Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites

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The human malaria parasite Plasmodium falciparum harbors a relict, nonphotosynthetic plastid of algal origin termed the apicoplast. Although considerable progress has been made in defining the metabolic functions of the apicoplast, information on the composition and biogenesis of the four delimiting membranes of this organelle is limited. Here, we report an efficient method for preparing highly purified apicoplasts from red blood cell parasite stages and the comprehensive lipidomic analysis of this organelle. Apicoplasts were prepared from transgenic parasites expressing an epitope-tagged triosephosphate transporter and immunopurified on magnetic beads. Gas and liquid chromatography MS analyses of isolated apicoplast lipids indicated significant differences compared with total parasite lipids. In particular, apicoplasts were highly enriched in phosphatidylglycerol, consistent with a suggested role for phosphoinositides in targeting membrane vesicles to apicoplasts. Apicoplast phosphatidylglycerol and other phospholipids were also enriched in saturated fatty acids, which could reflect limited acyl exchange with other membrane phospholipids and/or a requirement for specific physical properties. Lipids atypical for plastids (sphingomyelins, ceramides, and cholesterol) were detected in apicoplasts. The presence of cholesterol in apicoplast membranes was supported by filipin staining of isolated apicoplasts. Galactoglycerolipids, dominant in plant and algal plastids, were not detected in P. falciparum apicoplasts, suggesting that these glycolipids are a hallmark of photosynthetic plastids and were lost when these organisms assumed a parasitic lifestyle. Apicoplasts thus contain an atypical melanage of lipids scavenged from the host human, alongside lipid remodeled by the parasite cytoseplasm, and stable isotope labeling shows some apicoplast lipids are generated de novo by the organelle itself.

chloroplast | Apicomplexa | endosymbiosis | lipidome | FASII

Malaria is a major global health problem that affects 200–500 million people resulting in 0.7–1 million deaths each year (1). The disease is caused by parasites of the genus Plasmodium, with Plasmodium falciparum being the most lethal. There are currently no effective subunit vaccines and antimalarial efficacy has been undermined by the emergence of drug-resistant parasites (2). P. falciparum as well as most other members of the phylum Apicomplexa harbor a nonphotosynthetic plastid, termed the apicoplast, which is homologous to the plastids of plants and algae (3). Apicoplasts were acquired some 450 million years ago by secondary endosymbiosis before the divergence of Apicomplexa and dinoflagellate algae (4). Apicoplasts lack enzymes and pigments required for photosynthesis but retain a number of other anabolic pathways that are indispensable for parasite growth and viability (5, 6), including Fe-S cluster assembly (7, 8), fatty acid biosynthesis (9), haem synthesis (7, 10), and isoprenoid biosynthesis (11). Recent studies have shown that apicoplast isoprenoid precursor biosynthesis is essential for the viability of red blood cell (RBC) stages of P. falciparum (12), indicating that some of these pathways cannot be bypassed by salvage of lipids from the host cell. The apicoplast is surrounded by four membranes, reflecting the complex evolutionary origin of this organelle (13). Essentially nothing is known about the lipid composition of these membranes or their biogenic origin. Apicoplasts contain a prokaryotic-like fatty acid synthase (FASII) complex as well as key enzymes involved in phospholipid biosynthesis, indicating that de novo synthesized fatty acids may be incorporated into apicoplast membrane lipids (9). However, recent studies have shown that apicoplast FASII is not essential for the growth of axenic RBC parasite stages, suggesting that apicoplast fatty acids and other lipids can be derived from other parasite lipid biosynthetic pathways and/or salvage from the host (for review, see refs. 6, 14, 15, 16). Consistent with this hypothesis, it has recently been reported that several apicoplast membrane proteins are delivered to the apicoplast via membrane vesicles derived from the endomembrane system (17, 18).

The plastids of photosynthetic plants and algae characteristically contain high levels of the galactoglycerolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which are essential for plastid function (19, 20). MGDG and DGDG are also abundant in Chromera velia (21), a photosynthetic alga closely related to Apicomplexa. Galactoglycerolipids and galactoceramides have previously been reported in Toxoplasma gondii (22–24), but these glycolipids have not been localized to the apicoplast and no genes encoding MGDG or DGDG galactosyltransferases have been identified in Apicomplexa. Whether the nonphotosynthetic plastids of Apicomplexa contain galactoglycerolipids therefore remains an open question.

