $\pm$ SD), respectively.

Polyamine content of *T. gondii*

The polyamine content of *T. gondii* tachyzoites whole-cell homogenates was determined (Table 3). The concentrations of the physiological polyamines putrescine, spermidine and spermine were similar to those found in other protists that have been examined (Bacchi & Yarlett, 1995). In agreement with the enzymic analysis, determination of polyamine levels in cells incubated with L-[2,3-³H]ornithine or L-[U-¹⁴C]arginine did not produce detectable quantities of ³H-labelled or ¹⁴C-labelled polyamines, respectively. Additionally incubation with these polyamine precursors did not significantly alter basal polyamine levels in these cell extracts (Table 3), and total polyamine pools remained constant for all treatments: control, 369–799 nmol (mg protein)⁻¹; ornithine, 324–772 nmol (mg protein)⁻¹;

arginine, 340–718 nmol (mg protein)⁻¹. These results corroborate the enzyme analysis indicating that *T. gondii* is auxotrophic for polyamines.

Analysis of products from incubations containing L-[U-¹⁴C]arginine revealed that arginine is rapidly transported, and converted into citrulline, ornithine and carbamoyl phosphate (Table 4). These results demonstrate that the major products of arginine metabolism are citrulline, and equimolar amounts of ornithine and carbamoyl phosphate, consistent with the presence of an arginine dihydrolase pathway in this parasite. The addition of 5 mM DFMO to the incubation medium caused a 25 % increase in arginine uptake, with a concomitant 45 % increase in the amount of citrulline formed; however, 85 and 77 % reductions in ornithine and carbamoyl phosphate, respectively, were observed, indicating that DFMO interferes with ornithine carbamoyl transferase activity (Table 4). The addition of 5 mM DFMA to the incubation medium caused a 45 % reduction in arginine, indicating that DFMA effectively competes with the transport of arginine into the parasite. The reduced arginine resulted in an equimolar (43 %) reduction in the amount of citrulline formed; additionally, 68 and 40 % reductions in ornithine and carbamoyl phosphate, respectively, were observed (Table 4). Cells incubated with L-[2,3-³H]ornithine demonstrated that ornithine was also rapidly transported into the parasite, and that an anabolic function for ornithine

Table 4. Intermediates of the arginine dihydrolase pathway in *T. gondii*

Values (nmol ml⁻¹) were determined in whole-cell homogenates incubated with [U-¹⁴C]arginine or [U-¹⁴C]ornithine, in the presence and absence of 5 mM DFMO or 5 mM DFMA, as described in Methods. Values are means $\pm$ SD for triplicate samples.

Treatment	Arginine	Citrulline	Ornithine	Carbamoyl-PO ₄
Arginine	13.7 $\pm$ 2.7	14.3 $\pm$ 3.5	3.32 $\pm$ 1.7	3.04 $\pm$ 1.6
Arginine + DFMO	17.2 $\pm$ 3.2	20.8 $\pm$ 4.1	0.49 $\pm$ 0.1	0.69 $\pm$ 1.2
Arginine + DFMA	7.60 $\pm$ 2.1	8.20 $\pm$ 2.9	1.07 $\pm$ 0.2	1.81 $\pm$ 0.9
Ornithine	–	5.33 $\pm$ 3.2	20.6 $\pm$ 5.3	–
Ornithine + DFMO	–	1.60 $\pm$ 0.7	1.93 $\pm$ 1.2	–
Ornithine + DFMA	–	6.23 $\pm$ 3.8	22.7 $\pm$ 3.4	–

carbamoyl transferase was present that converts ornithine back to citrulline (Table 4). The addition of 5 mM DFMO to the incubation mix resulted in a 90% reduction in ornithine, indicating that DFMO effectively competes with the transport of ornithine into the parasite, resulting in a 70% reduction in citrulline (Table 4). The addition of 5 mM DFMA to the incubation medium did not have a significant effect upon the intracellular citrulline and ornithine concentrations (Table 4).

