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The glycosomal alkyl-dihydroxyacetonephosphate synthase *TbADS* is essential for the synthesis of ether glycerophospholipids in procyclic trypanosomes

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Abstract

Glycerophospholipids are the main constituents of the biological membranes in *Trypanosoma brucei*, which causes sleeping sickness in humans. The present work reports the characterization of the alkyl-dihydroxyacetonephosphate synthase *TbADS* that catalyzes the committed step in ether glycerophospholipid biosynthesis. *TbADS* localizes to the glycosomal lumen. *TbADS* complemented a null mutant of *Leishmania major* lacking alkyl-dihydroxyacetonephosphate synthase activity and restored the formation of normal form of the ether lipid based virulence factor lipophosphoglycan. Despite lacking alkyl-dihydroxyacetonephosphate synthase activity, a null mutant of *TbADS* in procyclic trypanosomes remained viable and exhibited normal growth. Comprehensive analysis of cellular glycerophospholipids showed that *TbADS* was involved in the biosynthesis of all ether glycerophospholipid species, primarily found in the PE and PC classes.

Graphical abstract

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Keywords

Trypanosoma brucei; Null mutant; Alkyl-dihydroxyacetonephosphate synthase; Ether glycerophospholipids; Glycosome

1. Introduction

Trypanosoma brucei is a flagellated protozoan parasite of the Kinetoplastida class that causes the diseases sleeping sickness in humans and nagana in cattle in Africa. The life cycle of this unicellular microbe comprises an insect stage, where it develops in the digestive system and salivary glands of the tsetse fly vector followed by a vertebrate host stage, where it multiplies in the bloodstream of an infected mammal.

Glycerophospholipids are the most abundant lipids in *T. brucei*, representing 80% of total cellular lipids (Smith and Butikofer, Patnaik, Field et al. 1993, Smith and Butikofer 2010). The five main classes are phosphatidylcholine (PC, 45-60%), phosphatidylethanolamine (PE, 10-20%), phosphatidylinositol (PI, 6-12%), phosphatidylserine (PS, <4%), and cardiolipin (<3% reviewed in (Smith and Butikofer 2010, Serricchio and Butikofer 2011, Farine and Butikofer 2013, Ramakrishnan, Serricchio et al. 2013). While most of the glycerophospholipids are ester glycerophospholipids that bear a fatty acid at position one of the glycerol backbone, a significant proportion of glycerophospholipids (mainly PE and PC species) in this parasite carry an ether linked aliphatic fatty alcohol (ether glycerophospholipids) instead (Patnaik, Field et al. 1993, Richmond, Gibellini et al. 2010) reviewed in (Smith and Butikofer 2010)). Beside their structural function as the main constituent of the biological membrane, glycerophospholipids are also involved in various essential biological processes. As second messengers of signaling pathways, they regulate key processes such as membrane trafficking, cell cycle progression, and dynamic of organelles such as mitochondria and endoplasmic reticulum (Coppolino, Dierckman et al. 2002, Krauss, Kukhtina et al. 2006, Santarius, Lee et al. 2006, Gibellini, Hunter et al. 2009, Signorell, Gluenz et al. 2009, Zhang, Wilson et al. 2009, Serricchio and Butikofer 2011, Farine and Butikofer 2013, Ramakrishnan, Serricchio et al. 2013). Glycerophospholipid-based molecules have also been shown to be implicated in virulence in *Leishmania* and *T.*

brucei (Spath, Epstein et al. 2000, Turco, Spath et al. 2001, Spath, Garraway et al. 2003, Zufferey, Allen et al. 2003, Ponte-Sucre 2016).

