Structural and functional divergence of the aldolase fold in *Toxoplasma gondii*

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Abbreviations: ADF, actin depolymerizing factor; ALD1, fructose 1,6-bisphosphate aldolase; AU, asymmetric unit; CSS, complexation significance score; DLS, dynamic light scattering; dR5P, 2-deoxyribose 5-phosphate; DPA, dR5P aldolase-like protein; GAPDH, α -glyceraldehyde phosphate dehydrogenase; PH, Pleckstrin homology; R_H , hydrodynamic radius; r.m.s.d., root mean square deviation; SEC, size exclusion chromatography; TPI, triose phosphate isomerase.

ABSTRACT

Parasites of the phylum Apicomplexa are highly successful pathogens of humans and animals world-wide. As obligate intracellular parasites, they have significant energy requirements for invasion and gliding motility that are supplied by various metabolic pathways. Aldolases have emerged as key enzymes involved in these pathways, and all apicomplexans express one or both of fructose 1,6-bisphosphate (F16BP) aldolase and 2-deoxyribose 5-phosphate (dR5P) aldolase (DERA). Intriguingly, *Toxoplasma gondii*, a highly successful apicomplexan parasite, expresses F16BP aldolase (*TgALD1*), d5RP aldolase (*TgDERA*), and a divergent dR5P aldolase-like protein (*TgDPA*) exclusively in the latent bradyzoite stage. While the importance of *TgALD1* in glycolysis is well established and *TgDERA* is also likely to be involved in parasite metabolism, the detailed function of *TgDPA* remains elusive. To gain mechanistic insight into the function of different *T. gondii* aldolases, we first determined the crystal structures of *TgALD1* and *TgDPA*. Structural analysis revealed that both aldolases adopt a TIM barrel fold accessorized with divergent secondary structure elements. Structural comparison of *TgALD1* and *TgDPA* with members of their respective enzyme families revealed that while the active site residues are conserved in *TgALD1*, key catalytic residues are absent in *TgDPA*. Consistent with this observation, biochemical assays showed that while *TgALD1* was active on F16BP, *TgDPA* was inactive on dR5P. Intriguingly, both aldolases are competent to bind polymerized actin *in vitro*. Altogether, structural and biochemical analyses of *T. gondii* aldolase and aldolase-like proteins reveal diverse functionalization of the classic TIM barrel aldolase fold.

INTRODUCTION

The phylum Apicomplexa comprises more than five thousand obligate intracellular parasitic protozoan species that are responsible for causing devastating diseases on a global scale. *Toxoplasma gondii* is of particular interest as it infects up to one third of the global human population with disease observed most visibly in immunocompromised patients and congenitally infected neonates [1, 2]. Additionally, there is some evidence that *T. gondii* cysts in the brain may lead to behavioural changes and neurological disorders in immunocompetent adults [3]. Importantly, chronic *T. gondii* infections are largely refractory to pharmacological intervention making it difficult, if not impossible, for the host to clear the parasite [4]. The success of *T. gondii* is attributed, in part, to a multi-stage, complex life cycle with each stage displaying distinct cellular morphology and biochemistry; tachyzoites are responsible for rapid dissemination within the host and for congenital transmission, while bradyzoites form latent tissue cysts in the muscles and brain, and sporozoites are the infectious product of the sexual cycle in felines.

The majority of apicomplexan parasites, including *T. gondii*, are obligate intracellular pathogens and thus commit significant energy resources to invading host cells [5]. It is widely believed that these parasites rely on glycolysis for energy metabolism, and a recent study has supported this in *T. gondii* [6, 7]. Several major steps in metabolic pathways are catalyzed by the aldolase family of enzymes that promote reversible cleavage of aldols. Aldolases are classified into four groups based on their substrates: dihydroxyacetone phosphate or fructose 1,6-bisphosphate (F16BP)-dependent, pyruvate- or phosphoenolpyruvate-dependent, acetaldehyde or 2-deoxyribose 5-phosphate (dR5P)-dependent, and glycine-dependent [8]. In addition to their metabolic functions, aldolases have also been shown to possess non-metabolic roles. F16BP aldolases, for example, may be involved in endocytosis, mRNA stability, and binding to cytoskeletal proteins, particularly F-actin, as F16BP aldolases have been shown to bind the F-actin cytoskeleton *in vivo* and cross-link F-actin into a gel or form rafts *in vitro* [9-12].

All apicomplexan parasites with sequenced genomes harbor an F16BP aldolase gene and a substantial proportion also have a dR5P aldolase (DERA) gene [13]. Interestingly, *T. gondii* harbors a divergent dR5P aldolase-like protein (*TgDPA*) in addition to F16BP aldolase (*TgALD1*) and dR5P aldolase (*TgDERA*) [14]. Transcriptomic analysis reveals that *TgALD1* is constitutively expressed at steady state levels in all developmental stages [15]. While *TgDERA* mRNA expression is marginally higher in the late bradyzoite and early oocyst stages, protein expression has been verified by mass spectrometry of the tachyzoite proteome [16] consistent with *TgDERA* also being present in all life cycle stages. In contrast, *TgDPA* appears to be specific to the bradyzoites [14]. Analysis of the KEGG pathway suggests that both *TgALD1* and *TgDERA* have metabolic activities linked through the pentose phosphate pathway, with *TgALD1* playing a major role in glycolysis [17]. *TgALD1* is also able to bind both F-actin and the intracellular domains of transmembrane adhesins *in vitro* and was, until recently, considered to bridge extracellular binding events to the actin-myosin motor [18, 19]. A recent study, however, has shown that *TgALD1* is not the obligate factor for gliding motility and host cell invasion but rather dedicated to energy metabolism [20]. The role of *TgDPA* remains unclear, but recent studies have suggested that *TgDPA* interacts with actin depolymerization factor (*TgADF*) to enhance its activity in regulating actin dynamics in the nonmotile, dormant bradyzoites [21].

