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The carbon and energy sources of the non-photosynthetic plastid in the malaria parasite

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This work is dedicated in memory of Dr. Kylie A. Mullin, whose ardor and fighting spirit will always be an inspiration.

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ABSTRACT

The malaria parasite harbours an indispensable plastid known as the 'apicoplast'. The apicoplast's exact role remains uncertain, but it houses components involved in fatty acid, isoprenoid and haem biosyntheses. These pathways offer opportunities to develop anti-malarials. In the absence of photosynthesis, how apicoplast anabolism is fuelled is unclear. Here we investigated plant-like transporters of the apicoplast and measured their substrate preferences using a novel cell-free assay system to explore the carbon and energy sources of the apicoplast. The transporters exchange triose phosphate and phospho*enol*pyruvate for inorganic phosphate, demonstrating that the apicoplast taps into host-derived glucose to fuel its metabolism.

1. Introduction

Plastids trap photons and use them to split water. Electrons released from the fission reaction generate reducing equivalents and are also used to establish a proton gradient that is harvested to generate ATP. Carbon is fixed from the atmosphere using this energy and reducing power providing a plant or alga with all its necessary fuel. Plastids export excess reduced carbon compounds to the cytosol during the day but can also import reduced carbon compounds, typically triose phosphates and phospho*enol*pyruvate, from the cytosol at night to power their activity [1]. Exchange of reduced carbon compounds between the plastid and cytosol is managed by a small family of transporters known as the plastidic phosphate translocators (pPTs), which are antiporters that exchange inorganic phosphate for phosphorylated C3, C5 or C6 compounds as counter substrates [1]. Plant plastids typically have a specific pPT for each substrate exchanged, e.g. triose phosphate transporter (TPT), phospho*enol*pyruvate (PEP) transporter (PPT), xylulose phosphate transporter (XPT) and glucose 6-phosphate (G6P) transporter (GPT) [1]. How the different pPTs discriminate between trioses, pentoses and hexoses and even the position of the phosphate on C3 compounds is unknown, and substrate preference cannot yet be predicted from the primary structure.

The relict plastid (apicoplast) of malaria parasites has lost the ability to trap photons by photosynthesis, but retains a suite of anabolic processes that need to be fuelled. Apicoplasts synthesize fatty acids, isoprene precursors and haem, and the parasite's reliance on these pathways make them attractive drug targets [2]. We hypothesized that apicoplasts would import reduced carbon compounds from the parasite cytosol using transporters homologous to those of plant and algal plastids. Indeed, *Plasmodium falciparum* has two putative triose phosphate/phosphate transporters, *Pf*oTPT and *Pf*iTPT, previously shown to reside in the outermost and innermost membranes of the four-membraned apicoplast [3]. To date, they are the only candidate metabolite transporters of the organelle and their activities and substrate preferences have not been determined. *P. falciparum* transporters are notoriously difficult to express and characterize in surrogate systems, probably due to strong nucleotide biases in their genes and atypical targeting motifs that confound foreign cell machinery [4]. Although several transporters of *P. falciparum* were successfully reconstituted using *Xenopus* oocytes [5–8], our previous attempts to express active *Pf*oTPT and *Pf*iTPT in oocytes were unsuccessful, as were

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attempts to express these genes in yeast (data not shown). Here, we utilise a novel, cell-free transporter assay system [9] to characterize the substrate preferences of *Pfo*TPT and *Pfi*TPT and demonstrate their potential roles as gatekeepers to the metabolism of the apicoplast.

2. Materials and methods

2.1. Cloning and constructs

Native *Pfo*TPT is unprocessed whereas mature *Pfi*TPT has an N-terminal targeting motif that is removed in the apicoplast [3]. The DNA sequences encoding the mature sequence of *Pfi*TPT (PlasmoDB accession number: *PF*E1510c) and the full length of *Pfo*TPT (PlasmoDB accession number: *PF*E0410w) were codon optimised for expression in *Saccharomyces cerevisiae* and synthesized in the absence of *Nco*I and *Bam*HI sites (GeneMaker®, Blue Heron Biotechnology, Inc., Bothell, USA). The sequences were cloned in frame with an N-terminal hexa-histidine tag into the *Nco*I and *Bam*HI sites of the pEU3a vector (CellFree Sciences, Matsuyama, Japan). It is noteworthy that several genes of *P. falciparum* have previously been translated by the wheat germ cell free system [10] but this report encompasses the first expression of membrane transporters by the system. A non-optimised version of *Pfo*TPT was also cloned into the pEU3a vector and was successfully produced by the system but was not used for the experiments described herein.

2.2. Cell-free expression of proteins

Full length *Pfo*TPT and mature *Pfi*TPT were synthesized by the bilayer method [11] using a cell-free system (Endext Technology, CellFree Sciences). In this method, a substrate mixture (50 μl) was overlaid carefully with a reaction mixture (200 μl) and incubated at 26 °C overnight (16 h). The former consisted of mRNA transcribed from the construct of interest (pEU3a-*Pfi* or pEU3a-*Pfo*), wheat germ extract supplying the translation machinery, creatine kinase, RNAse inhibitor (Promega, Madison, USA) and SUBAMIX®. Phosphatidylcholine (1%) and Brij-35 (0.04%) were supplemented for optimal production of the transporters. Each protein sample was desalted by gel filtration using a Sephadex G-25 column (NAP 5 column, GE Healthcare, catalogue number 17-0853-01) before reconstitution.