The isolation of apicoplasts and detailed analysis of their lipids in relation to those of whole parasites is an essential prerequisite for understanding the steps involved in apicoplast biogenesis and identifying lipids that are potentially important for apicoplast biosynthetic functions. Previous attempts to purify apicoplasts have been hampered by their physical connection to the mitochondrion (25). Here we report the use of an immunosolubilization...


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protocol similar to that used to purify subcellular organelles from other eukaryotes (26) for generating a highly enriched apicoplast fraction from blood stage trophozoites of *P. falciparum*. Lipidomic analysis of the purified organelles indicates that apicoplasts have a lipid composition atypical for plastids and that apicoplast biogenesis in RBC stages is likely dependent on uptake and intraorganelar transport of host lipids.

**Results and Discussion**

**Purification of Intact Apicoplasts.** Previous attempts to isolate apicoplasts from *P. falciparum* or *T. gondii* using density gradient centrifugation (25) or capillary zone electrophoresis (27) have resulted in low yields and/or poorly defined fractions. We therefore developed an alternative approach that allowed immunosolation of highly purified apicoplasts from parasite lines expressing HA-tagged versions of the *P. falciparum* plastid phosphate transporter or outer membrane triose phosphate transporter (*Pfo*o*TPT*). *Pfo*o*TPT* is a polytopic membrane protein involved in the import of reduced carbon compounds into the apicoplast (28, 29). It contains 10 transmembrane domains and is located in the outer membrane of the apicoplast with both the C- and N-termini orientated toward the cytoplasm (6). N- or C-terminally tagged *Pfo*o*TPT* are therefore ideal ligands for the immunopurification of the apicoplast. Synchronous cultures of *P. falciparum* were harvested at mid-trophozoite stage, coinciding with peak *Pfo*o*TPT* expression (24–30 h post-invasion, Fig. S1). Host erythrocytes were permeabilized by saponin to release free parasites, which were then lysed by osmotic shock (29). Nuclei and cellular debris were removed by low-speed centrifugation to generate an organelle fraction from which apicoplasts were retrieved using magnetic beads coated with an anti-HA monoclonal antibody (Fig. 1). Optimal yields of apicoplasts were obtained from parasites expressing the C-terminally tagged *Pfo*o*TPT*-HA. In contrast, no enrichment for apicoplast markers was observed when the organelle fraction from wild type (WT) parasites was incubated with anti-HA beads, confirming selective enrichment for *Pfo*o*TPT*-tagged apicoplasts.

Apicoplast purity was assessed by Western blotting with antibodies directed to protein markers for the apicoplast stroma, apicoplast outer membrane, mitochondrion, plasma membrane, food vacuole, and Golgi/endoplasmic reticulum (ER) (Fig. 2A). The final apicoplast fraction contained the apicoplast membrane marker *Pfo*o*TPT* (HA tagged) as well as the apicoplast acyl carrier protein (ACP) luminal marker and was essentially free of markers for the mitochondrion (heat shock protein 60, HSP60), the plasma membrane (nucleoside transporter 1, NT1), the food vacuole (multidrug resistance protein 1, NT1), and the Golgi/ER (endoplasmic reticulum retention defective 2, ERD2). The absence of mitochondrial protein markers in this fraction indicates that the close association between the apicoplast and mitochondrion observed in vivo (10, 30, 31) is readily disrupted under the relatively mild isolation conditions used here. The absence of significant contamination with mitochondrion membranes was further supported by the absence of detectable levels of the mitochondrial lipid cardiolipin, in the apicoplast fraction (Fig. 2H).

Apicoplast purity and integrity were further assessed by immunofluorescence and EM. Whole parasites (Fig. 2B) or beads carrying isolated apicoplasts (Fig. 2C) were labeled with antibodies against the apicoplast stromal marker ACP (red) and the apicoplast outer membrane marker [either *Pfo*o*TPT*-HA (green) or *Pfo*o*TPT*; Fig. S2]. In trophozoite stages, the apicoplast is a spherical organelle with a diameter of 200–500 nm (Fig. 2B). After purification, about 2–10 ACP and *Pfo*o*TPT*-HA–positive structures of such size were visible on each bead (Fig. 2C). Immunofluorescence assays using markers for other organelles did not identify any contaminating structures on beads, consistent with the absence of mitochondria, food vacuoles, plasma membrane, and Golgi/ER.