To investigate the functional roles of the different aldolases in *T. gondii*, we first determined the high-resolution crystal structures of *TgALD1* and *TgDPA*. Structural analysis revealed an architectural similarity between *TgALD1* and *TgDPA* with both proteins adopting a TIM barrel fold consistent with their classification as aldolases. Notwithstanding the overall conserved architecture, *TgDPA* showed no catalytic activity with dR5P as a substrate consistent with the absence of key active site residues, and yet was able to coordinate polymerized actin similar to other aldolases and glycolytic enzymes [9, 18]. The

high-resolution structures of *Tg*ALD1 and *Tg*DPA complemented with biochemical data provide mechanistic insight into the diverse roles of these aldolases in *T. gondii*.

EXPERIMENTAL PROCEDURES

Bioinformatics

Phylogenetic analysis for apicomplexan F16BP and dR5P aldolases based on the sequences in OrthoMCL DB [22], EuPathDB [13], NCBI and UniProt was performed in MEGA 6.0 [23] – a neighbor-joining tree with 500 bootstrap replicates was constructed for a representative sample of the apicomplexan F16BP aldolases and DERA/DPA ortholog groups. Accession numbers: *T. gondii* aldolase 1 (*Tg*ALD1; TGME49_236040), *T. gondii* aldolase 2 (*Tg*ALD2; TGME49_036050), *Neospora caninum* aldolase 1 (NCLIV_050380), *N. caninum* aldolase 2 (NCLIV_050370), *Eimeria tenella* aldolase 1 (ETH_00015505), *E. tenella* aldolase 2 (ETH_00008600), *Cryptosporidium hominis* aldolase (Chro.10335), *Babesia bovis* aldolase (XP_001609245.1), *Theileria annulata* aldolase (TA20060), *Plasmodium yoelii* aldolase (PY03709), *P. vivax* aldolase (PVX_118255), *P. falciparum* aldolase (*Pf*ALDO; PF14_0425), *T. gondii* DPA (*Tg*DPA; TGME49_318750), *T. gondii* DERA (*Tg*DERA; TGME49_270650), *N. caninum* DPA (NCLIV_010810), *N. caninum* DERA (NCLIV_036120), *P. yoelii* DERA (*Py*DERA; PY02252), *P. berghei* DERA (PBANKA_050580), *P. chabaudi* DERA (PCHAS_050590), *P. falciparum* DERA (PF10_0210), *P. vivax* DERA (PVX_001945), *P. knowlesi* DERA (PKH_060570)]. Sequences for *Oryctolagus cuniculus* aldolase A (Rabbit muscle aldolase, *Oc*ALDOA; NP_001075707.1), *Pf*Aldolase (*Pf*ALDO) and *Tg*ALD1, and for *Escherichia coli* DERA (*Ec*DERA; NP_757311.1), *P. yoelii* DERA, *Tg*DERA and *Tg*DPA were obtained from EuPathDB and NCBI [13] and aligned in Clustal Omega [24].

*Tg*ALD1 and *Tg*DPA cloning, protein production and purification

A construct encoding the conserved fructose-1,6-bisphosphate aldolase domain (residues Pro⁷⁹–Gly⁴¹⁷) was amplified from *T. gondii* Type II (Pru) cDNA and cloned into *Nco*I/*Not*I sites in a modified pET28a vector encoding an N-terminal hexa histidine tag and thrombin cleavage site. *Tg*ALD1 was produced recombinantly in *E. coli* BL21-CodonPlus cells (Stratagene) grown in autoinduction medium (Invitrogen) from a 1% inoculum. Following four hours of growth at 310 K and 16 hours at 303 K, the cells were harvested and the pellet resuspended in 20 mM Hepes pH 8.3, 1 M NaCl, 30 mM imidazole, 2.5 mM beta-mercaptoethanol. Cells in suspension were lysed using an EmulsiFlex-C5 (Avestin), insoluble material was removed by centrifugation, and the soluble fraction was applied to a HisTRAP FF 5 mL column (GE Healthcare). Bound *Tg*ALD1 was eluted with an increasing concentration of imidazole, the

His tag was subsequently removed by thrombin cleavage, and *TgALD1* was further purified by gel filtration chromatography on a Superdex 200 16/60 HiLoad column in HBS (20 mM Hepes pH 6.8, 150 mM NaCl) with 2 mM tris(2-carboxyethyl)phosphine. The purity of *TgALD1* was assessed at each stage by SDS-PAGE, and protein concentration was determined using a calculated extinction coefficient of $24410 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.

TgDPA sequence from ToxoDB (<http://ToxoDB.org>) was codon-optimized based on *E. coli* codon usage bias, the prediction of protein crystallizability (XtalPred, <http://ffas.burnham.org/XtalPred/cgi/xtal.pl>), and protein sequence analysis. The optimized *TgDPA* coding sequences (residues Ile⁶–Ser²⁸¹) was gene synthesized and subcloned into a pCCK-N-HisTEV expression vector (MCLAB, CA). The resulting pCCK-N-*TgDPA*6-281 vector was introduced into BL21(DE3) Magic *E. coli* and expression was induced at OD₆₀₀=1.0 with the addition of 0.5 mM IPTG overnight at 25 °C. Harvested cells were resuspended in a buffer containing 20 mM Tris-HCl pH 8.3, 500 mM NaCl, 10% glycerol, 5 mM β -mercaptoethanol (BME) and 10 mM imidazole. Cells were lysed by sonication. Recombinant *TgDPA* was purified using immobilized metal-affinity chromatography on a Ni-NTA agarose column and size-exclusion chromatography on a Superdex 200 16/60 HiLoad column. *TgDPA* was concentrated in a buffer containing 10 mM Tris-HCl pH 8.3, 500 mM NaCl and 5 mM BME. *TgDPA* concentration was determined by absorbance with a calculated extinction coefficient of $16960 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.

