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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

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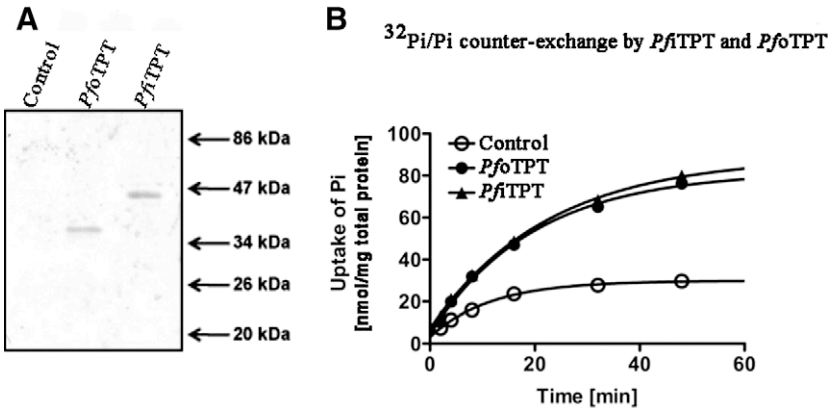

Fig. 1. (A) Anti-poly-His Western blot analysis of protein expression from cell-free system detected His-tagged *Pfo*TPT (41.7 kDa) and *Pfi*TPT (46.1 kDa). (B) Time kinetics of [^{32}P]-Pi uptake by *Pfo*TPT and *Pfi*TPT compared to control liposomes. A representative graph from three independent experiments is presented.

Pi mg total protein^{-1} and initial rates of 3.54 ± 0.47 and 3.22 ± 0.30 nmol Pi min^{-1} mg total protein^{-1} for *Pfo*TPT and *Pfi*TPT, respectively. Control liposomes also displayed some Pi uptake but the background uptakes were consistently less than the uptakes observed in the test proteoliposomes. These observations were consistent in three independent experiments. Thus [^{32}P]-Pi uptakes by both the transporters were considered significant.

To define the substrate specificities of *Pfo*TPT and *Pfi*TPT, proteoliposomes were preloaded with saturating concentrations (i.e. 30 mM) of various substrates (Pi, PEP, DHAP, 3PGA, G6P) and the initial rates of [^{32}P]-Pi uptake were calculated. The specific uptake of [^{32}P]-Pi by each transporter for each test counter-substrate was compared to the corresponding control.

As illustrated in Fig. 2A, the uptakes of [^{32}P]-Pi into *Pfo*TPT proteoliposomes preloaded with PEP, DHAP and 3PGA were consistently threefold or higher than those in corresponding controls. In contrast, uptakes were consistently less than onefold higher than controls when the proteoliposomes were loaded with G6P, which we interpret as a non-relevant substrate for *Pfo*TPT. *Pfo*TPT has an apparent K_m value of 1.64 ± 0.03 mM for Pi (Table 1). The affinity of *Pfo*TPT for PEP was high as reflected by the low K_i value of 0.22 ± 0.03 mM while DHAP and 3PGA competitively inhibited [^{32}P]-Pi at higher concentrations of 1.53 ± 0.03 mM and 3.72 ± 0.40 mM, respectively.

Similar to *Pfo*TPT, *Pfi*TPT demonstrated significant uptake of PEP, DHAP and 3PGA but not for G6P (Fig. 2A). *Pfi*TPT has an apparent K_m value of 3.63 ± 0.47 mM for Pi (Table 1). *Pfi*TPT mirrored *Pfo*TPT in its high affinity for PEP and was competitively inhibited in [^{32}P]-Pi uptake with K_i values of 1.76 ± 0.20 mM for DHAP and 2.54 ± 0.45 mM for 3PGA.

*Pfi*TPT and *Pfo*TPT thus have equivalent substrate specificity profiles with high affinities for PEP, a lesser, but still significant, affinity for DHAP and 3PGA, and essentially no affinity for G6P. This is the first instance of pPTs transporting both PEP and DHAP (Fig. 2B and Table 1). It is noteworthy that the demonstrated transport activities show that *Pfo*TPT and *Pfi*TPT are misnomers since they are more than just triose phosphate transporters. They should be pPTs and be named as *Pfo*pPT and *Pfi*pPT. *Pfo*pPT resides in the outermost of the four membranes that bound the apicoplast whereas *Pfi*pPT is thought to be located in the innermost membrane based on analogy to plant pPTs and the fact that its N-terminal targeting motif is removed [3,15]. The equivalent substrate preferences of *Pfo*pPT and *Pfi*pPT suggest that the pair of transporters operates in tandem to transport PEP, DHAP and 3PGA across the apicoplast membranes. As yet there are no candidate metabolite transporters in the middle pair of apicoplast membranes. Because the C3 metabolites are charged, passive diffusion through the lipid bilayers seems unlikely, so how reduced C3 compounds cross these two membranes remains unclear. The second outermost (periplastid) membrane originated from the plasma membrane of the engulfed red alga while the third outermost membrane is analogous to the outer envelope of the chloroplast [16]. In chloroplasts the 21 kDa outer envelope protein (OEP21) translocates anionic substances (including DHAP and PEP) [17], but there is no obvious homologue of OEP21 in *P. falciparum*. In the apicoplast of another apicomplexan *Toxoplasma gondii* it has been argued that one transporter (*Tg*APT1) might reside in multiple membranes [18], which could resolve the issue of transport. We cannot yet exclude the possibility that *Pfi*pPT could also be localized in all three of the inner apicoplast membranes, but there is no precedent in mitochondria or chloroplasts for targeting a protein to multiple concentric membranes of an organelle [19].

