Carotenoid Biosynthesis in Intraerythrocytic Stages of Plasmodium falciparum

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Carotenoids are widespread lipophilic pigments synthesized by all photosynthetic organisms and some nonphotosynthetic fungi and bacteria. All carotenoids are derived from the C40 isoprenoid precursor geranylgeranyl pyrophosphate, and their chemical and physical properties are associated with light absorption, free radical scavenging, and antioxidant activity. Carotenoids are generally synthesized in well defined subcellular organelles, the plastids, which are also present in the phylum Apicomplexa, which comprises a number of important human parasites, such as Plasmodium and Toxoplasma. Recently, it was demonstrated that Toxoplasma gondii synthesizes ascorbic acid. We therefore asked if Plasmodium falciparum is also capable of synthesizing carotenoids. Herein, biochemical findings demonstrated the presence of carotenoid biosynthesis in the intraerythrocytic stages of the apicomplexan parasite P. falciparum. Using metabolic labeling with radioisotopes, in vitro inhibition tests with norflurazon, a specific inhibitor of plant carotenoid biosynthesis, the results showed that intraerythrocytic stages of P. falciparum synthesize carotenoid compounds. A plasmodial enzyme that presented phytoene synthase activity was also identified and characterized. These findings not only contribute to the current understanding of P. falciparum evolution but shed light on a pathway that could serve as a chemotherapeutic target.

Human malaria is caused by four species of the parasitic protozoan genus Plasmodium. Of these four species, Plasmodium falciparum is responsible for the vast majority of the 300–500 million episodes of malaria worldwide and accounts for 0.7–2.7 million annual deaths (1). Given the genetic flexibility and the resulting rapid development of resistance to almost every drug, a comprehensive understanding of plasmodial metabolic pathways is essential for the development of new chemotherapeutic strategies.

Millions of years ago, an ancestor of the phylum Apicomplexa gained a plastid by secondary endosymbiosis of a photosynthetic eukaryote (2, 3). This chloroplast was retained, and although some chloroplastid genes were lost during evolution, many were transferred to the Apicomplexan nucleus, ultimately giving rise to the apicoplast organelle, a structure essential for parasite survival (4).

In the case of malaria parasites, especially the most virulent species, P. falciparum, a series of new “plantlike” enzymes was recently discovered. Some of these enzymes are associated with the apicoplast (5), whereas the nature of the others and the pathways they are involved in remain elusive to current bioinformatics approaches.

The plant and algae plastids are the site for many essential biochemical pathways; some of them were already found in P. falciparum. It is possible that other metabolic routes were retained and incorporated in the parasite metabolism (6).

Among these biochemical pathways, the carotenoid biosynthesis is an attractive target for investigation, because it is essential in algae, higher plants, bacteria, and fungi but absent in mammals, and its products are involved in many important metabolic functions (7).

All carotenoids are derived from the isoprenoid biosynthesis pathway and possess a polyisoprenoid structure, a long conjugated chain of double bonds, and an almost bilateral symmetry around the central double bond. Their biosynthesis starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene, the initial C40 carotenoid skeleton. Different carotenoids are derived essentially by modifications in the base structure such as cyclization of the end groups and by introduction of oxygen functions, resulting in their characteristic colors and antioxidant properties (8).

Our group has previously demonstrated (9) that intraerythrocytic stages of P. falciparum biosynthesize dolichol of 11–12 isoprenic units and certain unknown compounds when [1-3H]geranylgeranyl pyrophosphate was used as a metabolic substrate.

The abbreviations used are: GGPP, geranylgeranyl pyrophosphate; [1-3H]GGPP, [1-14C]IPP, [1-14C]ISopentenyl pyrophosphate ammonium salt; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; PDA, photodiode array; ESI, electrospray ionization; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; THF, tetrahydrafuran; BHT, 2,6-di-tert-butyl-4-methylphenol; MOPS, 4-morpholinepropanesulfonic acid; HMM, hidden Markov model.