Dynamic Light Scattering (DLS)

DLS measurements of *TgALD1* and *TgDPA* were performed using a DynaPro-TC-04 dynamic light scattering instrument (Wyatt Technology, Santa Barbara, CA). Prior to DLS analysis, all solutions were centrifuged at 13,000 rpm for 15 min and filtered (0.22 μm pore). A concentration-gradient (1-100 μM) was used to determine the optimal concentration for subsequent analyses (10 μM). Data were collected using the following parameters: temperature stabilized at 298 K, wavelength of 834 nm, auto-attenuated laser power, solvent refractive index of 1.33, and 10 acquisitions of 5 sec each. All data was analyzed in Dynamics 7.1 software to determine the mean hydrodynamic radius (R_h), polydispersity (P_d), and molecular weight using a globular polymer model. The analyzed data are an average of triplicate measurements.

Crystallization

Crystals of *TgALD1* were initially identified in the Structure screen (Molecular Dimensions) using sitting drop at 291 K. The final drops consisted of 1.5 μl of *TgALD1* at 15 mg ml^{-1} with 1.5 μl of reservoir solution and were equilibrated against 100 μl of reservoir solution. Diffraction quality crystals grew overnight in 0.1 M Sodium Chloride, 0.1 M Hepes pH 7.5, 1.6 M Ammonium Sulfate. A single

crystal was looped, cryopreserved in 2 M Lithium Sulfate for 20 seconds, and flash-cooled to 100 K directly in the cryostream.

Crystals of *TgDPA* were initially identified in the PACT Suite screen (Qiagen, Valencia, CA, USA) using sitting drops at 295 K. The final drops consisted of 1 μ l of *TgDPA* at 12.7 mg ml⁻¹ in 10 mM Tris-HCl pH 8.3 plus 500 mM NaCl and 5 mM BME plus 1 μ l of a reservoir solution equilibrated against 90 μ l of reservoir solution. Diffraction quality crystals grew in 0.2 M NaCl, 0.1 M MES buffer pH 6.0, 20% (w/v) PEG 6000. For X-ray data collection, a crystal was cryopreserved in the well solution and flash-frozen in liquid nitrogen.

Data collection and structure determination

Diffraction data for *TgALD1* crystals were collected on beamline 9-2 at Stanford Synchrotron Radiation Laboratory (SSRL) at a wavelength of 0.9795 Å and processed to 1.75 Å resolution using HKL-2000 [25]. Initial phases were obtained by molecular replacement using PHASER [26] with one unit of the tetrameric aldolase from *Babesia bovis* (Protein Data Bank (PDB) ID **3KX6**, 73% identity over 334 residues) pruned with CHAINSAW [27]. Solvent molecules were selected using Coot [28, 29] and refinement carried out using Refmac 5 [30]. Stereo-chemical analysis performed with PROCHECK and SFCHECK in the CCP4 suite [31] showed excellent stereochemistry with more than 90% of the residues in the favored conformations and no residues modeled in disallowed orientations of the Ramachandran plot.

Monochromatic X-ray data for *TgDPA* crystals were collected on beamline 21-ID-G (λ = 0.9785 Å), at Life Sciences Collaborative Access Team (LS-CAT), Advance Photon Source (APS), Argonne National Laboratory (ANL) and processed to 1.37 Å resolution using HKL-2000 [25]. Initial phases were obtained by molecular replacement using PHASER [26] from the CCP4 package [31] with the crystal structure of *TgDPA* in complex with sucrose as the search model (PDB ID **3QYQ**). Solvent molecules were added running *ARP/wARP* [32]. The model was corrected in Coot [28, 29] and refined with Refmac v.5.5 [30]. The Translation-Libration-Screw (TLS) groups from the TLS Motion Determination (TLSMD; <http://skuld.bmsc.washington.edu/~tlsmd/>; [33, 34]) server were introduced at the final stages of the refinement. The quality of the structure was checked with ADIT validation server (<http://deposit.pdb.org/validate/>) and MolProbity server [35, 36]. Diffraction images for the deposited *TgDPA* structure are available at the CSGID website (<http://www.csgid.org/csgid/pages/home>). Data collection and refinement statistics for both structures are presented in Table 1.

Structure images were prepared with PyMOL [37]. Structures were deposited in the PDB under the accession numbers **4D2J** (*TgALD1*) and **4EIV** (*TgDPA*).

Enzyme Activity Assay

In vitro TgALD1 and TgDPA activities were determined using a well established assay [38]. Briefly, activities were spectrophotometrically assayed at 298 K in the presence of triose phosphate isomerase (TPI) and α -glyceraldehyde phosphate dehydrogenase (GAPDH) and measuring NADH oxidation as a function of decreasing absorption per minute at 340 nm. The assay mixture (0.2 ml) contained (final concentration) Tris (87 mM), F16BP (1.9 mM), NADH (0.13 mM) and 5 U of α GAPDH/TPI. Similar conditions were used for TgDPA assay with the exception of 2-deoxyribose 5-phosphate (DR5P) (1.9 mM) substituted for F16BP. Specific activity was measured in triplicate. One unit of enzyme activity was defined as the amount of TgALD1/TgDPA required to convert 1 μ M of F16BP or DR5P per minute at 298 K at pH 7.4.