Can PEP, DHAP and 3PGA fuel apicoplast anabolism? Apicoplast metabolism requires carbon skeletons, ATP and NADH to drive the biosyntheses of isopentenyl di-phosphate (IPP) and fatty acids [2]. Imported DHAP could be converted to glyceraldehyde-3-phosphate (GA3P) by apicoplast resident triose phosphate isomerase (TPI), which would generate one substrate required for the IPP pathway (Fig. 3). The other requisite IPP substrate, pyruvate, would be provided by import of PEP and its modification to pyruvate by an apicoplast-localized pyruvate kinase [20,21]. Substrate-level phosphorylation of ADP using the high-energy phosphate from carbon 2 of PEP would also provide a welcome source of ATP in the apicoplast. Pyruvate could also be fed into fatty acid biosynthesis via the plastidic pyruvate dehydrogenase complex (PDHC) [22], which would generate acetyl-CoA and the reducing equivalent NADH. Pyruvate is thus a branch point in the apicoplast carbon import chain. By importing phosphorylated, reduced carbon compounds and then dephosphorylating and partially oxidizing them, the apicoplast provides for its carbon requirements while supplementing its energy and reducing power needs. Just as there are two fates for the PEP imported, there are two channels for imported DHAP to take. In addition to the aforementioned conversion of DHAP to GA3P by TPI, DHAP could also be converted to glycerol-3-phosphate (G3P) by the plastidic glycerol-3-phosphate dehydrogenase (GpdA). G3P would be the acceptor backbone for apicoplast synthesized fatty acids to generate phospholipids. The apicoplast thus survives by importing glycolytic intermediates from the parasite cytosol (Fig. 3). Glucose for this glycolysis is scavenged by the parasite from the red cells and serum of the human host, so the plastid taps directly into this carbon/energy source.

The ability of *Pfi*pPT and *Pfo*pPT to transport 3PGA is at face value somewhat puzzling. We cannot envisage any sink or modification for imported 3PGA based on the repertoire of apicoplast

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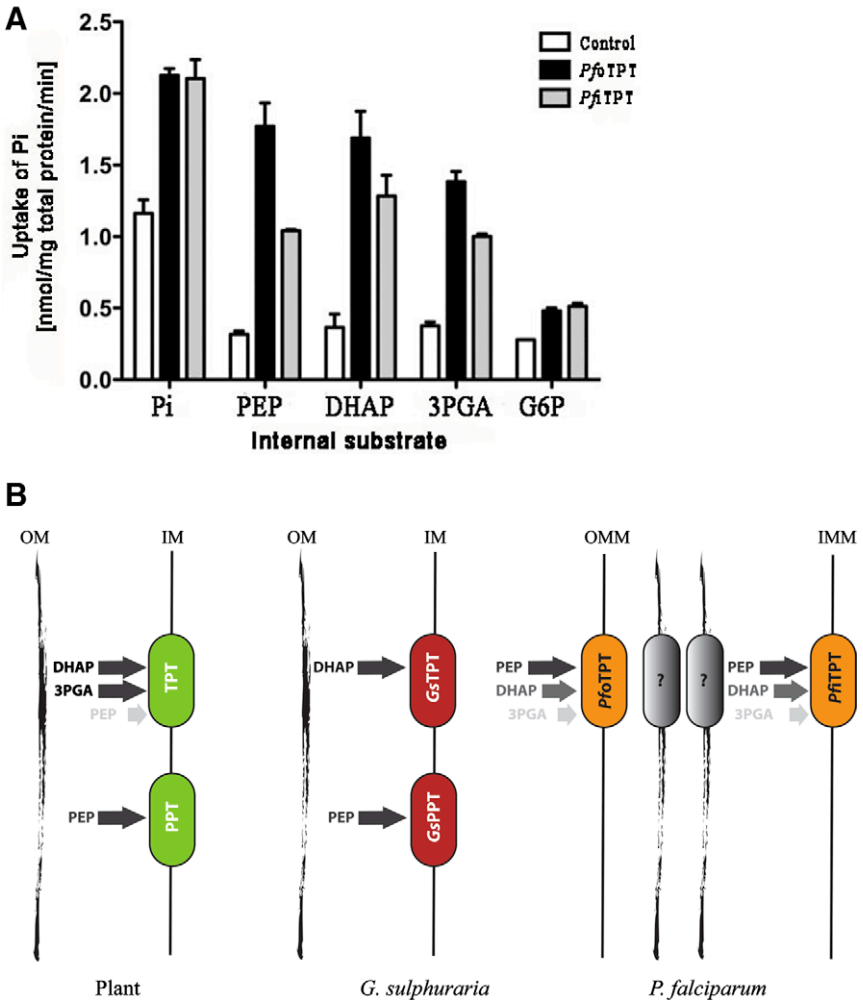