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precursor (10). Given that plant and algae plastids are sites for many essential biochemical pathways, we asked if \textit{P. falciparum} was able to produce carotenoids, once these compounds have GGPP as precursor (Fig. 1).

Using metabolic labeling with radioisotopes, \textit{in vitro} inhibition tests with a specific inhibitor of plant carotenoid biosynthesis, and bioinformatics analysis, we demonstrated, for the first time, that the intraerythrocytic stages of \textit{P. falciparum} in fact synthesize carotenoid compounds. The first enzyme of the pathway of the carotenoid biosynthesis was cloned, expressed, and biochemically characterized. The results introduced here not only contribute to the understanding of the evolution of the \textit{P. falciparum} but also introduce a possible new point of study for treatments of malaria.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Percoll® was purchased from Amersham Biosciences. [1-3H]Geranylgeranyl pyrophosphate triammonium salt ([1-3H]GGPP) (16.5 Ci/mmol), [1-14C]isopentenyl pyrophosphate ammonium salt ([14C]IPP), and L-[35S]methionine (>1,000 Ci/mmol) were obtained from Amersham Biosciences. Invitrogen supplied Albumax I. All sol-
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vents used were of HPLC grade or better. Syngenta Inc. (Basel, Switzerland) generously donated the herbicide norflurazon. Authentic standards of dolichol and polypropenol were kindly supplied by Dr. Tadeusz Chojnacki (Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland). Lithium iodide hydrate was purchased from Fluka. Sigma provided farnesyl pyrophosphate ammonium salt, geranylgeranyl pyrophosphate ammonium salt, all-trans-lutein, all-trans-β-carotene, and all other biochemical reagents. Carotenoid standards were donated by DSM Nutritional Products (Basel, Switzerland).

Plasmodium Falciparum Culture

The P. falciparum clone 3D7 was cultured in vitro according to Trager and Jensen (11) with the modification that parasites were grown in a 40-ml volume in an atmosphere of 5.05% CO₂, 4.93% O₂, and 90.2% N₂. In some experiments, an atmosphere of 5.05% CO₂, 20% O₂, and 74.95% N₂ was applied. The cultures were initially synchronized in ring stage (1–10 h after invasion) by two treatments with 5% (w/v) D-sorbitol solution in water, and the parasites were maintained in culture until the development of trophozoite (20–24 h after reinvasion) or schizont (30–35 h after reinvasion) stages. Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears.

Metabolic Labeling

Synchronous cultures of P. falciparum were labeled in the ring, trophozoite, or schizont stages with [1-³H]GGPP (3.125 μCi/ml) in normal RPMI 1640 medium for 16 h. Subsequently, parasites were then recovered, showing 25–30% parasitemia, in trophozoite, schizont, and ring stages, respectively. After labeling, the parasites cultures were centrifuged at 2,000 g for 10 min at room temperature, and each pellet was resuspended in 10 ml of phosphate-buffered saline (0.007 M Na₂HPO₄, 0.01 M NaCl) containing 0.1% of saponin in order to separate the parasites from erythrocytes. After three washes with phosphate-buffered saline at 10,000 g for 10 min, the parasites were lyophilized and stored in liquid nitrogen. Another protocol described by us (12) was used to evaluate protein biosynthesis in synchronous cultures of P. falciparum.