Co-sedimentation assay

Rabbit muscle actin (>99% pure from Cytoskeleton Inc) shares 83% sequence identity with *T. gondii* actin and is a well-established substitute in co-sedimentation experiments [39]. Rabbit skeletal muscle G-actin was made up to a concentration of 10 μ M in 1X G-Buffer [5 mM Tris (pH 8.0), 0.2 mM CaCl₂, 0.5 mM DTT, 0.2 mM ATP]. To remove aggregated protein, G-actin was incubated on ice for 45 min, followed by centrifuging at 100,000 x g for 1 hour in a Beckman Optima TL ultracentrifuge using a TL-100 rotor. 1/10 total volume 10X F-Buffer (500 mM KCl, 20 mM MgCl₂ and 10 mM ATP) was added to the supernatant and the reaction mixture incubated at 298 K to produce the polymerized F-actin. Purified His-tagged TgDPA was added to F-actin in a 1:10 molar ratio, incubated for 1 hour at 298 K, and centrifuged at 100,000 x g for 1 hour at 298 K. The pellet was dissolved in sample buffer, resolved by 12% SDS PAGE, transferred to polyvinylidene fluoride (PVDF) membrane and TgDPA was detected by Western Blot analysis using anti His antibodies (Sigma).

RESULTS AND DISCUSSION

***TgALD1* is a canonical F16BP aldolase, while *TgDPA* is a highly divergent member of the apicomplexan dR5P aldolase family**

Apicomplexans generally express at least one F16BP aldolase; in *T. gondii*, TgALD1 belongs to the F16BP aldolase family, responsible for the reversible conversion of fructose 1,6-bisphosphate to D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate through a Schiff base intermediate (Fig. 1A, left) [40]. Additionally, the majority of apicomplexans harbor a single DERA [22], an enzyme that catalyzes the reversible transition of dR5P into D-glyceraldehyde 3-phosphate and acetaldehyde through a

Schiff base intermediate (Fig. 1A, right). An expansion of this family in *T. gondii* and the closely related apicomplexan *N. caninum* has resulted in the acquisition of a divergent dR5P aldolase-like protein identified as DPA [14]. To establish the evolutionary relationship between *TgALD1*, *TgDERA*, *TgDPA* and their respective enzyme families, we performed phylogenetic and sequence analyses.

Phylogenetic analysis of representative apicomplexan F16BP aldolases confirms the close clustering of these aldolases and mimics the established relationships between genera, with *T. gondii* aldolases closely related to other coccidian aldolases (*Neospora caninum*, *Eimeria tenella*) and more distantly related to the *Plasmodium* aldolases (Fig. 1B). Expectedly, the DERA/DPA sequences cluster in a distinct clade from the F16BP aldolases, and intriguingly, *T. gondii* and *N. caninum* DPA and DERA sequences showed only a distant connection, consistent with evolutionary divergence (Fig. 1B).

Sequence alignment of *TgALD1* with rabbit muscle aldolase (*Oryctolagus cuniculus* - *OcALDOA*) and *Plasmodium falciparum* aldolase (*PfALDO*) indicates that all of the active site residues, including the critical lysine involved in Schiff base formation, and substrate binding residues are completely conserved (Fig. 1C, top). Comparison of *TgDPA* and *TgDERA* sequences with the well characterized *E. coli* DERA (*EcDERA*) and the only structurally characterized apicomplexan DERA (*Plasmodium yoelii* DERA (*PyDERA*) [41]) reveals that although *TgDPA* maintains approximately the same level of sequence identity (~30%) with *EcDERA*, *PyDERA* and *TgDERA*, the residues implicated in substrate binding and the key catalytic residue are not well conserved (Fig. 1C, bottom). These observations suggest that, in contrast to other dR5P aldolase family members, *TgDPA* does not retain catalytic activity. The possibility exists, however, that in the spatial context of the active site additional residues could compensate for the lysine to glutamine substitution and rescue activity.

Both *TgALD1* and *TgDPA* adopt the TIM barrel fold

To further investigate the expanded repertoire of aldolases in *T. gondii*, we determined the high-resolution crystal structures of *TgALD1* and the divergent dR5P aldolase-like protein, *TgDPA*. The aldolase domains of *TgALD1* (Fig. 2A, top) and *TgDPA* (Fig. 2B, top) were expressed as soluble hexahistidine fusions in *E. coli*, and purified by Ni-affinity and size exclusion chromatography (SEC).

TgALD1 crystallized with one molecule in the asymmetric unit (AU) and adopts an α_8/β_8 TIM barrel fold classically associated with members of the aldolase family [42] (Fig. 2A, dark blue helices and magenta strands). The aldolase fold was confirmed by DALI analysis of the *TgALD1* structure showing its close similarity to the F16BP aldolases with the top scoring hit of *PfALDO* (Z-score of 58; PDB ID [1A5C](#)) [42]. Similar to other F16BP aldolases, *TgALD1* accessorizes the core TIM barrel with 2 ancillary β -strands (Fig. 2A, light pink), 4 ancillary helices (Fig. 2A, light blue), and the N-terminal helix, which closes off the back end of the beta-barrel (Fig. 2A, marine helix, yellow arrow).

TgDPA crystallized with two molecules in the AU, and the two chains can be overlaid with 0.29 Å root mean square deviation (r.m.s.d.) over 191 Ca. While *TgDPA* also adopts the α_8/β_8 TIM barrel fold (Fig. 2B), it differs from that of *TgALD1* by a fewer number of ancillary secondary structure elements, with just a single N-terminal helix bordering the TIM barrel (Fig. 2B, marine helix, yellow arrow). Despite a distant evolutionary relationship *TgDPA* and the DERAs are structurally homologous; a DALI analysis showed strongest similarity to *PyDERA* (Z-score of 35), and Z scores of over 25 relative to other structurally characterized DERA enzymes [42]. We also determined the structure of *TgDPA* in a different crystal form (data not shown but structure deposited with identifier PDB ID **3QYQ**), which revealed different conformations for the $\alpha 7$ -helix: partially disordered, bent, or elongated (Fig. 2B, bottom, black box). Whether these observations are functionally relevant or simply a consequence of crystal-packing interactions will require further examination. However, preliminary sequence and structural comparison of several dR5P aldolases (r.m.s.d. <2.0 Å and sequence homology as low as 27%) with *TgDPA* revealed a unique twelve residue insertion in *TgDPA* that facilitates the different lengths and organizations of the $\alpha 7$ -helix, suggesting a possible functional relevance for this expanded region.