The apicoplast fraction contained a homogeneous assemblage of ~200-nm diameter organelles when analyzed by transmission EM (Fig. 2D). As expected, these structures were bounded by four membranes (Fig. 2F and G) and bore *Pfo*o*TPT* on the outermost membrane (Fig. 2E). They also contained numerous small particles, tentatively identified as 70S ribosomes based on
their size (Fig. 2 F and G), consistent with previous ultrastructural observations of apicoplasts in intact parasites (3, 7, 13). Small electron lucent zones were observed in the center of the purified apicoplasts (Fig. S3). These zones have not been previously reported in apicoplasts and are reminiscent of nucleoids that contain the organelle genomes in plant and algal plastids (32). Collectively, these analyses indicate that our method can be used to prepare highly purified, intact apicoplasts.

**Apicoplast Is Enriched in Saturated Fatty Acids.** The fatty acid composition of purified apicoplast was determined by GC-MS of the released fatty acid methyl esters (Fig. 3). Compared with the total cellular fatty acid composition of trophozoite-stage parasites, the apicoplast preparations were enriched in the long chain saturated fatty acid C18:0 and depleted in corresponding monounsaturated (C18:1) and di-unsaturated (C18:2) fatty acids. The levels of some other unsaturated fatty acids (C20:4) were also depleted in apicoplasts, as were the levels of some very long fatty acids (C24:0). Overall, the apicoplasts were highly enriched in saturated fatty acids (~90% of all fatty acids) compared with whole parasites (~65% of all fatty acids).

We have recently shown that the majority of the long chain saturated fatty acids in the related Apicomplexa parasite, T. gondii, are synthesized by the apicoplast FASII (33). The fatty acids detected in the apicoplast fraction could therefore reflect both the composition of membrane phospholipids as well as de novo synthesized free or CoA-linked fatty acids. These fatty acids are exported from the apicoplast and further elongated/modified by ER-located fatty acid elongases and desaturases (33, 34). The absence of an apicoplast isoform of the stearoyl-CoA Δ9 desaturase in the *P. falciparum* genome could account for the paucity of unsaturated C18 fatty acids in the apicoplast. This differs markedly from the situation in plant and algal plastids in which the majority of FASII-produced C18:0-ACP is desaturated into C18:1-ACP by a stromal stearoyl-ACP Δ9 desaturase (34, 35).

The enrichment for saturated over unsaturated fatty acids in the apicoplast fractions (12.8 compared with 1.8 in whole parasites, Fig. 3A), might be required to maintain the multilaminate membrane structure and/or minimize oxidative damage to apicoplast lipids. Blood cell-stage parasites are exposed to high oxidative levels of endogenous reactive oxygen species as a result of hemoglobin digestion in the digestive vacuole and may have
evolved a membrane composition that is intrinsically resistant to oxidative stress (36). The high levels of saturated fatty acids in Plasmodium, and particularly in apicoplast membranes, would complement other well-characterized antioxidant and redox regulatory systems (16, 36–38).

The apicoplast fraction also contained low levels of the odd-chain fatty acid, C17:0 (Fig. 3A and Fig. S4). This fatty acid could, in principal, be synthesized by either the type II FAS complex or by fatty acid elongases using propionyl-CoA instead of acetyl-CoA (39). Propionyl-CoA, a toxic metabolite-inhibiting cell growth, can be generated during the catabolism of amino acids, such as Val, Leu, Thr, and Met (40–44). The terminal steps in propionyl-CoA catabolism occur in the mitochondrion (38, 39), and it is likely that odd chain fatty acids are exported from the mitochondrion to the ER fatty acid elongases and subsequently incorporated into phospholipids that are transported to the apicoplast. The synthesis of odd chain fatty acids may be elevated in P. falciparum because these parasites lack enzymes required for the 2-methylcitrate cycle that detoxifies these intermediates in T. gondii (42).