Fig. 2. (A) For each sample, [^{32}P]-Pi uptake up to 16 min was measured and linear regression was applied to calculate the rate of uptake. The histogram represents data from three independent experiments. *Pfo*TPT and *Pfi*TPT proteoliposomes displayed significant [^{32}P]-Pi uptake in exchange with PEP, DHAP and 3PGA but not G6P. (B) A schematic depiction of the broadened substrate specificities of the *Plasmodium* pPTs relative to those in plants and the red alga *G. sulphuraria*. OM, outer membrane; IM, inner membrane, OMM, outermost membrane; IMM, innermost membrane.

Table 1
Relative substrate affinities of *Pfo*TPT, *Pfi*TPT to the plant and algal TPTs and PPTs. Data from plants are taken from Flugge [1] while data from *G. sulphuraria* are quoted from Linka et al. [26].

Kinetic constants (mM)	Plant TPTs	Plant PPTs	GsTPT	GsPPT	*Pfo*TPT	*Pfi*TPT
K_m (Pi)	1	0.8	0.33 ± 0.07	0.76 ± 0.08	1.64 ± 0.03	3.63 ± 0.47
K_i (DHAP)	1	8	0.5 ± 0.04	7.93 ± 0.23	1.53 ± 0.03	1.76 ± 0.20
K_i (PEP)	3.3	0.3	8.75 ± 0.40	0.36 ± 0.04	0.22 ± 0.03	0.18 ± 0.04
K_i (3PGA)	1	4.6	7.71 ± 0.47	3.95 ± 0.1	3.72 ± 0.40	2.54 ± 0.45

enzymes [4]. Indeed, the apicoplast of *T. gondii* is probably able to generate 3PGA from GA3P using apicoplast resident glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) [23]. An attractive hypothesis is that rather than importing 3PGA apicoplast pPTs export this carbon compound in exchange for DHAP in a redox shuttle that operates in reverse to that normally seen in plants thereby generating further NAD(P)H and ATP in the apicoplast [2,23]. However, *P. falciparum* appears to lack GAPDH and PGK in its plastid, which casts doubt on the presence of such a shuttle in the malaria parasite. The ability of *Pfi*pPT and *Pfo*pPT to transport 3PGA could be a redundant, vestigial function given that all plant TPTs can transport 3PGA [1,24,25].

The ability of *Pfo*pPT and *Pfi*pPT to efficiently transport both triose phosphate and PEP is unique amongst functionally characterized pPTs [1,24–26]. We believe that a widening of substrate specificity has allowed a rationalization of the ancestral transporter array to service a simplified organelle anabolism such that only one pPT type is required in the outer and inner membranes of the apicoplast (Fig. 2B). In a broad sense, it appears that the pPTs of the relict plastid of malaria parasites have undergone a reversal of roles. Whereas reduced carbon compounds undergo a net efflux from autotrophic plastids of photosynthetic plants and algae, the parasite plastid must import all carbon and energy and the transporters act as importers. Redox power must also be imported and the hypothetical reverse version of the plant shuttle that brings in electrons would meet this demand. Intriguingly, much of this import activity mirrors the way that plants deal with metabolic demands at nighttime when their photosynthesis machinery is quiescent. We propose that the malaria parasite can be likened to a *plant in dark* with respect to how it maintains its plastid. An