DISCUSSION

The polyamines spermidine and spermine are essential to the growth and development of all known cells. These molecules play a critical role in cell proliferation and differentiation, and biosynthesis of macromolecules. Because of the significance of these molecules to cell survival, they are obvious targets for the development of chemotherapeutic agents designed to block cell growth. The lead and rate-controlling enzyme of polyamine biosynthesis in the majority of eukaryotic cells is either ODC or ADC, producing putrescine, to which an aminopropyl group is sequentially added forming first spermidine, and then spermine (Cohen, 1998; Bacchi & Yarett, 2002). *T. gondii* is a member of the apicomplexa, a diverse group of intracellular parasites that have in common a multistage life cycle (Bush *et al.*, 2001). In this study, we demonstrate that *T. gondii* lacks ODC and ADC activity, and therefore is auxotrophic for polyamines. These results agree with the observed growth requirement of *T. gondii* for host polyamines reported by others (Moraes *et al.*, 2004; Seabra *et al.*, 2004), but distinguish *T. gondii* from other members of the apicomplexa that have the ability to synthesize polyamines (Keithly *et al.*, 1997; Wrenger *et al.*, 2001). *T. gondii* has previously been shown to have a high-affinity putrescine transporter, demonstrating the ability to scavenge host-derived putrescine (Seabra *et al.*, 2004). We show that the parasite also has the ability to retro-convert spermine to spermidine, and spermidine to putrescine, via an active SSAT. This ability would not only provide the parasite with a mechanism for production of spermidine from spermine, but also provide a mechanism for control of intracellular polyamines by acetylation, and subsequent removal from the cell (Wallace *et al.*, 2003; Fig. 2). The various members of the apicomplexa have different methods for polyamine biosynthesis: *E. tenella* and *P. falciparum* have an active ODC (Keithly *et al.*, 1997; Wrenger *et al.*, 2001), whereas *C. parvum* has an ADC (Keithly *et al.*, 1997); these differences may be the result of different intracellular localizations of these parasites. Most information is available for the malaria parasite *P. falciparum*, which has been shown to possess a bifunctional ODC-adenosylmethionine decarboxylase that is responsible for polyamine biosynthesis (Wrenger *et al.*, 2001). The activity of the *P. falciparum* enzyme has been shown to be significantly blocked by putrescine analogues that exhibited 1300-fold higher activity toward the parasite compared with DFMO (Das Gupta *et al.*, 2005). Depletion of putrescine by these

Fig. 2. Polyamine and arginine interconversion by *T. gondii* tachyzoites. Arginine, ornithine and putrescine transporters; Based upon the results shown in Table 4, DFMA is taken up via the arginine transporter, and DFMO is taken up via the ornithine transporter. These fluorinated amino acid analogues act as non-functional substrates for enzymes of the arginine dihydrolase pathway, resulting in the metabolic changes noted in the text. In the absence of these analogues, arginine is metabolized to ornithine and carbamoyl phosphate via the arginine dihydrolase pathway. 1, Arginine deiminase; 2, ornithine carbamoyl transferase (catabolic); 3, ornithine carbamoyl transferase (anabolic); 4, carbamate kinase. Spermine is taken up from the host cell, and metabolized to spermidine by the combined action of SSAT (5) and PAO, and to putrescine by the combined action of SAT (6) and PAO.

analogues results in an increase in spermine, and this is concluded to be due to the activity of a non-specific spermidine synthase (Das Gupta *et al.*, 2005; Haider *et al.*, 2005).

It has been reported that *T. gondii* has a higher infection rate in younger embryos, which exhibit a greater ODC activity, and have a higher intracellular putrescine content, indicating the important contribution of host cell polyamines to parasite growth and development (Moraes *et al.*, 2004). Previous studies have indicated that the specific irreversible inhibitor DFMO is only effective at inhibiting growth of *T. gondii* when used at concentrations that are toxic to the host-cell monolayers (Hofflin *et al.*, 1985; Derouin & Chastang, 1988; Moraes *et al.*, 2004). These results contrast with the intestinal parasite *Eimeria*, which has an active ODC. Growth of *Eimeria* is blocked by DFMO administration at concentrations that are non-toxic to the host (Hanson *et al.*, 1982; San Martin-Nuñez *et al.*, 1988).

It is significant that the total polyamine content of *T. gondii* remains constant after incubation with radiolabelled arginine or ornithine, and that no label is incorporated into parasite intracellular polyamines. Radiolabelled arginine was, however, converted via the arginine dihydrolase pathway into citrulline, and equimolar amounts of ornithine and carbamoyl phosphate (Fig. 2). Interestingly, the specific irreversible inhibitor of arginine decarboxylase, DFMA, significantly reduced the uptake of arginine, resulting in a proportional reduction of the products of the arginine dihydrolase pathway. DFMO did not interfere with the uptake of arginine, but significantly reduced the synthesis of radiolabelled ornithine from arginine, indicating that DFMO exhibits an inhibitory effect towards ornithine carbamoyl transferase. DFMO did significantly compete with the uptake of ornithine, and caused a concomitant reduction in citrulline formation. The transport and metabolism of host-derived arginine via the ADH pathway would have an important role in parasite ATP formation (Fig. 2). It is also proposed that this mechanism would divert host arginine away from NO synthesis, and hence be responsible for protection from this host-derived cytotoxic defence mechanism (Seabra *et al.*, 2004). It is likely that the parasite ADH pathway is a rational target for the development of chemotherapeutic agents to treat disease caused by *T. gondii*.