Ether glycerophospholipid biosynthesis initiates with the acylation of DHAP by a dihydroxyacetonephosphate (DHAP) acyltransferase (DHAPAT) to yield acyl-DHAP. The latter is then converted to 1-alkyl-DHAP by an alkyl-DHAP synthase (ADS), which removes the acyl group and replaces it with a fatty alcohol, thus introducing the ether linkage. The alkyl-DHAP synthase catalyzes the committed step as its product, 1-alkyl-DHAP, serves as the obligate precursor for the production of all ether glycerophospholipids. Then, an NADPH dependent alkyl/acyl-DHAP reductase converts 1-alkyl-DHAP to 1-alkyl-glycerol-3-phosphate (1-alkyl-G3P). The DHAPAT enzyme can also contribute to the production of ester glycerophospholipids when its product, 1-acyl-DHAP, is directly reduced to 1-acyl-G3P by the acyl/alkyl-DHAP reductase (Jones and Hajra 1983, Liu, Nagan et al. 2005). All three enzymes are associated with the peroxisomes. In *T. brucei*, DHAPAT activity is mediated by two enzymes, *TbDAT* and to a lesser extent, *TbGAT* (Fig. 1 Patel, Pirani et al. 2016, Zufferey, Pirani et al. 2017). *TbDAT* and *TbGAT* exhibit slightly different specificities regarding the fatty acyl-CoA donor and localize in different subcellular compartments *TbGAT* resides in the endoplasmic reticulum, while *TbDAT* localizes to peroxisome-related organelles, called glycosomes in protozoan parasites of the family Trypanosomatidae (Oppendoes 1984, Zomer, Oppendoes et al. 1995, Heise and Oppendoes 1997, Vertommen, Van Roy et al. 2008, Patel, Pirani et al. 2016). Both enzymes are dispensable for normal growth, but only *TbDAT* is important for ether glycerophospholipid biogenesis.

Despite their lower abundance compared to that of ester glycerophospholipids, ether glycerophospholipids fulfill essential cellular functions (reviewed in (Gorgas, Teigler et al. 2006)). In mammals, they play an important role in membrane trafficking, in the release and composition of exosomes, and in angiogenesis for retina development (Thai, Rodemer et al. 2001, Saab, Buteau et al. 2014, Phuyal, Skotland et al. 2015). In the neuronal system, they regulate exocytosis and efflux of neurotransmitters in synapses, and play an important role in myelination (Teigler, Komljenovic et al. 2009, Brodde, Teigler et al. 2012). Furthermore, ether glycerophospholipids are essential for spermatogenesis and development of the optic nerve (Rodemer, Thai et al. 2003). In *T. brucei*, ether PE have been shown to be important for the integrity of the inner membrane of the mitochondria and completion of cytokinesis/cell division (Signorell, Rauch et al. 2008, Signorell, Gluenz et al. 2009, Farine and Butikofer 2013).

ADS enzymes from several diverse living organisms have been characterized genetically and biochemically (reviewed in (van den Bosch and de Vet 1997)). ADS is a luminal, surface peroxisomal membrane associated enzyme that utilizes a FAD cofactor to exchange the acyl with an alkyl group (Hardeman and van den Bosch 1989, Zomer, de Weerd et al. 1993, Zomer, Oppendoes et al. 1995, de Vet, Biermann et al. 1997, de Vet, Hilkes et al. 2000, Razeto, Mattioli et al. 2007, Nenci, Piano et al. 2012). In higher eukaryotes, lack of ADS leads to cataract resulting from defective lens fiber cells, and male sterility due to impaired sperm biogenesis (Liegel, Chang et al. 2011, Liegel, Ronchetti et al. 2014). Human mutations in the *ADS* gene are responsible for a pathological condition called rhizomelic

chondrodysplasia punctata form 3, which is characterized by skeletal dysplasia and mental retardation (Itzkovitz, Jiralerspong et al. 2012, Noguchi, Honsho et al. 2014). Increased ADS activity, and consequently higher levels of ether glycerophospholipids levels, has been associated with cancer conversely, ablation or depletion of *ADS* resulted in reduced cell survival and proliferation, and decreased cancer aggressiveness and tumor growth (Lee, Fitzgerald et al. 1980, Benjamin, Cozzo et al. 2013, Zhu, Liu et al. 2014, Zhu, Zhu et al. 2014). The ADS enzyme is also important for virulence in the protozoan parasite *Leishmania major*, as the ether glycerophospholipid based virulence factor lipophosphoglycan was impaired in a mutant strain lacking this enzyme (Zufferey, Allen et al. 2003). In *T. brucei*, the alkyl-DHAP synthase activity was characterized and its gene was cloned (Zomer, Oppendoes et al. 1995, Zomer, Michels et al. 1999). However, its role in parasite's biology was not investigated yet. The present work focuses on the biochemical characterization and role of the ether glycerophospholipid committed enzyme *TbADS* in growth and lipid metabolism of procyclic trypanosomes.