2.3. Immunoblot analysis of expressed proteins

Proteins were separated using SDS–PAGE [12] and transferred onto a PVDF nitrocellulose membrane (Amersham) via semi-dry transfer (Carl Roth GmbH, Karlsruhe, Germany). To visualise hexa-histidine tag-containing fragments, the membrane was blocked in western blocking buffer and incubated in anti-poly-His tag antibody (Qiagen, Hilden, Germany) diluted 1:2500 in blocking solution. After washing with western wash buffer, the membrane was probed with secondary alkaline phosphatase-conjugated antibody (Promega) in blocking buffer. Protein bands were detected using the substrates for alkaline phosphatase (Promega), according to the manufacturer's protocol.

2.4. Production of proteoliposomes

Phospholipids were prepared by sonicating a 6% (w/v) acetone-washed L-α-phosphatidylcholine (Sigma–Aldrich) solution containing 200 mM Tricine_KOH, 40 mM potassium gluconate (Sigma–Aldrich) and 60 mM counter-substrate (PEP/3-phosphoglycerate (3PGA)/G6P/dihydroxyacetone (DHAP); Sigma, Germany). Sonication was performed for 5 min on ice with a sonifer-type Branson Sonicator 250, equipped with a microtip using 30% duty cycle and output power control of three. 0.5 ml of the translation mixture and the same volume of the phospholipid suspension were mixed by vortexing. Desalted membrane proteins were incorporated into the liposomes by freezing in liquid nitrogen and thawing at room temperature [13]. Resulting suspensions were sonicated for 15 pulses (30% duty cycle, output control 3) prior to uptake assays to seal the vesicles leading to the formation of proteoliposomes. Control experiments involved liposomes incorporated with desalted translation reactions that did not have any mRNA added.

To remove external counter-substrates not incorporated into the proteoliposomes, the suspensions were passed through Sephadex G-25 columns (PD-10 column, GE Healthcare, catalogue number 17-0851-01) pre-equilibrated with 100 mM sodium gluconate, 40 mM potassium gluconate, and 10 mM Tricine–KOH (pH 7.5).

2.5. Uptake assays

Transport assays were initiated by adding radio-labeled [^{32}P]-orthophosphate (Pi) at a final concentration of 0.5 mM (Hartmann Analytic, Braunschweig, Germany). Assays were performed at room temperature for various times and for each data point 0.2 ml proteoliposomes were collected and passaged through a AG-1X8 Resin (acetate form, 200–400 mesh; Bio-Rad) pre-equilibrated with 150 mM sodium acetate. This filtration step effectively removed all external Pi by strong anion-exchange chromatography and stopped the uptake reaction. Proteoliposomes were eluted into water and the uptake of [^{32}P]-Pi was measured by liquid scintillation counting (Beckmann LS6000 counter, Beckman Coulter Inc., Fullerton, CA).

2.6. Data analyses

Kinetic constants were determined by measuring the initial velocity of each experiment. The Michaelis–Menten kinetic constant (K_m), which describes the affinity of a particular substrate by the protein of interest, is analysed with a minimum of six external phosphate concentrations ranging from 0.05 mM to 12.5 mM. The inhibitor constant, K_i, described by Dixon [14] was also evaluated to assess competitive inhibition of [^{32}P]-Pi transport. Experiments were done in a concentration range from 0.001 mM to 12.5 mM. The GraphPad-Prism software was used for non-linear regression analyses of all the enzyme kinetic data.

3. Results and discussion

To enhance transporter syntheses and functionality, translation reactions were supplemented with liposomes and Brij-35 detergent to provide an environment conducive for the folding and insertion of membrane proteins. Immunoblot analysis with anti-poly-His tag antibody demonstrated that *Pfo*TPT and *Pfi*TPT were expressed compared to control where no transcript was introduced in the translation reaction (Fig. 1A). Presence of recombinant transporter proteins was checked by western blot for all replicates prior to reconstitution and the transporters were inserted into liposomes by a freeze-thaw step [13].

pPTs catalyse homo-exchange of Pi in addition to their characteristic substrates [1]. We first tested for homo-exchange of Pi by both *Pfo*TPT and *Pfi*TPT to verify their functionality. *Pfo*TPT and *Pfi*TPT proteoliposomes were preloaded with 30 mM unlabelled Pi, then radio-labeled [^{32}P]-Pi was added and uptake kinetics measured over time. Exchange equilibrium was achieved for both transporters after 60 min using an external Pi concentration of 0.5 mM (Fig. 1B). Pi transport followed a first-order kinetic and reached an isotopic equilibrium of 82.0 ± 3.8 and 89.0 ± 2.8 nmol