ACKNOWLEDGEMENTS

This research was supported by grant AI 49785 (N. Y.). The following apicomplexan genome databases were searched for polyamine metabolic genes: *T. gondii* at <http://CryptoDB.org/cryptodb>, which contains genomic data provided by the Institute for Genomic Research [supported by the National Institutes of Health (NIH) grant AI05093], the Sanger Institute (Wellcome Trust), and Washington University (NIH grant 1R01AI045806-01A1); *P. falciparum* at <http://PlasmoDB.org/plasmo/home.jsp>, funded by NIH grant 1R01AI058515-01; *C. parvum* at <http://CryptoDB.org/cryptodb>, funded by NIH contract N01-AI-40037 (HHSN266200400037C); and *E. tenella* at www.sanger.ac.uk/Projects/E_tenella, funded by the Biotechnology and Biological Sciences Research Council (UK).

REFERENCES

- Abrahamsen, M. S., Templeton, T. J., Enomoto, S., Abrahante, J. E., Zhu, G., Lancto, C. A., Deng, M., Liu, C., Widmer, G. & other authors (2004). Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* **304**, 441–445.
- Bacchi, C. J. & Yarlett, N. (1995). Polyamine metabolism. In *Biochemistry and Molecular Biology of Parasites*, pp. 119–131. Edited by J. J. Marr & M. Muller. New York: Academic Press.
- Bacchi, C. J. & Yarlett, N. (2002). Polyamine metabolism as a chemotherapeutic target in protozoan parasites. *Mini Rev Med Chem* **2**, 553–563.
- Boyde, T. R. & Rahmatullah, M. (1980). Optimization of conditions for the colorimetric detection of citrulline using diacetyl monoxime. *Anal Biochem* **107**, 424–431.
- Bush, A. O., Fernandez, J. C., Esch, G. W. & Seed, J. R. (2001). *Parasitism: the Diversity and Ecology of Animal Parasites*, pp. 66–94. Cambridge: Cambridge University Press.
- Cai, X., Fuller, A. L., McDougald, L. R. & Zhu, G. (2003). Apicoplast genome of the coccidian *Eimeria tenella*. *Gene* **321**, 39–46.
- Cohen, S. S. (1998). *A Guide to the Polyamines*, pp. 122–230. New York: Oxford University Press.
- Coombs, G. H., Denton, H., Brown, S. M. A. & Thong, K.-W. (1977). Biochemistry of the coccidia. *Adv Parasitol* **39**, 141–226.
- Das Gupta, R., Krause-Ihle, T., Bergmann, B., Müller, I. B., Khomutov, A. R., Müller, S., Walter, R. D. & Lüersen, K. (2005). 3-Aminooxy-1-aminopropane and derivatives have an antiproliferative effect on cultured *Plasmodium falciparum* by decreasing intracellular polyamine concentrations. *Antimicrob Agents Chemother* **49**, 2857–2864.
- Derouin, F. & Chastang, C. (1988). Enzyme immunoassay to assess effect of antimicrobial agents on *Toxoplasma gondii* in tissue culture. *Antimicrob Agents Chemother* **32**, 303–307.
- Entzeroth, R., Mattig, F. R. & Werner-Meier, R. (1998). Structure and function of the parasitophorous vacuole in *Eimeria* species. *Int J Parasitol* **28**, 1015–1018.
- Fichera, M. & Roos, D. S. (1997). A plastid organelle as a drug target in apicomplexan parasites. *Nature* **390**, 407–409.
- Furtado, G. C., Cao, Y. & Joiner, K. A. (1992). Lamrin on *Toxoplasma gondii* mediates parasite binding to the β -1 integrin receptor α -6 β -1 on human foreskin fibroblasts of Chinese hamster ovary cells. *Infect Immunol* **60**, 4925–4931.
- Haider, N., Eschbach, M.-L., de Souza Dias, S., Gilberger, T.-W., Walter, R. D. & Lüersen, K. (2005). The spermidine synthase of the malaria parasite *Plasmodium falciparum*: molecular and biochemical characterization of the polyamine synthesis enzyme. *Mol Biochem Parasitol* **142**, 224–236.
- Hamana, K. & Matsuzaki, S. (1992). Polyamines as a chemotaxonomic marker in bacterial systematics. *Crit Rev Microbiol* **18**, 261–283.
- Hanson, W. L., Bradford, M. M., Chapman, W. L., Waits, V. B., McCann, P. P. & Sjoerdsma, A. (1982). α -Difluoromethylornithine: a promising lead for preventative chemotherapy for coccidiosis. *Am J Vet Res* **43**, 1651–1653.
- Hempelmann, E., Ling, I. & Wilson, R. J. (1981). S-antigens and isozymes in strains of *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg* **75**, 855–858.
- Hofflin, J. M., Guptill, D. R., Araujo, F. G. & Remington, J. S. (1985). Difluoromethylornithine and formycin B in toxoplasmosis. *J Infect Dis* **152**, 1101.
- Keithly, J. S., Zhu, G., Upton, S. J., Woods, K. M., Martinez, M. P. & Yarlett, N. (1997). Polyamine biosynthesis in *Cryptosporidium parvum* and its implications for chemotherapy. *Mol Biochem Parasitol* **88**, 35–42.
- Kohler, S., Delwiche, C. F., Denny, P. W., Tilney, P., Webster, P., Wilson, R. J. M., Palmer, J. D. & Roos, D. S. (1997). A plastid of probable green algal origin in apicomplexan parasites. *Science* **275**, 1485–1489.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Moraes, A. M. M., Pessôa, C. N., Vommaro, R. C., De Souza, W., de Mello, F. G. & Hokoç, J. N. (2004). Cultured embryonic retina systems as a model for the study of underlying mechanisms of *Toxoplasma gondii* infection. *Invest Ophthalmol Vis Sci* **45**, 2813–2821.
- Riordan, C. E., Ault, J. G., Langreth, S. G. & Keithly, J. S. (2003). *Cryptosporidium parvum* Cpn60 targets a relict organelle. *Curr Genet* **44**, 138–147.