2. Material and methods

2.1. Strains and growth conditions

The wild-type strain of procyclic forms of *T. brucei* used in this work is stock 427-60 and was cultivated in SDM-79 medium supplemented with 10 % heat inactivated fetal bovine serum as described in (Brun and Schonenberger 1979). Growth, transformation, and limiting dilution of parasites were carried out as previously described (Patel, Pirani et al. 2016). Transgenic lines were selected in the presence of 5 µg/ml blasticidin, 2 µg/ml puromycin, and 3 µg/ml phleomycin as appropriate. Growth curves were performed by seeding the culture at a cell density of 1×10^6 cells/ml. Parasites were counted with a hemocytometer as a function of time.

Promastigotes of *L. major* Friedlin V1 strain (MHOM/IL/80/Friedlin) were propagated in liquid M199-based medium (Zufferey, Allen et al. 2003). The null mutant *Imads/ Imads* and complemented line *Imads/ Imads/LmADS* were described previously (Zufferey, Allen et al. 2003). Transfection was performed according to Ngo and colleagues, and selection was accomplished in the presence of 50 µg/ml puromycin (Ngo, Tschudi et al. 1998).

2.2. Plasmids

pXGPAC.TbADS (Ec885) for complementation of *Leishmania* null mutant *Imads/ Imads* was constructed by PCR-amplifying the *TbADS* gene with the oligonucleotides 507 (5' - GGATCCATGGATAAGAGAATGATTACTGATGC-3') and 508 (5' - GGATCCTCAGAGGTGAGCCTGAAGAG-3') and *T. brucei* genomic DNA as template. The resulting product was digested with BamHI and ligated in sense orientation into the BamHI site of pXGPAC (Ha, Schwarz et al. 1996) to give pXGPAC.TbADS.

To generate the plasmids for inactivation of the *TbADS* alleles, the following plasmids pUC.TbADS:BSD (Ec867) and pUC.TbADS:PAC (Ec875) were created. Two antibiotic resistance cassettes, *BSD* and *PAC*, were flanked by approximately 200 bp of *TbADS* untranslated regions (UTR) to promote double crossing over by homologous recombination.

First pUC.TbADS53U (Ec865) was created by PCR amplification of the 5' UTR and 3' UTR with primers 509 (5'-AGCTTCCATCCCTGATCCTCTGTTG-3') and 510 (5'-TCTAGATTGTTGTTTTGATTTTAAATTGCGGTAACGG-3'), and 511 (5'-TCTAGAGGGGTCATATGTATTTATGCACAC-3') and 512 (5'-AAGCTTCCCGTTGTCGGTTGCATGC-3'), respectively. The amplified DNA pieces were digested with XbaI and HindIII, and triple ligated into the HindIII sites of pUC19 to give pUC.TbADS53U. The *BSD* and *PAC* cassettes were then inserted in sense orientation into the XbaI site of pUC.TbADS53U to yield pUC.TbADS:BSD and pUC.TbADS:HYG, respectively.

For complementation of the *T. brucei* null mutant, *TbADS* was inserted in sense orientation into the BamHI site of the plasmid pHD309PUR/BLEO.V5 to give pHD309BLEO.V5:TbADS (Ec871 (Patel, Pirani et al. 2016)). The *V5* encoding DNA was then deleted by cutting with HindIII and BamHI (partial) to create pHD309BLEO.TbADS (Ec884). All PCR-amplified DNAs were verified by sequencing.