It has recently been proposed that the apicoplast FASII pathway is inactive in asexual RBC stages because genetic disruption of this enzyme complex has little effect on parasite growth (15). Consistent with this conclusion, we found little evidence of de novo fatty acid biosynthesis when parasite cultures were grown in the presence of [U-13C]-glucose. Triose phosphates generated from [13C]-glucose catabolism provide the carbon backbones used by the FASII apicoplast complex, and de novo synthesis can be monitored by the appearance of fatty acid isotopeomers containing variable levels of [13C] (33). Under standard culture conditions, only C14:0 fatty acids (myristate) exhibited any detectable labeling (Fig. 3F). Remarkably, when parasites were transferred into a minimal medium lacking exogenous lipids but supplemented with the two fatty acids species required for in vitro culture (45), highly efficient labeling of C14:0 was observed (Fig. 3G). The predominant C14:0 isotopeomer labeled under these conditions was fully labeled (m/z = 256) consistent with de novo synthesis rather than elongation of unlabeled short chain fatty acid precursors. These data support the presence of an active FASII in the apicoplast in RBC stages that appears to be strongly regulated by the availability of exogenous fatty acids. Under lipid-rich conditions, most of the parasite fatty acids in asexual stages are salvaged from the medium/host (15), whereas de novo synthesis is markedly up-regulated under fatty acid–limiting conditions. Because this response occurs shortly after transfer to lipid-free medium, it is likely to reflect posttranscriptional regulation of FASII activity.

Apicoplast Lipid Composition. The membrane lipid composition of the purified apicoplasts was further analyzed using liquid chromatography-tandem MS (MS/MS). Lipids were detected using full-ion scanning in positive and negative modes (Fig. 3B), and the molecular species composition of specific lipid classes was confirmed by targeted analysis via precursor ion scanning and neutral loss scanning (Table S1). A total of 184 lipid molecular species belonging to 10 major lipid classes were identified in apicoplasts and total parasite lipid extracts (Fig. S5). Major phospholipids were quantified by multiple reaction monitoring and normalization against selected internal standards (Fig. 3B and Fig. S5). The apicoplast membranes contained a similar phospholipid composition to the total parasite membrane, with phosphatidylycholine (PC) and phosphatidylethanolamine (PE) being major species. However, modest but significant differences were observed in the molecular species composition of these major phospholipid species, consistent with the GC/MS analysis. In particular, apicoplasts were enriched in molecular species containing fully saturated or monosaturated fatty acids [PC (34:0), PC (34:0), and PE (34:0)] (Fig. S5). The predominance of PC and PE molecular species containing 34:0 and 34:1 acyl compositions is consistent with the finding that asexual RBC stages require exogenous supply of oleic and palmitic acid for normal growth (45). PC and PE species containing alkyl-acyl and alkenyl-acyl lipid moieties were also detected (Fig. S5). These plasmodalogen species are commonly found in human tissue, supporting the notion that asexual stages actively salvage a range of lipids from the host.

The levels of several other apicoplast phospholipids differed dramatically from those of the total parasite membrane (Fig. 3F). Specifically, phosphatidylserine and sphingomyelin (SM) were depleted in the apicoplast compared with total cellular membranes. In contrast, phosphatidylinositol (PI) represented nearly 15% of apicoplast phospholipids but only 5% of phospholipids of whole parasites. This enrichment was confirmed by a significant increase of myo-inositol in the apicoplast lipid fraction, as determined by GC-MS analysis (Fig. S5). The major molecular species of PI in both whole parasite extracts and apicoplasts were enriched in C18:0, C18:1, or C18:2 fatty acids, compared with other phospholipid classes, resulting in enriched levels of PI (36:2) and PI (36:3) (Fig. 3C). Molecular species containing C18:3 could originate from a putative plant-like α-linolenic (C18:3 synthesizing) pathway recently described in P. falciparum (46). Because PI is present at very low levels in uninfected erythrocytes, all of the PI detected in parasite and apicoplast membranes must have been synthesized de novo. In addition to its role as a membrane lipid, PI is a precursor for glycosylphosphatidylinositol anchors and phosphoinositides such as phosphatidylinositol 3-phosphate (PI3P) (40). PI3P is essential in Plasmodium blood stages (17) and has been localized to the apicoplast and surrounding vesicles in T. gondii, suggesting a role in vesicle-mediated transport either to or from the apicoplast (18). The elevation of PI in apicoplasts (Fig. 3B) could reflect continuous delivery of ER membrane containing both PI and PI3P to the apicoplast and the subsequent dephosphorylation of the latter.