Carotenoid Extraction

Lyophilized parasites were extracted with four volumes of ice-cold acetone three times followed by centrifugation at 8,000 × g for 5 min. The pooled extracts were dried under a nitrogen stream and stored in liquid nitrogen.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Protocol I—The acetone extracts of each parasite stage obtained from metabolically labeled parasites were resuspended in 300 μl of acetonitrile, filtered through a 0.45-μm nylon filter (Advantec MFS, Inc., Dublin, CA), and then analyzed with a Phenomenex Luna C18 column (250 × 4.6 × 5 μm). A gradient elution system was used, with acetonitrile/ethyl acetate/water (88:2:10, v/v/v) as solvent A and acetonitrile/ethyl acetate/water (85:15:0) as solvent B. We applied the following gradient program: 0–15 min, 0–100% B; 15–45 min, 100% B; 45–50 min, 100 to 0% B; 50–55 min, 0% B. The flow rate was adjusted to 1 ml/min, and the column was maintained at 29 °C. The UV detector was set at 450 nm, and fractions were collected at 1-min intervals using a Gilson HPLC 322 pump (Gilson, Villiers-le-Bel, France) and also a gradient module connected to a 152 UV-visible detector, an 831 temperature regulator, and an FC203B fraction collector. We co-injected the samples with carotenoid extracts from six Amazon fruits previously characterized (13).

Protocol II—The acetone extracts of each parasite stage were resuspended in 20 μl of methyl tertiary-butyl ether, and then analyzed using a YMC C₁₈ polymeric column (4.6 × 250 mm, 3 μm and/or 5 μm) (YMC Inc.). We utilized a gradient elution system with methanol, 0.1% triethylamine (v/v) as solvent A and methyl tertiary-butyl ether as solvent B. The following gradient program was applied: 0–30 min, 95–70% A; 30–50 min, 70–50% A; 50–80 min, 50% A. The flow rate was set to 1 ml/min, and the column was maintained at 30 °C. Fractions were collected at 1-min intervals. In the experiments with radioactively labeled parasites, 20 μl of carotenoid extract mixture (obtained from Amazon fruits) in methyl tertiary-butyl ether that served as standard were co-injected. We carried out the analyses in an HPLC-photodiode array (PDA) equipped with a model 600 quaternary solvent delivery system (Waters, Milford, MA) and an on-line degasser, a Rheodyne injection valve with a 20-μl loop, and an external oven coupled to the model 996 PDA detector (Waters). We used the Millenium Waters software for data acquisition and processing. The PDA was set at 450, 346, and 288 nm for the analysis of carotenoids (13).

For purification and identification of dephosphorylated polyisoprenoid products (40 or more carbons), the other compounds made by PfB0130w, we carried out HPLC as described previously (14, 15).

In all experiments with radioactively labeled parasites, the resulting fractions were dried using a SpeedVac, resuspended in 500 μl of liquid scintillation mixture (PerkinElmer Life Sciences), and monitored with a Beckman LS 5000 TB β-counter.

Mass Spectrometry Analysis

We used a Finnigan LCQ-Duo ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) to collect the ion trap electrospray ionization-mass spectrometry (ESI-MS) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) data. The samples, which were purified by RP-HPLC (Protocol I) and corresponded to the all-trans-β-carotene and all-trans-lutein standards, were dried under a nitrogen stream and resuspended in 30 μl of chloroform/methanol (1:1, v/v), containing 2 mM lithium iodide. The samples (10 μl) were directly infused into the ESI probe across a 10-μl loop using an OMNIFIT N₂ pressure system (Omnifit Ltd., Cambridge, UK) with 10 p.s.i. pressure at a 10 μl/min flow rate. The all-trans-β-carotene and all-trans-lutein standards were run in the ESI positive ion mode with spray voltage, capillary voltage, and capillary temperature set to 4.52 kV, 17 V, and 250.0 °C, respectively. For ESI-MS/MS, relative collision energy of 40% (2 eV) was applied, and the sheath (N₂) and collision helium gas pressures were set to 1.5
millitorrs and 4 p.s.i., respectively. The MS spectra were acquired in full ion as well as in single ion monitoring modes.

The lithium iodide was used to improve the ionization of the carotenoid molecules in the ESI positive mode. Different from what was observed for the noncyclic polyisoprenoid compounds (16), the ions formed from carotenoid compounds did not present an addition of 7 Da, corresponding to the molecular mass of lithium. This ionization process improved the limit of detection of all-trans-lutein and all-trans-β-carotene from 1 nM and 2.5 nM, respectively, to ~100 pm.