2.3. Creation of the null mutant *tbads/ tbads* and complemented line *tbads/ tbads/ TbADS* and *tbads/ tbads/V5:TbADS*

The null mutant of *TbADS* was created by transformation of the *TbADS:PAC* cassette that was amplified by PCR with primers 507 and 512 using pUC.TbADS:PAC as a template. The PCR product was used directly for transformation of wild-type procyclic parasites followed by selection in the presence of puromycin. The resistant, heterozygous clones were verified by PCR and then subjected to a second round of transformation with the *TbADS:BSD* cassette, which was also PCR-amplified with primers 507 and 512. Parasites resistant to both puromycin and blasticidin were selected, and their genomic *TbADS* locus analyzed by PCR. The complemented line was generated by transformation of the null mutant *tbads/ tbads* with NotI linearized plasmids pHD309BLEO.TbADS or pHD309BLEO.V5:TbADS to give *tbads/ tbads/TbADS* and *tbads/ tbads/V5:TbADS*, respectively. Expression of *TbADS* and *V5:TbADS* was maintained in the presence of 1 µg/ml phleomycin. The proper chromosomal integrations were verified by PCR (Fig. 4C). The presence of the *TbADS* gene was confirmed with primer pair 507 (5'-GGATCCATGGATAAGAGAATGATTACTGATGC-3') and 508 (5'-GGATCCTCAGAGGTGAGCCTGAAGAG-3'). Oligonucleotides 514 (5'-TCTCTGTTTGGGTGCGTTATG-3') and 54 (5'-GCTCTAGATTAGCCCTCCACACATAAC-3'), and 53 (5'-GCTCTAGATGCCTTTGTCTCAAGAAGAATC-3') and 515 (5'-CCGAGGAAACCTCCTGTAAAT-3') were used for the verification of the *BSD* cassette integration, while primers 514 and 369 (5'-TCTAGATCAGGCACCGGGCTTGCG-3'), and 367 (5'-TCTAGATGACCGAGTACAAGCCCACG-3') and 515 were applied for the confirmation of the *PAC* marker integration. Oligonucleotides 484 (5'-TGATCGACTCTGTGCTCGATGTGT3'), 525 (5'-GTCGATCATACCACGCCTTAC-3'), 461 (5'-TGGTTTGTGTTGCCGGATCAAGAGC-3'), and 485 (5'-GCACGTTTCAGCATCTGCTCATCAA-3') were used to confirm the proper integration of the plasmids pHD309BLEO.TbADS while primers 484, 44 (5'-

ACCGAGGAGAGGGTTAGGGAT-3'), 461, and 485 were applied to confirm the insertion of the episome pHD309 BLEO.V5:TbADS into the tubulin locus.

2.4. Enzymatic assays

Leishmania and *Trypanosoma* whole cell extracts were prepared as described previously (Zufferey and Ben Mamoun 2006). Protein concentration was determined by the bicinchoninic acid assay using bovine serum albumin as a standard.

The alkyl-DHAP synthase assay was performed with whole cell extracts similarly as described in (Heise and Opperdoes 1997) in the presence of [¹⁴C]octadecanol (American Radiolabeled Chemicals, Inc., St Louis specific activity of 50-60 mCi/mmol). Briefly, 0.75 mg of proteins were incubated in 0.5 ml containing 50 mM TrisHCl pH 7.5, 150 μM palmitoyl-CoA, 1 mM dithiothreitol, 4 mM NaF, 4 mM MgCl₂, 0.5 mM DHAP, 1 mg/ml fatty acid free BSA, 0.1% CHAPS at 30°C. After 15 min, 25 μM of radioactive [¹⁴C]hexadecanol were added and the assay was incubated for 1 hr. The reaction was stopped by addition of 30 ul of 6 M HCl and extracted with 1.2 ml of chloroform:methanol (1:1, v/v). The organic phase was air dried, spotted onto Silica gel 60 plates, and resolved in a solvent made of chloroform, methanol, acetic acid, 5% sodium bisulfite (100:40:12:4, v/v/v/v). The thin layer chromatography plate was scanned with a Typhoon 950, and the radioactivity in bands with an *RF* of 3.5 was quantified by scintillation counting. The enzymatic assays were carried out at least twice in duplicate.