Overall structural homology (Z-score of 13.6 [42]) is observed for *TgALD1* and *TgDPA* as they both adopt the α_8/β_8 TIM barrel fold, however the organization of these elements is notably different. For *TgALD1*, the central beta-strands tend to be longer than for *TgDPA*, and they are arranged in a more tapered fashion leading to the front of the *TgALD1* beta-barrel being more open than the back (Fig. 2A). In contrast, the *TgDPA* beta-barrel is more circular in cross-section and more cylindrical throughout its length (Fig. 2B). These architectural differences combined with variances in the distribution of the surrounding secondary structure elements highlight the potential for dissimilar functions for these two *T. gondii* aldolases.

Homomultimeric assemblies observed for both *TgALD1* and *TgDPA*

Previous studies have shown that F16BP aldolases exist predominantly as tetramers [43], while DERAs have been shown to adopt various assemblies from monomer to tetramer [44-46], with *PyDERA* (the only characterized apicomplexan DERA) likely existing as a dimer based on an assessment of the crystal packing using PISA [46]. To investigate the multimeric assemblies of *TgALD1* and *TgDPA*, we employed SEC, dynamic light scattering (DLS) and crystal packing analysis.

While *TgALD1* eluted as a dimer (74 kDa; expected monomer weight is 37 kDa) from a size-exclusion column (data not shown), analysis of the crystal packing using PISA clearly revealed a dimer-of-dimers assembly generated by crystallographic symmetry (Fig. 2C) [46]. Subunit interfaces result in an overall buried surface area of 6,432 Å² for the four interfaces stabilized by a total of 40 hydrogen bonds and salt bridges. Assessment of the individual interfaces reveals two distinct pairs, with one clearly more

stable than the other (Fig. 2C, solid vs dotted rectangles). Each primary dimer-forming interface buries $1855 \text{ \AA}^2$ and is stabilized by twelve hydrogen bonds/salt bridges while each secondary dimer-forming interface (referred to hereafter as a tetramer-forming interface) buries $1361 \text{ \AA}^2$ and is stabilized by eight hydrogen bonds/salt bridges. Consistent with these observations, the PISA complexation significance score (CSS, scale of 0.00 to 1.00, with 1.00 indicating a critical role in complex formation) was determined to be 1.00 for each dimer-forming interface and 0.48 for each tetramer-forming interface. In support of the crystal packing analysis, a tetramer was observed by DLS (hydrodynamic radius (R_H) of $5.24 \pm 0.27 \text{ nm}$ corresponding to a globular molecular weight of $153 \pm 3 \text{ kDa}$ (Fig. 2E)). Altogether, analysis of the inter-molecular interfaces in the crystal is consistent with the strong dimer component observed by SEC and the stable tetramer observed by DLS.

Size exclusion chromatography, DLS, and crystal packing analysis consistently showed *TgDPA* to be a dimer with a DLS measured R_H of $3.24 \pm 0.08 \text{ nm}$ corresponding to a molecular weight of $58 \pm 6 \text{ kDa}$ (expected monomer weight is 31 kDa), and buried surface area of $2,386 \text{ \AA}^2$ corresponding to a PISA CSS of 1.00 (Fig. 2D and E, and data not shown). Interestingly, the dimer interface of *TgDPA* forms around the flipped out N-terminal helix (Fig. 2B), similar to the dimerization interface predicted for *PyDERA* [46], but significantly different than the dimer and tetramer-forming interfaces observed for other dR5P aldolases [44, 47, 48]. Additionally, no salt bridges and only one hydrogen bond were present at the interface consistent with a largely hydrophobic-based assembly.

Homomultimeric assembly has been shown to play important roles in aldolase stability and function. For example, homotetramer formation is critical for thermal stability of F16BP and dR5P aldolases, but does not seem to be important for catalytic activity [44, 49]. In addition, the homotetrameric assembly of F16BP aldolases is suspected to be essential for their ability to crosslink F-actin [12]. While the defined role of homomeric assembly of the aldolases in *T. gondii* has not been investigated, the tetrameric assembly of F16BP and dimeric assembly of DPA are likely to be important for stability and possibly for generating composite interfaces to support assembly of macromolecular complexes.

Aldolase activity measured for *TgALD1*, but not for *TgDPA*

To explore the molecular basis for the function of these *T. gondii* aldolases, we first compared the structures of *TgALD1* and *TgDPA* with members of their respective enzyme families and complemented these analyses with biochemical assays for enzymatic activity.

Structural superposition of *TgALD1* and *OcALDOA* in a Schiff base intermediate with F16BP showed an r.m.s.d. of $0.46 \text{ \AA}$ over 287 Ca atoms (Fig. 3A), indicating strong conservation of the core protein fold between these distantly related F16BP aldolases. The largely positive electrostatic surface observed in *OcALDOA* and required for F16BP binding is conserved in *TgALD1*, and sulfate ions from the *TgALD1*

crystallization solution structurally mimic the phosphate groups of F16BP (Fig. 3A, top two insets). Close inspection of the side chains involved in substrate binding and catalytic activity revealed clear spatial conservation, particularly for the Schiff base forming lysine residue (Fig. 3A, bottom left inset) consistent with previous studies showing retention of F16BP aldolase enzyme function by *Tg*ALD1 [18]. Using an established *in vitro* assay for aldolase activity [38], glycolytic activity was confirmed for *Tg*ALD1 with F16BP as a substrate resulting in a specific activity of 10 +/- 0.33 U/mg of protein (Fig. 3A, bottom right).