**HPLC and LC-Atmospheric Pressure Chemical Ionization (APCI)-MS/MS Analyses**

Carotenoids were analyzed using C<sub>30</sub> columns, and the carotenoid standards used were obtained from six Amazon fruits previously characterized by LC-PDA and LC-PDA-APCI-MS/MS comparing with HPLC grade standards from DSM Nutritional Products (Basel, Switzerland) to guarantee the identity of the compounds present in each chromatographic fraction (13). Analyses of all-trans-β-carotene and all-trans-lutein from unlabeled schizont stages were performed in the same way as described above.

**Inhibition Tests with the Herbicide Norflurazon and Chloroquine**

We first diluted norflurazon in ethanol and then in RPMI medium to the following concentrations: 1000, 100, 10, 1, and 0.1 µM. Chloroquine was diluted directly in the RPMI 1640 medium, yielding concentrations of 20, 10, 5, 1, and 0.5 nM. Inhibition tests were carried out in flat-bottomed microtiter plates. We applied the method proposed by Desjardins et al. (17) to determine the 50% inhibitory concentrations (IC<sub>50</sub> values). The IC<sub>50</sub> chloroquine value used was lower than the one described by Cremer et al. (18).

The IC<sub>50</sub> values for growth inhibition were calculated by Probit Analysis (Minitab Statistical Software 13.30 TM; Minitab Inc.). After determination of the IC<sub>50</sub> values for norflurazon (25 ± 1.0 µM) and chloroquine (10 ± 0.5 nM), the parasites in ring stage were treated with either 25 µM norflurazon or 10 nM chloroquine for 24 h and were metabolically labeled with [1-<sup>3</sup>H]GGPP in the presence of the drug. Carotenoid compounds were then extracted and analyzed by HPLC (Protocol II) to determine the pattern of carotenoid compounds synthesized by the treated and untreated parasites.

**Rescue Assay**

Lycopene was solubilized in THF, 10 mM BHT and then diluted to 0.2 mM with human serum. Next, we added the THF/human serum-lycopene preparation to synchronous P. falciparum cultures treated with 25 µM norflurazon to yield a final lycopene concentration of 200 nM; the parasitemia was read every 24 h.

**Enzyme Assays of Crude Parasite Extracts**

We diluted 1–2 mg/ml Triton X-100 schizont extracts in an incubation buffer containing 2 mM NADP, 0.02 mM FAD, and 10 µM [1-<sup>3</sup>H]GGPP in a total volume of 1 ml. The extracts were then incubated at 30 °C for 3 h in the dark with agitation (19).
RESULTS

Biosynthesis of Carotenoids by the Intraerythrocytic Stages of P. falciparum—In a first approach, we tried to identify de novo synthesized carotenoids in vitro cultivated blood stage parasites. For this, $2.7 \times 10^9$ parasites from each stage (ring, trophozoite, and schizont) were metabolically labeled with [1-3H]GGPP, lysed by saponin treatment and extracted with acetone, and then analyzed on a C$_{18}$ reverse phase column (Protocol I). A radioactive fraction with retention times coincident with all-trans-lutein standard (12 min) was detected in all intraerythrocytic stages, whereas the radioactive fraction coincident with all-trans-β-carotene standard (38 min) was only detected in
Carotenoids in *P. falciparum*—In order to confirm the presence of carotenoid biosynthesis in the intraerythrocytic stages of *P. falciparum*, we performed an *in vitro* enzymatic activity assay using Triton X-100 crude extracts of 5 × 10^10 schizont parasites separated from erythrocytes by treatment with 0.1% saponin. Following the addition of [1-^3H]GGPP as a substrate and subsequent HPLC analysis (C_{30} column, 5 μm; Protocol II) of the acetone extracts, radioactive fractions with retention times coincident to all-trans-lutein (14 min), phytoene (20 min), all-trans-phytofluene (24 min), all-trans-β-carotene (35 min), cis-γ-carotene (39 min), and cis-δ-carotene (44 min) standards were observed (Fig. 3C). Under identical experimental conditions, extracts of uninfected erythrocytes did not lead to the production of any radioactive fractions (data not shown).