- San Martín-Núñez, B. V., Ordoñez-Escudero, D. & Alunda, J. M. (1988).** Preventative treatment of rabbit coccidiosis with α -difluoromethylornithine. *Vet Parasitol* **30**, 1–10.
- Seabra, S. H., Da Matta, R. A., de Mello, F. G. & de Souza, W. (2004).** Endogenous polyamine levels in macrophages are sufficient to support growth of *Toxoplasma gondii*. *J Parasitol* **90**, 455–460.
- Slapeta, J. & Keithly, J. S. (2004).** *Cryptosporidium parvum* mitochondrial-type HSP70 targets homologous and heterologous mitochondria. *Eukaryot Cell* **3**, 483–494.
- Smith, T. A. (1983).** Arginine decarboxylase (oat seedlings). *Methods Enzymol* **94**, 176–180.
- Thompson, R. C., Olson, M. E., Zhu, G., Enomoto, S., Abrahamsen, M. S. & Hijawi, N. S. (2005).** *Cryptosporidium* and cryptosporidiosis. *Adv Parasitol* **59**, 77–158.
- Wallace, H. M., Fraser, A. V. & Hughes, A. (2003).** A perspective of polyamine metabolism. *Biochem J* **376**, 1–14.
- Wrenger, C., Lüersen, K., Krause, T., Müller, S. & Walter, R. D. (2001).** The *Plasmodium falciparum* bifunctional ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, enables a well balanced polyamine synthesis without domain-domain interaction. *J Biol Chem* **276**, 29651–29656.
- Yarlett, N. & Bacchi, C. J. (1988).** Effect of DL- α -difluoromethylornithine on polyamine synthesis and interconversion in *Trichomonas vaginalis* grown in semi-defined medium. *Mol Biochem Parasitol* **31**, 1–9.
- Yarlett, N., Goldberg, B., Moharrami, M. A. & Bacchi, C. J. (1992).** Inhibition of *Trichomonas vaginalis* ornithine decarboxylase by amino acid analogs. *Biochem Pharmacol* **44**, 243–250.
- Yarlett, N., Martinez, M. P., Goldberg, B., Kramer, D. L. & Porter, C. W. (2000).** Dependence of *Trichomonas vaginalis* upon polyamine backconversion. *Microbiol* **146**, 2715–2722.
- Yarlett, N., Wu, G., Waters, W. R., Harp, J. A., Wannemuehler, M. J., Morada, M., Athanasopoulos, D., Martinez, M. P., Upton, S. J. & other authors (2007).** *Cryptosporidium parvum* spermidine/spermine N¹-acetyltransferase exhibits different characteristics to the host enzyme. *Mol Biochem Parasitol* (in press).

Edited by: J. Tachezy