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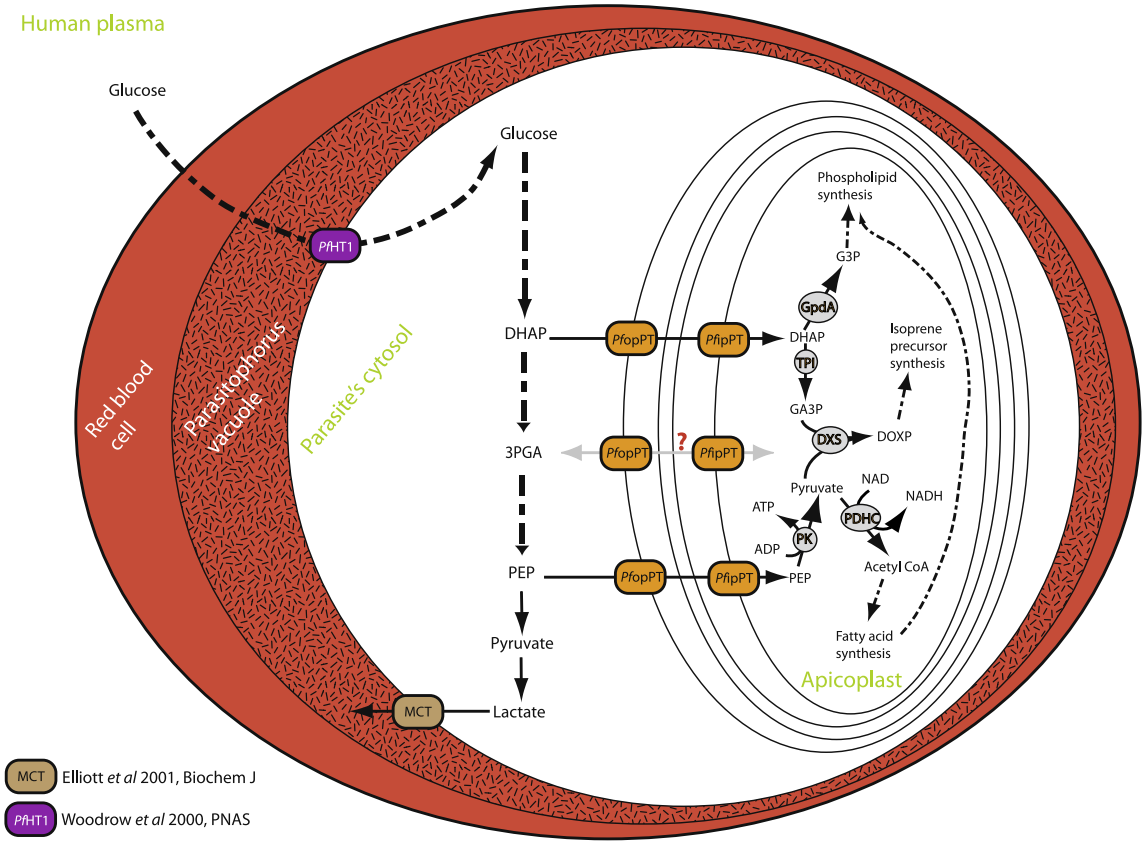

Fig. 3. The metabolic dependence of the apicoplast on host-derived glucose for carbon, reducing power and energy. In this study, we demonstrate that *Pf*opPT and *Pf*ipPT can import the 3-carbon glycolytic intermediates DHAP and PEP to fuel the biosyntheses of isoprenoid and fatty acid in the apicoplast. DHAP, dihydroxyacetone phosphate; DOXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DOXP synthase; G3P, glycerol-3-phosphate; GA3P, glyceraldehyde-3-phosphate; GpdA, glycerol-3-phosphate dehydrogenase; MCT, monocarboxylate transporter; 3PGA, 3-phosphoglyceric acid; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TPI, triose phosphate isomerase; *Pf*HT1, *P. falciparum* hexose transporter 1; *Pf*ipPT, *P. falciparum* innermost plastidic phosphate translocator and *Pf*opPT, *P. falciparum* outermost plastidic phosphate translocator.

attractive scenario would be that modification of the transporters' polarity and localization occurred as parasitic apicomplexans relinquished their ancestral ability to photosynthesize. An investigation of pPTs in the *Chromera* plastid might shed light on how the apicoplasts became non-photosynthetic and/or reveal candidate transporters in the second and third membranes of the apicoplast [27]. Just as the parasite itself learned to sup at the table of its host and steal glucose, the plastid had to learn to sup at the table of its host and import reduced carbon compounds in order to continue its essential biosynthetic roles. Inserting transporters into the membranes of the first autotrophic endosymbionts was a key evolutionary step allowing eukaryotic host cells to domesticate cyanobacteria and capture the photosynthesis process [28]. Our data indicate that reversing the transport direction was a key innovation to fuel a plastid on which the parasites had become reliant even though the "lights had been turned out". Malaria parasites are heavily reliant on these transporters making them attractive targets for therapeutic drugs and the cell-free assays described here could help identify inhibitors.

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