**Norflurazon Treatment Inhibits the P. falciparum Carotenoid Biosynthesis**—Next, we investigated whether carotenoid biosynthesis is essential for parasite survival or solely represents an evolutionary remnant. To this end, parasites were treated with norflurazon, a well known bleaching herbicide that inhibits carotenoid biosynthesis in higher plants and microalgae. Parasite growth was inhibited in a concentration-dependent manner (Fig. 4A) with an IC_{50} value of 25 ± 1.0 μM at 48 h of treatment. At this IC_{50}, no inhibition of protein biosynthesis was observed (Fig. S4). Interestingly, the inhibitory effect of norflurazon was partially reversed (77 ± 8.0%) by the addition of 200 nM lycopene (Fig. 4B), a downstream product in the carotenoid pathway. This finding suggests that the carotenoid biosynthesis pathway is commonly used by the parasite and that norflurazon affects it specifically. The effect of norflurazon on carotenoid biosynthesis in the schizont stage was further investigated. Consistent with the hypothesis that blockade of the phytene desaturase enzyme leads to accumulation of precursors and depletion of downstream products, the treatment of parasites with 25 μM norflurazon increased phytene content and diminished other carotenoid compound levels (Fig. 4C).

**Characterization of the P. falciparum Phytene Synthase, a Bifunctional Enzyme**—Since no carotenoid synthesis-related genes were specified in the *P. falciparum* genome, we
attempted to identify possible *Plasmodium* genes associated with the carotenoid biosynthesis pathway. An exhaustive bioinformatic search, based on HMMs and BLAST analyses, identified PfB0130w as a potential candidate for the *P. falciparum* phytoene synthase. Interestingly, our group previously characterized PfB0130w as an octaprenyl pyrophosphate synthase.

**FIGURE 3.** Analysis of carotenoid biosynthesis in intraerythrocytic stages of *P. falciparum* by RP-HPLC (C30 column), LC-APCI-MS, and LC-APCI-MS/MS.

A, radioactive elution profile of RP-HPLC analysis (C30 column, 3 μm; Protocol II) of the acetone extracts from the each intraerythrocytic stage (ring, trophozoite, and schizont; 2.7 × 10⁷ parasites of each stage) of *P. falciparum* metabolically labeled with [1-3H]GGPP. 1, all-trans-lutein; 2, phytoene; 3, all-trans-phytofluene; 4, all-trans-β-carotene; 5, cis-β-carotene; 6, cis-β-zeacarotene; 7, cis-γ-carotene. B, LC-APCI-MS and LC-APCI-MS/MS analysis of the acetone extracts of unlabeled schizont parasites (5.0 × 10¹¹). In the fractions with retention times coincident to all-trans-lutein and all-trans-β-carotene standards, matching ionization profiles (matching the respective HPLC grade standards from DSM Nutritional Products (Basel, Switzerland)) were observed, with all-trans-lutein and all-trans-β-carotene identified by the [M + H]⁺ ion at m/z 569 and the [M + H – H₂O]⁺ ion at m/z 551 and the [M + H]⁺ ion at m/z 537, respectively. The molecular structures were confirmed by comparing the APCI-MS/MS spectra of the ions with m/z 569 and m/z 537 from *P. falciparum* with those of the standards; identical dissociation patterns were found. C, carotenoid *in vitro* formation using Triton X-100 crude extracts of 5 × 10¹⁰ schizont parasites, separated from erythrocytes by treatment with 0.1% saponin, following the addition of [1-3H]GGPP as a substrate and subsequent HPLC analysis (C30 column, 5 μm; Protocol II). The mixture of carotenoid standards was co-chromatographed. 1, all-trans-lutein; 2, phytoene; 3, all-trans-phytofluene; 4, all-trans-β-carotene; 5, cis-β-carotene; 6, cis-γ-carotene.
(14). However, no candidates for other carotenoid synthesis-associated enzymes could be unambiguously identified (data not shown). Similar results were recently obtained by Nagamune et al. (23) in an attempt to identify absicic acid synthesis genes in T. gondii. The protein sequence that most corresponded to PbB0130w was the predicted phytoene synthase of the purple bacterium Rubrivivax gelatinosus (NCBI accession number BA949032, 21% amino acid identity after pairwise alignment) (Fig. S5). Although not confirmed by biochemical evidence, the Rubrivivax protein was predicted to contain a trans-isoprenyl diphosphate synthase head-to-tail domain similar to the one found in PbB0130w protein but distinct from the head-to-head domains present in other phytoene synthases (24).