Apicoplasts contain more lysophosphatidylcholines than whole parasites (Fig. 3B), which could have been generated during the purification/extraction procedure but could also reflect continuous remodeling of apicoplast phospholipids by lysophospholipases and acylglycerol-phosphate acyltransferases with neighboring ER (6, 47).

The apicoplast fraction had appreciable levels of diacylglycerol (DAG), which contained the highest enrichment for C16:0 and C18:0 of all lipid species (Fig. 3D). DAG is a precursor for the synthesis of most phospholipids and for triacylglycerols. Both DAGs and triacylglycerols are present in lipid droplets and may play an important role in hemoglobin formation in the food vacuole (48). DAG synthesis is thought to increase in trophozoites, possibly providing a pool of precursors for TAG synthesis in schizont stages (49). DAG can be synthesized from phosphatidic acid (PA) via phosphatidic acid phosphatases that are predicted to be located in both the cytosol/ER (PF3D7_0625000, PF3D7_0303200) and the apicoplast (PF3D7_0807500). Alternatively, DAG can be generated by the action of phospholipases C. Gene deletion studies have suggested that a PIP-specific phospholipases C is essential, highlighting the potential importance of this pathway and homeostasis of DAG levels (50).

Cholesterol was detected in both whole parasites and apicoplasts. Molecular species were detected by precursor ion scanning for m/z by 369.1 and coelution with authentic standard (Fig. A4). Cholesterol has previously been detected in P. falciparum–infected erythrocytes (51), but not in apicoplasts. To exclude the possibility that the cholesterol in apicoplasts was derived from erythrocyte plasma membrane contamination, purified apicoplasts were labeled with filipin, a fluorescent dye with high affinity to sterol-rich membranes. Bead-purified apicoplasts were strongly stained by filipin and PFOPTP, indicating that cholesterol is indeed embedded in one or more apicoplast membranes (Fig. 4B).
Precursor ion scanning at \text{m/z} also allowed detection of cholesterol-ester (CE) species (Fig. S6). However, further MS/MS analysis showed that only one species putatively corresponded to CE (18:0), whereas others corresponded to DAG species. Because Apicomplexa lack enzymes needed for sterol and CE synthesis, these lipids must have been acquired from the host cell. Cholesterol has previously been detected in \textit{P. falciparum} blood stages (49, 50) and is believed to be present in lipid bodies and/or rhoptries (48, 52, 53). The incorporation of host cholesterol and SM into the apicoplast membranes could contribute changes in multimembrane properties, affecting both the permeability to small molecules and, potentially, the activity of integral membrane transporter proteins.

All of the detected Cer contained a regular sphinganine base covalently bound to different fatty acid chains ranging from 14 to 24 carbons, predominantly saturated or monounsaturated (Fig. S5). The most abundant Cer contained C24 fatty acids, especially C24:1. Because neither C24:1 nor C24:0 were detected in apicoplasts by GC/MS (Fig. S4), Cer is likely a very minor component of apicoplast lipids (Fig. 3E). Cer can be synthesized from host SM by the parasite sphinomyelinase (49) and can induce \textit{Plasmodium} growth inhibition (54). Thus, Cer needs to be maintained at low physiological levels, as observed in our analysis (Fig. S5).

The purified apicoplasts lacked detectable levels of the plant-like galactolipids MGDG and DGDG. These glycolipids can be readily detected by neutral loss of \text{m/z} 179 ion and \text{m/z} 341 ion (21). MGDG and DGDG are the major membrane components of plant and algal plastid membranes (20, 33) including the photosynthetic plastids of \textit{C. velia}, a recently discovered algal relative of \textit{Plasmodium} and \textit{Toxoplasma} (20). Despite previous reports of plant-like hexosyl(galactosyl)glycerolipids and hexosyl (galactosyl)ceramides in \textit{T. gondii} (22), we could not detect any ion with a mass corresponding to chloroplast-like galactolipids in \textit{P. falciparum}. Thus, plant-like galactoglycerolipids are either completely absent or are undetectable in both whole parasites and purified apicoplasts using highly sensitive mass spectrometric methods. Galactoglycerolipids are critical for photosynthesis (55, 56) and appear to have been lost or remodeled after conversion to parasitism in Apicomplexa, apparently being replaced by PC and PE.