2.5. Proteinase K assay and electrophoresis

Proteinase K assay was carried out by incubating 5×10⁶ cells in a volume of 100 μl with 50 μg/ml proteinase K, 12 μg/ml digitonin, 5 mM PMSF, and 1% detergent as appropriate on ice for 30 min. The reaction was stopped by addition of 5 mM PMSF. Proteins were precipitated using 15% trichloroacetic acid and washed with 1% trichloroacetic acid in acetone before loading on a SDS-PAGE gel.

SDS-PAGE (12.5%/4%) followed by Western blot analyses in the presence of antibodies specific to BiP (Bangs, Uyetake et al. 1993), arginase (Hai, Kerkhoven et al. 2015), glycosomes (Parker, Hill et al. 1995), lipophosphoglycan (WIC79.3 (Tolson, Turco et al. 1989)) or to the V5 epitope (Thermo Fisher Scientific, Inc.) were carried out as previously described (Zufferey and Ben Mamoun 2006, Zufferey, Al-Ani et al. 2009).

2.6. Lipid isolation and mass spectrometry

Parasites were grown in quadruplicate cultures to mid log phase (1×10⁷/ml) and washed three times in cold PBS. Total cellular lipids were purified following a Folch-based protocol (Schneider and Daum 2006). Lipids were profiled by electrospray ionization tandem mass spectrometry (ESI-MS/MS) using the method described by (Zufferey, Al-Ani et al. 2009), except that internal standards were (with some small variation in amounts in different batches of internal standards): 0.6 nmol di12:0-PC, 0.6 nmol di24:1-PC, 0.6 nmol 13:0-lysoPC, 0.6 nmol 19:0-lysoPC, 0.3 nmol di12:0-PE, 0.3 nmol di23:0-PE, 0.3 nmol 14:0-lysoPE, 0.3 nmol 18:0-lysoPE, 0.3 nmol 14:0-lysophosphatidylglycerol (lysoPG), 0.3 nmol 18:0-lysoPG, 0.3 nmol di14:0-PA, 0.3 nmol di20:0 (phytanoyl)-PA, 0.2 nmol di14:0-PS, 0.2

nmol di20:0(phytanoyl)-PS, 0.23 nmol 16:0–18:0-PI, 0.3 nmol di14:0-PG, and 0.3 nmol di20:0(phytanoyl)-PG. In addition to the scans previously described (Zufferey, Al-Ani et al. 2009), a scan for PG as $[M+NH_4]^+$ in the positive mode with NL 189.0 was performed with collision energy of 20 V, declustering potential of 100 V, and exit potential of 14 V. Ether linked (alk(en)yl, acyl) lipids were quantified in comparison to the diacyl compounds with the same head groups without correction for response factors for these compounds as compared to their diacyl analogs.

2.7. Immunofluorescence assay

Immunofluorescence assay was carried out as described previously (Zufferey, Allen et al. 2003). The monoclonal antibody specific to V5 (Thermo Fisher Scientific Inc.) and polyclonal anti-glycosome antiserum (generous gift of M Parsons (Parker, Hill et al. 1995)) were used at a 1:300 and 1:100 dilution, respectively. Images were taken with a Nikon microscope.

2.8. Statistical analyses

Two-tailed Student's t-test was used for statistical analysis which was performed using GraphPad Prism Software (version 5.0). *P* values ≤ 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. TbADS complements a null mutant of *L. major* lacking ADS activity

ADS activity has been reported in *T. brucei* and the encoding gene (Tb927.6.1500) responsible for this activity has previously been cloned (Zomer, Oppendoes et al. 1995), but its role in the parasite's physiology and lipid metabolism has not been established. To assess whether *TbADS* functions similarly to the previously described alkyl-DHAP synthase encoding gene *LmADS* of *Leishmania major*, *TbADS* was introduced into the *Imads/Imads* null mutant, lacking the orthologous gene *LmADS* (Zufferey, Allen et al. 2003). This strain was devoid of ADS activity and consequently, produced a slower migrating form of the ether PI derived virulence factor lipophosphoglycan (McConville, Bacic et al. 1987, Orlandi and Turco 1987, Zufferey, Allen et al. 2003). The *TbADS* gene restored the wild-type electrophoretic behavior of lipophosphoglycan and led to the reappearance of ADS activity in the *Imads/Imads* null mutant to levels similar to that of the complemented line *Imads/Imads/LmADS*, used here as a positive control (Fig. 2A, B). This data demonstrates that *TbADS* is properly expressed and complements the genetic defect of the *Imads/Imads* mutant strain, and thus, fulfills a similar role as the orthologous gene *LmADS* of *L. major*.