To verify the existence of a phytoene synthase activity of the PbB0130w protein, we conducted in vitro enzymatic assays using [1-³H]GGPP as a substrate, followed by HPLC analysis (Protocol II). Only one radioactive fraction with a retention time coincident with the phytoene standard was observed (Fig. 5A). LC-APCI-MS/MS analysis of the product resulting from the enzymatic reaction using GGPP revealed the presence of the phytoene [M + H]⁺ ion at m/z 545 (Fig. 5B). We detected the same [M + H]⁺ ion when subjecting the phytoene standard to LC-APCI-MS (Fig. 5B). The molecular identity was confirmed by comparing the LC-APCI-MS/MS of the ion at m/z 545 from the enzymatic reaction (Fig. 5B) with that of the standard. Both spectra yielded identical and structurally diagnostic dissociation profiles.

When the truncated version of PbB0130w recombinant protein, previously described by our group (14), was used with the same substrate [1-³H]GGPP and under identical reaction conditions as for the analysis of phytoene synthase activity, no products with retention times corresponding to phytoene were found, indicating that the carboxyl-terminal domain of the enzyme is essential for the phytoene synthase function (data not shown). To investigate if the octaprenyl pyrophosphate synthase activity was present in the full-length PbB0130w gene product, we incubated the enzyme with farnesyl pyrophosphate ammonium salt and [14C]IPP under the conditions optimal for polyisoprenol synthesis and then detected products containing 40, 45, and 50 carbons (Fig. S6), a finding similar to that obtained with the truncated enzyme. The kinetic experiments showed that upon increase of the GGPP concentration in the full-length PbB0130w gene product assay mixture, a corresponding increase of the phytoene formation was observed, and saturation was achieved at GGPP concentrations above 300 μM. The Lineweaver-Burk plot of substrate-velocity data yielded a linear relationship with an apparent Kₘ of 21.7 ± 3.3 μM for GGPP and an apparent Vₘₐₓ of 34.6 ± 5.2 nmol mg⁻¹ protein h⁻¹ (Fig. 6). Since the recombinant full-length enzyme PbB0130w shows specific activity and Michaelis-Menten kinetics similar to those of the other phytoene synthases (20, 21, 25), a time kinetic study was performed. Similar to results published by Iwata-Reuyl et al. (20), phytoene production was detected after 15 min of incubation, and no significant increase after this was observed.

If the phytoene synthase activity of the PbB0130w protein is analogous to the one found in the carotenoid pathway of plants, then we expect this protein to be located in the apicoplast of the parasite. However, when scanning the peptide sequence with five different programs for intracellular location prediction, the related protein is localized in a mitochondrial compartment. In order to identify the localization, we conducted immunofluorescence assays using antibodies against the truncated version of this protein (14) (Fig. 5G). The PbB0130w protein was localized to the cytoplasm of P. falciparum late trophozoite stages, and the fluorescence partially overlapped with that occupied by the mitochondrial marker and anti-GFP (apicoplast) (Fig. S8).