Structural overlays of *Tg*DPA with *Ec*DERA [45, 50] and a high confidence homology model of *Tg*DERA generated using Modeller [51] in Chimera [52] with a *Py*DERA template (PDB ID **2A4A**), allowed for a comparison of the substrate binding pocket and catalytic residues (Fig. 3B). Viewing the coordination of a dR5P reaction intermediate (carbinolamine intermediate; step just prior to generation of the Schiff base intermediate) in complex with *Ec*DERA reveals binding in a deep, electropositive tunnel (Fig. 3B, top left inset). While the model of *Tg*DERA shows a tunnel with similar shape and charge (Fig. 3B, top middle inset), the structure of *Tg*DPA reveals a hydrophobic tunnel nearly completely blocked by an electronegative surface (Fig. 3B, top right inset). These observations suggest that while *Tg*DERA is likely able to coordinate the dR5P substrate, *Tg*DPA is not. Furthermore, as predicted by the sequence analysis (Fig. 1B), the catalytic lysine, Lys¹⁶⁷, in *Ec*DERA is retained as Lys¹⁷⁶ in *Tg*DERA, but substituted by a glutamine, Gln¹⁷³, in *Tg*DPA (Fig. 3B, bottom insets), and no appropriately positioned compensatory lysine residues are present in *Tg*DPA. Together, these analyses support the previously established activity of *Ec*DERA [45] and suggest that *Tg*DERA is likely a functional dR5P aldolase while indicating that *Tg*DPA has diverged to the point of losing dR5P enzymatic activity. Indeed, no activity was observed for *Tg*DPA using dR5P as a substrate (Fig. 3B, bottom right), consistent with a previous study showing inactivity of a GST-fused *Tg*DPA construct [14]. Given the inability of *Tg*DPA to cleave its expected substrate, and the complete restructuring of the “active site,” *Tg*DPA may be a pseudoaldolase – defined here as a protein that adopts the TIM barrel aldolase fold but lacks the Schiff-base forming lysine residue required for catalytic activity. As a pseudoaldolase, *Tg*DPA has likely repurposed the aldolase fold for a function that does not involve intrinsic catalytic activity, such as serving as an allosteric regulator to coordinate/activate additional biomolecules. Indeed, the protozoan kinetoplastids have been shown to harbor catalytically dead proteins that play important biological roles by forming heteromultimeric complexes with homologous enzymes to enhance the activity of the catalytically competent homolog [53, 54].

A putative role for *TgDPA* in regulating actin dynamics

Transcriptomic and proteomic analyses have previously shown that *TgALD1* and *TgDERA* are expressed across all lifecycle stages, while *TgDPA* expression is strongly associated with the bradyzoite stage [14, 15, 55]; these data are consistent with *TgALD1* and *TgDERA* having glycolytic functions that are required for the metabolic pathways of all parasite lifecycle stages, and *TgDPA* having a currently unknown bradyzoite specific function. While a role in allosteric regulation of biologically important enzymes is possible for *TgDPA*, a previous study suggested that *TgDPA* is involved in regulating actin dynamics based on its interaction with actin depolymerizing factor (*TgADF*) [21]. The presence of *TgDPA* almost exclusively in the bradyzoites could be required to enhance the activity of *TgADF*, which is constitutively expressed throughout *T. gondii*'s lifecycle stages, at least at the mRNA level [15], and may offer strategic advantages to the quiescent bradyzoite. This preliminary finding of *TgDPA* indirectly affecting actin assembly and disassembly, combined with the fact that other TIM barrel enzymes (the F16BP aldolases) are well known to directly bind actin, led us to investigate the possibility of a direct interaction between actin and *TgDPA*. Using an established co-sedimentation assay, we showed that *TgDPA* co-sedimented with F-actin (Fig. 4A), though the biological significance of this interaction will require further investigation. While both *TgDPA* and *TgALD1* bind actin [18], they are unlikely to coordinate this molecular partner in the same way; despite the core similarity of the TIM barrel fold, the restructured active site of *TgDPA* implies that any molecular interaction events observed for F16BP aldolases overlapping with the concentrated basic patch in the substrate binding region, such as actin binding, are unlikely to be spatially conserved (Fig. 3). It is likely that *TgDPA* has functionalized a different region of its surface to engage molecular partners such as actin and ADF.

A side view of the *TgDPA* dimer shows a deep groove between the two subunits (Fig. 4B), and the fact that the extended $\alpha 7$ helix and unique $\alpha 7$ - $\beta 6$ loop of *TgDPA* are located on the inner side of the cap-shaped dimer (Fig. 4A) implicates this region in a *TgDPA*-specific function. Moreover, the flexibility of this region (Fig. 2B, bottom) suggests that *TgDPA* may use a partial induced fit mechanism for ligand binding. Our structural studies provide a possible mechanism for *TgDPA* stabilization of *TgADF*: the solution structure of *TgADF* overlayed on a rabbit ADF-actin co-structure shows a region of *TgADF* exposed for potential interaction with another binding partner (Fig. 4C) [56, 57], and the *TgDPA* dimer could cap the surface of *TgADF* not involved in complex formation with actin. Importantly, while the interplay between *TgDPA*, *TgADF*, and actin has not been definitively shown, it is tempting to speculate based on our structural analysis that *TgDPA* binding to a molecular partner, such as *TgADF* or actin, is mediated by the base of its cap-like dimeric assembly.