3.2. A null mutant of TbADS is viable but lacks ADS activity

To assess the role of *TbADS* in parasite viability and lipid metabolism, a null mutant was created by successive transformation and selection in the presence of appropriate antibiotics as described in Materials and methods. A null mutant was obtained that bears replacement of both *TbADS* alleles with two different antibiotic cassettes: *BSD* that mediates blasticidin resistance and *HYG* that relieves hygromycin sensitivity (Fig. 3B). PCR was carried out

using appropriate primers to confirm the proper integration of the antibiotic resistance cassettes into the *TbADS* locus by double crossing over (Fig. 3A, B, C). A complemented strain was created by re-introducing the wild-type *TbADS* gene as well as a V5 tagged version of the gene into the β -tubulin locus. The absence of *TbADS* gene expression in the null mutant and the re-expression of the *TbADS* and *V5:TbADS* gene versions in the complemented lines were confirmed by reverse transcriptase polymerase chain reaction (Fig. 3D). The null mutant *tbads/ tbads* exhibited normal growth as the wild type and complemented line (Fig. 4A), demonstrating that *TbADS* is dispensable for the viability and growth of procyclic parasites.

ADS activity of *T. brucei* null mutant of *TbADS* was then quantified. This enzymatic activity was present in the wild type but absent in null mutant cell extracts (Fig. 4B), indicating that *TbADS* is the sole alkyl-DHAP synthase enzyme in procyclic parasites, a situation reminiscent of that of *L. major* (Zufferey and Ben Mamoun 2006). As expected, re-expression of the *TbADS* or *V5:TbADS* genes into the null mutant background restored ADS activity to almost wild-type levels, demonstrating that the V5 epitope did not disturb *TbADS* activity, and that *TbADS* and *V5:TbADS* were not over-expressed (Fig. 4B).

3.3. *TbADS* localizes to the lumen of glycosomes

TbADS possesses a putative C-terminal peroxisomal targeting signal sequence suggesting that it may reside in the glycosomes, which are peroxisome-related organelles in trypanosomatids (Opperdoes 1984). To confirm its subcellular localization, the *TbADS* was tagged with a V5 epitope at its N terminus (*V5:TbADS*) and expressed in the null mutant background. Western blot analysis in the presence of the monoclonal V5 specific antibodies confirmed the expression of the tagged enzyme as a band of an apparent molecular mass of 75 kDa, which was visible in the *tbads/ tbads/V5:TbADS* strain while this signal was absent in the null mutant background (Fig. 5A). Immunofluorescence assay performed on cells expressing *V5:TbADS* in the presence of V5 specific antibodies gave a punctate signal throughout the cell, suggesting a glycosomal localization (Fig. 5B, panel ii). This was corroborated with antibodies specific to glycosomes, which gave an overlapping signal (Fig. 5B, panels iii and iv (Parker, Hill et al. 1995)).

Proteinase K assay was carried out to determine whether *TbADS* localizes on the luminal or cytosolic side of the glycosomal membrane. When cells were permeabilized in the presence of low concentration of digitonin, which affects only the plasma membrane, proteinase K degraded the cytosolic protein arginase but not *V5:TbADS* (Fig. 5C (Parker, Hill et al. 1995)). The latter was only sensitive to proteinase K in the presence of the detergent Triton X-100, similarly to luminal glycosomal proteins aldolase and glyceraldehydephosphate dehydrogenase (Parker, Hill et al. 1995). These results demonstrate that *V5:TbADS* localizes within the lumen of glycosomes.