Probable Antioxidant Action of Carotenoids in P. falciparum—To test if the carotenoids provide essential antioxidant protection for P. falciparum parasites, we treated the parasites with chloroquine (10 nM for 24 h), a drug that inhibits ferriprotoporphyrin IX degradation, causing the generation of reactive oxy-
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FIGURE 5. Recombinant full-length PfB0130w gene product activity and subcellular localization. A, RP-HPLC (C\(_{30}\) column, 5 \(\mu\)m; Protocol II) analysis of in vitro enzymatic reaction products of the recombinant full-length PfB0130w protein, showing phytoene to be the only product formed when [1-\(^{3}H\)]GGPP was used as a substrate with appropriate reaction conditions for phytoene production. B, LC-APCI-MS analysis of an in vitro enzymatic reaction using full-length PfB0130w protein and GGPP as substrate, showing the presence of phytoene as the [M + H]\(^+\) ion at \(m/z\) 545. LC-APCI-MS/MS analysis of the ions at \(m/z\) 545 illustrates matching diagnostic dissociation profiles for the product and phytoene standard.

The unambiguous identification of molecules is commonly conducted by the usage of at least two biochemical analysis procedures. The carotenoids biosynthesized by the intraerythrocytic stages of *P. falciparum* were shown by metabolic labeling with the direct precursor [1-\(^{3}H\)]GGPP and identified by two HPLC methods, suitable for these type of molecules (28), and confirmed by ESI-MS/MS and LC-APCI-MS/MS analyses, excluding any uncertainty about the molecular nature of the detected compounds. Apparently, the schizont stages contained the highest quantities of carotenoids when compared with rings and trophozoites. This indicates that carotenoid synthesis starts in ring stage and accumulates in schizont stage. Importantly, neither of these compounds was detected in uninfected erythrocytes or in RPMI culture medium and subsequent tests in *P. falciparum* extracts showed that the parasite comes with the machinery to synthesize carotenoids.

Our results carry us to an important question. Are the carotenoids important for the parasite metabolism, or do they only represent an evolutionary vestige? To verify this, we treated the parasites with norflurazon, a well known bleaching herbicide that inhibits carotenoids biosynthesis in higher plants and microalgae by competitive inhibition of the phytoene desaturase (29). Benz-Amotz et al. (30) colleagues showed that norflurazon treatment of the halotolerant green alga *Dunaliella bardawil* blocks the production of all-trans-carotene and provokes, in certain conditions, the accumulation of massive amounts of phytoene, a result that was also observed in other carotenogenic organisms.

In our hands, *P. falciparum* reacted quite comparably with *D. bardawil* and showed growth inhibition upon norflurazon treatment. Likewise, the inhibition could be partially reverted by the addition of lycopene, which seems to be readily taken up, thus providing the products of the norflurazon-inhibited step in the carotenoid pathway. The norflurazon inhibition was not completely reversed, most likely because lycopene is unstable in solution, and the solvent (THF) indicated for carotenoid delivery to cells is cytotoxic (31).

The genes related to several important activities exerted by *Plasmodium*, such as transcriptional control (32) or the shikimate metabolism, continue to be elusive to bioinformatic approaches (33). We tried to identify candidate sequences genes for the carotenoid biosynthesis in *P. falciparum*, search-
ing for known sequences involved in this pathway in other organisms against a Plasmodium local data base using BLAST (34) and HMMER (35) methods. Our in silico analyses suggest the presence of a candidate for phytoene synthesis. Intriguingly, the candidate gene encoding the enzyme phytoene synthase that synthesizes the first product of the carotenoid biosynthesis, phytoene, was previously characterized by our group as an octaprenyl pyrophosphate synthase (14). The plasmodial enzyme is a rare example of a carotenogenic enzyme with a continuous line of evolution from archaea to bacteria (via cya-

FIGURE 6. Substrate velocity plots depicting P. falciparum phytoene synthase activity dynamics to varying GGPP concentrations to determine kinetic parameters. A, rectangular hyperbola; B, Lineweaver-Burk plot.