CONCLUSIONS

Nearly all apicomplexan parasites harbour a gene for one or both of F16BP aldolase and DERA, but in *T. gondii* and very closely related coccidian parasites the DERA family has undergone an expansion to include a divergent dR5P aldolase-like protein (DPA). Our structural characterization of *TgALD1* and *TgDPA* confirms architectural conservation of the two aldolases, with both adopting the TIM barrel fold. Comparison of these high-resolution structures with other aldolases and with a homology model of *TgDERA*, combined with biochemical assays, reveals a canonical *TgALD1* active on F16BP and a divergent *TgDPA* inactive on dR5P, likely enabled by the presence of the canonical *TgDERA*. Beyond the primary catalytic functions of *TgALD1* and *TgDERA*, there are many questions that remain to be answered about the biological roles of *T. gondii*'s panel of aldolases. For example, does *TgALD1* retain some of the moonlighting roles identified for related F16BP aldolases with respect to participating in macromolecular assemblies? While it has recently been established that *TgALD1* does not directly bridge the cytoplasmic tails of transmembrane adhesins with F-actin during parasite invasion of the host cell [20], our structure reveals strong conservation of the binding region that overlaps with the substrate binding site; taking into consideration the plasticity of this region and its demonstrated ability to bind a variety of ligands in other systems (e.x. SNX9 and WASP [58, 59]), does *TgALD1* play a role in protein coordination? Furthermore, a recent study revealed an interaction between human F16BP aldolase and the Pleckstrin Homology (PH) domain of the multimodular protein ARNO [60]; PH domain proteins have recently been characterized in *T. gondii* [61] and several putative PH-domain containing proteins are annotated in the *T. gondii* genome, leading to the question, "Is there a *TgALD1*-PH domain moonlighting interaction important for *T. gondii* biology?" While the specific role of *TgDPA* remains enigmatic, it's potential to engage molecular partners such as *TgADF* and actin may provide a mechanism to regulate actin dynamics in the *T. gondii* bradyzoite stage. Further investigation incorporating genetic approaches will likely be required to fully elucidate the function of *TgDPA* and establish its role in *T. gondii* biology.

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FIGURE LEGENDS

Figure 1. Phylogenetic and sequence analysis of F16BP and dR5P aldolases reveals canonical F16BP and d5RP aldolases in *T. gondii*, in addition to a divergent dR5P-like aldolase. (A) Left: reaction sequence for F16BP aldolases. Right: reaction sequence for dR5P aldolases. (B) Phylogenetic tree of representative sample of apicomplexan F16BP aldolase ortholog group and for the complete ortholog group of apicomplexan DERAs and DPAs based on the sequences in OrthoMCL DB [22]. (C) Top: sequences for rabbit muscle aldolase (*OcALDOA*; **NP_001075707.1**), *Pf*Aldolase (*PfALDO*; **PF14_0425**), and *TgALD1* (**TGME49_236040**) were aligned in Clustal Omega [24], and the residues involved in substrate binding, lining the active site (underlined), and catalysis (bold blue) were extracted. Bottom: sequences for *EcDERA* (**NP_757311.1**), *PyDERA* (**PY02252**), *TgDERA* (**TGME49_270650**), and *TgDPA* (**TGME49_318750**) were aligned and extracted in a similar fashion.

Figure 2. Tertiary and quaternary structures of *TgALD1* and *TgDPA* reveal different ancillary elements and oligomeric states (A) Top: recombinant *TgALD1* construct (residues Pro⁷⁷ to Gly⁴¹⁹). Top view (middle) and side view (bottom) of the tertiary structure of the *TgALD1* monomer, colored grey (coil), dark blue (helix within the TIM barrel core), marine (ancillary helix), light blue (N-terminal helix), magenta (strand within the TIM barrel core), light pink (ancillary strand). Yellow arrow indicates positioning of the N-terminal helix. (B) Top: recombinant *TgDPA* construct (residues Ile⁶ to Pro²⁸⁶). Middle and bottom show tertiary structure of the *TgDPA* monomer as in (A). Bottom inset: overlay of the different orientations observed for *TgDPA* helix 7 between different crystal forms: *P1* (PDB ID **3QYQ**) and *P2₁2₁2₁* (PDB ID **4EIV**). (C) Surface representation of *TgALD1* tetramer generated around crystallographic symmetry axes, colored by chain. Black box, tight dimer forming interface; dotted box, unique portion of the tetramer forming interface. CSS score for each interface is indicated. (D) Surface representation of *TgDPA* dimer colored by chain. Black box, dimer forming interface. (E) DLS measurements of *TgALD1* and *TgDPA* samples at 10 μ M were performed using a DynaPro–TC–04 dynamic light scattering instrument (Wyatt Technology, Santa Barbara, CA). Data were analyzed in Dynamics 7.1 software to determine the mean hydrodynamic radius (R_H) and molecular weight (MW) using a globular polymer model. Values are an average of triplicate measurements with standard deviation.

Figure 3. Comparison of *TgALD1* and *TgDPA* active site. (A) Left - superimposition of *TgALD1* (marine) with rabbit muscle aldolase, *OcALDOA* (grey), bound to F16BP in a Schiff base intermediate (PDB ID **1ZAI**); F16BP is shown in element-coded green ball-and-stick. Inset: top left, electrostatic

surface of the binding site of F16BP in *OcALDOA*; top right, electrostatic surface of the predicted substrate binding site of *TgALD1* with coordinated sulfate ions shown as ball-and-stick; bottom left, zoom-in of the overlay of the Lys residue involved in Schiff base formation. Right – assay for enzymatic activity of *TgALD1* on F16BP: activity was spectrophotometrically assayed in the presence of TPI and GAPDH, with NADH oxidation measured as a function of decreasing absorption per minute at 340 nm. (B) Left - superimposition of *TgDPA* (orange) with a high confidence homology model of *TgDERA*, and with *EcDERA* (grey) bound to 1-hydroxy-pentane-3,4-diol-5-phosphate (HDP) (PDB ID [1JCL](#)); HDP is shown in green ball-and-stick. Inset: top, electrostatic surfaces of *EcDERA* (left), *TgDERA* (middle) and *TgDPA* (right), indicating the positively charged binding tunnel of *EcDERA* and *TgDERA* contrasted with the acidic surface of *TgDPA*; bottom, orthogonal view of the amino acid involved in Schiff base formation (note the substitution of Lys for Gln in *TgDPA*). Right – assay for enzymatic activity of *TgDPA* on dR5P as described in (A).