Our results are in accordance with ADS activity being present in the lumen of glycosomes of *T. brucei* or in the lumen of peroxisomes in mammals such as guinea pig, human or rat (Shinojima, Ono et al. 1981, Gunawan, Rabert et al. 1990, Singh, Beckman et al. 1993, Zomer, Opperdoes et al. 1995, de Vet, Biermann et al. 1997). Furthermore, proteomics

analysis of *T. brucei* glycosomes corroborated the presence of *TbADS* in this organelle (Colasante, Voncken et al. 2013).

How does alkyl-DHAP exit the glycosomes to be further metabolized on the cytosolic side of the glycosomal membrane by the acyl/alkyl-DHAP reductase (Heise and Oppendoerfer 1997)? Analysis of the proteome of the glycosomal membrane revealed only a few integral membrane proteins, raising the question whether transport systems are available to shuttle products/metabolic intermediate across the glycosomal membrane (Colasante, Voncken et al. 2013). Inspection of the lipidome of glycosomes established that the lipid composition of this organelle does not differ from that of the plasma membrane and thus, the authors concluded that transport systems must exist to ferry metabolic intermediates across the glycosomal membrane (Colasante, Voncken et al. 2013). The identity and nature of these transport systems remain to be discovered.

3.4. *TbADS* is essential for the biosynthesis of ether glycerophospholipids

The consequence of *TbADS* deletion on ester and ether glycerophospholipid composition of the null mutant *tbads/ tbads* was then evaluated. Total cellular lipids were isolated and analyzed by comprehensive mass spectrometry as described in Materials and methods. Levels of ester glycerophospholipids PE, PS, and PI were unaffected in the null mutant (Fig. 6). In contrast, amounts of ether glycerophospholipids, which are primarily found in the PC, PE and PS species (reviewed in (Patnaik, Field et al. 1993, Smith and Butikofer 2010)), were severely decreased in the *tbads/ tbads* mutant, indicating that *TbADS* plays a crucial role in ether glycerophospholipid biosynthesis in this parasite. Similar results were obtained in a homologous mutant of *L. major* (Zufferey and Ben Mamoun 2006). However, the null mutant produced slightly more PC to seemingly compensate for the decreased levels of ether glycerophospholipids. This effect is not unusual as cells typically compensate for the lack or decreased levels of certain glycerophospholipid classes by overproducing other type(s) (Signorell, Rauch et al. 2008, Patel, Pirani et al. 2016, Zufferey, Pirani et al. 2017). In conclusion, *TbADS* is dispensable for the viability and growth of *T. brucei* procyclic forms, but is essential for ether glycerophospholipid production. As bloodstream forms also produce ether glycerolipids, future work will investigate the role of *TbADS* in this cell stage ((Patnaik, Field et al. 1993, Richmond, Gibellini et al. 2010) reviewed in (Smith and Butikofer 2010)).

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Abbreviations

ADS   alkyl-dihydroxyacetonephosphate synthase

DHAP	dihydroxyacetonephosphate
DHAPAT	DHAP acyltransferase
ESI-MS/MS	electrospray ionization tandem mass spectrometry
G3P	glycerol-3-phosphate

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HIGHLIGHTS

- *Tb*ADS exhibits alkyl-dihydroxyacetonephosphate synthase activity
- *Tb*ADS is important for ether lipid biosynthesis in procyclic trypanosomes
- *Tb*ADS localizes to the glycosomal lumen
- *Tb*ADS is dispensable for procyclic trypanosomes' viability

Fig. 1. Putative glycerophospholipid biosynthetic pathways of *T. brucei*

AGAT, 1-acyl-G3P acyltransferase; ADR, acyl/alkyl-DHAP reductase; PA, phosphatidic acid.

Fig. 2. *TbADS* restores expression of normal form of lipophosphoglycan in the *L. major* *Imads/Imads* null mutant that lacks alkyl-DHAP synthase activity

(A) Western blot in the presence of the monoclonal WIC79.3 antibody specific to the disaccharide mannose-phosphate-galactose of lipophosphoglycan (Tolson, Turco et al. 1989). Approximately 1×10^7 cells were loaded in each lane. 1, *Imads/Imads/LmADS*; 2, *Imads/Imads*; 3, *Imads/Imads/TbADS*. (B) ADS assay was carried out as described in Material and methods' section. *TbADS* is expressed in *L. major* null mutant *Imads/Imads* ($\Delta/\Delta/TbADS$). The assay was performed in the presence of [^{14}C] octadecanol. Values are expressed as percentages of the positive control *Imads/Imads/LmADS* ($\Delta/\Delta/LmADS$), which corresponds to 36.36 nmol/hrxmg. The assay was performed at least twice in duplicate and standard deviations are shown.