FIGURE 7. Biochemical evidence for the antioxidant action of carotenoids in P. falciparum. A, synchronous parasite cultures in ring stage were treated with 10 nM chloroquine for 24 h and then metabolically labeled with [1-3H]GGPP for an additional 16 h in the presence of the drug. The effect of chloro-

Biochemical pathways localize to both organelles (40, 41). Also, mitochondrial metabolism is not common in this parasite; once this organelle does not present crests, it is not associated with the energy production, and the function of the tricarboxylic acid cycle in the mitochondrion is unclear (40). This allows for a possibility that carotenoid biosynthesis may take place in both
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organelles, like the heme biosynthesis that occurs in both the mitochondrion and plastids (37).

If one considers that the first enzymatic function of the PfB0130w protein results in an isoprenic compound of 40 carbons, probably the isoprenic chain of *P. falciparum* ubiquinone, the localization of this enzyme associated with the mitochondrial membrane would be expected. A problem arises regarding the phytoene synthase activity, which in plants is normally localized to chloroplasts (42). It may be questioned if this reaction can occur in the mitochondrial environment. The daffodil model of carotenoid biosynthesis can answer this question (43), and according to this model, the biosynthesis of carotenoids takes place in a membrane-associated enzyme complex, but different reactions and enzymes require different microenvironmental conditions, including membrane-resident electron acceptors and donors and anaerobic environments (44). We speculate that the daffodil model could represent a good approach to describe the carotenoid biosynthesis in *P. falciparum*; nevertheless, the detection of other carotenoid-related enzymes and knowledge about their exact localization are indispensable prerequisites for the description of a model.

Regarding the timing of expression, the PfB0130w protein is present in trophozoites and schizonts, coinciding with previous findings, which showed that (i) PfB0130w transcription occurred mainly in ring and trophozoite stages (14) and (ii) the highest levels of carotenoid (this study) and other isoprenoid derivatives (12, 45) were found in schizont stages.

The carotenoids apparently are important for the parasite metabolism, but what are their functions? Considering that the main purpose of carotenoid compounds, even in the photosynthesis, is antioxidant, it has been suggested that they possess specific tasks in the antioxidant network, such as protecting lipophilic compartments or scavenging reactive species generated in photooxidative processes, neutralizing mainly singlet molecular oxygen and peroxyl radicals (8). In the case of *P. falciparum*, during the evolutionary progression from mixotrophy to parasitism, the ancestral pathogen probably encountered an increase in oxidative stress. Perhaps, as an adaptive response to this increase in oxidative stress, *Plasmodium* gradually developed into a fermentative organism (46). On the other hand, the parasites live in a pro-oxidant environment that contains oxygen and iron, the key prerequisite for the formation of reactive oxygen species via the Fenton reaction, and it is not surprising that *P. falciparum* are heavily dependent on efficient antioxidant systems (47–52).

To verify if carotenoids could be involved in the antioxidant systems of the parasite, we induced oxidative stress by chloroquine treatment, since Ginsburg and Kruglik (53) showed that chloroquine inhibits the ferroprotoporphyrin IX polymerization, causing the generation of reactive oxygen species due to the Fe$^{2+}$ ion, which is not detoxified.

The treatment with chloroquine demonstrated an increase in the carotenoid levels, indicating that carotenoids could be involved in the antioxidative defenses of *P. falciparum*. Additionally, a more efficient inhibitory effect of norfuran on the growth of parasites under higher levels of O$_2$ (20%) and no differences with residronate treatment support this hypothesis. Obviously, these results are not sufficient to prove the involve-