**Conclusions**

We have developed a unique method for purifying the apicoplasts of \textit{P. falciparum} asexual stages and provide a detailed lipidomic analysis of this organelle. Compared with bulk parasite membranes, the apicoplast membranes appear to be enriched in saturated fatty acids and the phospholipid PI. They also contain a range of lipid species (cholesterol, SM, and Cer) that likely represent uptake of host lipids and lack detectable galactolipids, a hallmark of plant/algal plastids. Together with [U-\text{13C}]glucose labeling studies, these analyses indicate that the biogenesis of intracelluar organelles in these parasites is normally dependent on the utilization of salvaged precursors. However, asexual stages retain the capacity to synthesize fatty acids via the apicoplast FASII complex when exogenous fatty acids are limiting. The development of the apicoplast purification method will now allow further dissection of the relative contribution of de novo versus salvage pathways in the biogenesis of apicoplast membranes.

**Materials and Methods**

**Parasite Cell Culture and Removal of RBC.** \textit{P. falciparum} strains (3D7) were cultured in T175 culture flasks (Dulbecco) in 75 mL of culture media as described (57). Cultures were tightly synchronized (2x in sorbitol lysis of late-stage parasites), fed daily, and harvested when the culture reached 15–35% parasitemia at mid trophozoite stage (24–30 h post-invasion). RBCs were removed by saponin lysis (0.15% in PBS) to release free whole parasites.

**Apoplast Purification.** Buffers and reagents were depleted of lipids. Parasites expressing either PfTPT-HA or HA-PfTPT (29) and WT parasites were tested for purifications. Saponin-released parasites were washed 3x in PBS and kept at 4 °C thereafter. Protein concentration of the released parasites was determined by Bradford assay. Trypsin digestion was performed with 25 μg of trypsin for every 1 mg of cell proteins for 5 min at 37 °C. The reaction was stopped by 40 μL of protease inhibitor mixture (Roche). Free parasites were washed in PBS containing protease inhibitor (Roche) followed by one wash in hypotonic buffer (1 mM Hepes NaOH, pH 7.4, 50 μg/mL DNase, Sigma and protease inhibitors; Roche). Parasites were lysed by suspension in hypotonic buffer and 20 passages through a 27-gauge needle. The lysate was made isotonic by the addition of 1/4 volume of 4x assay buffer (200 mM Hepes NaOH, pH 7.4, 200 mM NaCl, 8 mM EDTA, 6% fatty acid free BSA; Sigma). Unlysed parasites and nuclei were removed by centrifugation (1,500 g, 10 min, 4 °C). The organelle-enriched fraction including apicoplasts (supernatant) was precleared with unlabeled Dynabeads (5 μL) and activated (Dynabeads + 10 μL anti-rabbit Dynabeads/1 mL of sample; Invitrogen). The precleared beads were removed with a magnetic particle collector (Invi- trogen), and the remaining organelles incubated overnight at 37 °C with tosyl-activated Dynabeads (Invitrogen) precoated with mouse anti-HA antibody (Roche). Bound apicoplasts were collected with a magnetic particle collector followed by three washes in 1x assay buffer (10 min, 4 °C) and one PBS wash. The unbound fraction was collected for further experiments. Purified apicoplasts bound to beads were either stored at −80 °C or immediately used to further analysis.

**Lipid Metabolic Labeling, Extraction, and MS.** Infected RBCs were labeled with [U-\text{13C}]glucose under standard in vitro culture conditions or in minimal lipid depleted medium (45) as previously described (33). Total lipids were extracted and fatty acid composition was determined by GC-MS (21, 58). Lipid species were determined and quantified by LC-MS/MS (SI Materials and Methods).

**Western Blotting, Immunofluorescence Assay, and EM.** Samples were prepared and analyzed as described in SI Materials and Methods.

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