Figure 4. Possible mechanism for divergent *TgDPA* role in coccidian bradyzoites. (A) Western blot analysis of *TgALD1* and *TgDPA* cosedimentation with monomeric (G) or polymerized (F) actin. Supernatant (S) and Pellet (P) fractions were probed following ultracentrifugation; black arrow indicates proportion of *TgDPA* pulled down by F-actin. (B) Side view of the *TgDPA* dimer, with one chain shown as surface representation and one chain shown with cartoon and semi-transparent surface for orientation. Inner surface of the dimer cap is delineated by the thick black line; yellow arrow indicates $\alpha 7$ helix with unique properties in *TgDPA*. (C) Overlay of *TgADF* solution structure (red cartoon; PDB ID [2L72](#)) on complex of *OcADF* (dark grey cartoon) with *OcActin* (light grey surface) (PDB ID [3DAW](#)).

Tables

Table 1. Data collection and refinement statistics

	<i>Tg</i> ALD1	<i>Tg</i> DPA
<u>A. Data collection</u>		
Synchrotron source	SSRL 9-2	APS 21-ID-G
Space group	<i>P</i> 4 ₂ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.81, 109.81, 54.36	71.98, 73.28, 96.38
$\alpha = \beta = \gamma$ (°)	90.00	90.00
Wavelength (Å)	0.9795	0.97856
Temperature (K)	100	100
Resolution range (Å)	50.00–1.75 (1.78–1.75)	29.42–1.37 (1.39–1.37)
Measured reflections	458,250 (22,029)	107,282 (5,346)
Unique reflections	33,917 (1,644)	16,012 (798)
Redundancy	13.5 (13.4)	6.7 (6.7)
Completeness (%)	99.4 (100.0)	99.4 (100.0)
<i>I</i> / σ (<i>I</i>)	44.0 (4.0)	28.0 (2.9)
<i>R</i> _{merge} ^a (%)	4.8 (50.4)	4.7 (60.2)
<u>B. Refinement Statistics</u>		
Resolution (Å)	36.44–1.75 (1.80–1.75)	29.4–1.37(1.41–1.37)
<i>R</i> _{cryst} ^b / <i>R</i> _{free} ^c (%)	19.0(23.8)/23.1(26.8)	15.1(20.8)/18.5(23.7)
No. of atoms		
Overall	2,784	4,587
Protein	2,575	3,883
Solvent/Heterogen atoms	185/24	688/16
Mean temperature factor (Å ²)		
Overall	31.9	34.4
Protein	31.0	31.0
Solvent/Heterogen atoms	35.3/47.2	52.9/70.6
r.m.s. deviation from ideality		
Bond lengths (Å)	0.011	0.010
Bond angles (°)	1.36	1.43
Ramachandran statistics ^d		
Most favoured (%)	93.4	95.0
Allowed (%)	6.6	5.0
Generously allowed (%)	0.0	0.0
Disallowed (%)	0.0	0.0
Values in parentheses are for the highest resolution shell		
^a <i>R</i> _{merge} = $\sum_{hkl} \sum_i I_{hkl,i} - [I_{hkl}] / \sum_{hkl} \sum_i I_{hkl,i}$, where $[I_{hkl}]$ is the average of symmetry related observations of a unique reflection		
^b <i>R</i> _{cryst} = $\sum F_{obs} - F_{calc} / \sum F_{obs}$, where <i>F</i> _{obs} and <i>F</i> _{calc} are the observed and the calculated structure factors, respectively.		
^c <i>R</i> _{free} is <i>R</i> using 5% of reflections randomly chosen and omitted from refinement		
^d Ramachandran statistics were determined using PROCHECK		

Figure 1

A Fructose 1,6-bisphosphate aldolase 2-deoxyribose 5-phosphate aldolase

B

C

<i>Oc</i> ALDOA	D ³⁴	KR ⁴³	K ¹⁰⁸	K ¹⁴⁷	R ¹⁴⁹	E ¹⁸⁸	E ¹⁹⁰	K ²³⁰	LSG ²⁷³	GR ³⁰⁴
<i>Pf</i> ALDO	D ⁴⁰	KR ⁴⁹	K ¹¹³	K ¹⁵²	R ¹⁵⁴	E ¹⁹⁵	E ¹⁹⁷	K ²³⁷	LSG ²⁸⁰	GR ³¹⁰
<i>Tg</i> ALD1	D ¹⁰⁴	KR ¹¹³	K ¹⁷⁷	K ²¹⁶	R ²¹⁸	E ²⁵⁹	E ²⁶¹	K ³⁰¹	LSG ³⁴⁴	GR ³⁷⁴
<i>Ec</i> DERA	D ¹¹⁰	K ¹⁷⁵	GK ¹⁸⁰	K ²⁰⁹	GGVR ²¹⁵	G ²⁴⁴	SS ²⁴⁷			
<i>Py</i> DERA	D ¹⁰⁵	K ¹⁷¹	GK ¹⁷⁶	K ²¹⁰	GGIS ²¹⁶	G ²⁴⁵	SS ²⁴⁸			
<i>Tg</i> DERA	D ¹⁰⁸	K ¹⁷⁶	GK ¹⁸¹	K ²¹⁴	GGVK ²²⁰	G ²⁴⁹	SS ²⁵²			
<i>Tg</i> DPA	E ¹⁰⁷	Q ¹⁷³	GL ¹⁷⁸	K ²²⁴	VGDV ²³⁰	G ²⁵⁹	GF ²⁶²			

Figure 2

Figure 3

Figure 4

Graphical Abstract