Fig. 3. Generation of the *T. brucei* *TbADS* null mutant and complemented line

Organization of the wild-type *TbADS* allele (A) and mutated alleles (B). The position of all oligonucleotides used in (C) are depicted in panels (A) and (B), and the sizes of the resulting PCR products are shown. *BSD*, blasticidin, light grey arrow; *HYG*, hygromycin, dark grey arrow. (C) Agarose gel electrophoresis of PCR products with oligonucleotides described in (A) and (B). 1, wild type; 2, *tbads/ tbads*; 3, *tbads/ tbads/TbADS*; 4, *tbads/ tbads/V5:TbADS*. The DNA ladder is shown on the left. (D) Quantification of *TbADS* transcript by RT-PCR. RT, reverse transcriptase reaction; “–”, no reverse transcriptase reaction; “+”, with reverse transcriptase reaction. 1, wild type; 2, *tbads/ tbads*; 3, *tbads/ tbads/TbADS*; 4, *tbgat/ tbgat/V5:TbADS*. Oligonucleotides 539 (5′-ACGCCAAGCTAATACATGAACC-3′) and 540 (5′-TATTTCTCAGGCTCCCTCTCC-3′), and primers 507 and 25 (5′-GTCGATCATACCACGCCTTAC-3′) are specific to the *18S*

rRNA gene and the *TbADS* locus, respectively. 1, wild type; 2, *tbads*/*tbads*; 3, *tbads*/*tbads*/*TbADS*; 4, *tbads*/*tbads*/*V5:TbADS*.

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Fig. 4. *TbADS* is dispensable for growth

(A) Growth curves. Black circles, wild type; white circles, *tbads/ tbads*; grey circles, *tbads/ tbads/TbADS*. The assay was carried out twice in duplicate. Standard deviations were omitted and were within 10% of the average values. (B) The null mutant *tbads/ tbads* lacks ADS activity. The assay was carried out twice in duplicate and standard deviations are shown. WT, wild type; Δ , *tbads/ tbads*; Δ TbADS, *tbads/ tbads/TbADS*; Δ V5:TbADS, *tbads/ tbads/V5:TbADS*. Activities are expressed in percentage of the wild type value, which corresponds to 31.99 nmol/hrxmg.

Fig. 5. *TbADS* localizes to the glycosomal lumen

(A) Expression of a V5-tagged *TbADS* enzyme in the *tbads/ tbads* line. Western blot analysis in the presence of anti V5 monoclonal antibodies to reveal V5: *TbADS*, and anti-BiP as a loading control. Approximately 1×10^7 cells were loaded per lane. 1, *tbads/ tbads*; 2, *tbads/ tbads/V5:TbADS*. (B) Immunofluorescence assay in the presence of anti V5 monoclonal antibodies (panel ii) and glycosomes specific antiserum (panel iii). i, DIC; iv, overlay of panels ii and iii. The scale bar is shown. (C) V5: *TbADS* localizes to the lumen of the glycosomes. Proteinase K assay. Approximately 2×10^6 cell equivalent were loaded in each lane. The protein marker is shown on the left.

Fig. 6. *TbADS* is important for ether glycerophospholipid biosynthesis

Lipid composition of the *tbads*^{-/-} *tbads* null mutant. Lipid classes are represented as percentages of total glycerophospholipids. The assay was carried out in quadruplicate and standard deviations are shown. Black bars, wild type; white bars, *tbads*^{-/-} *tbads*; grey bars, *tbads*^{-/-} *tbads* *TbADS*. *, the *p* value resulting from the comparison between the wild type and *tbads*^{-/-} *tbads* was < 0.05.