Ketocarotenoid Biosynthesis Outside of Plastids in the Unicellular Green Alga Haematococcus pluvialis*

Received for publication, July 19, 2000, and in revised form, November 10, 2000
Published, JBC Papers in Press, November 20, 2000, DOI 10.1074/jbc.M006400200

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The carotenoid biosynthetic pathway in algae and plants takes place within plastids. In these organelles, carotenoids occur either in a free form or bound to proteins. Under stress, the unicellular green alga Haematococcus pluvialis accumulates secondary carotenoids, mainly astaxanthin esters, in cytoplasmic lipid vesicles up to 4% of its dry mass. It is therefore one of the favored organisms for the biotechnological production of these antioxidative compounds. We have studied the cellular localization and regulation of the enzyme β-carotene oxygenase in H. pluvialis that catalyzes the introduction of keto functions at position C-4 of the β-ionone ring of β-carotene and zeaxanthin. Using immunogold labeling of ultrathin sections and Western blot analysis of cell fractions, we discovered that under inductive conditions, β-carotene oxygenase was localized both in the chloroplast and in the cytoplasmic lipid vesicles, which are (according to their lipid composition) derived from cytoplasmic membranes. However, β-carotene oxygenase activity was confined to the lipid vesicle compartment. Because an early carotenogenic enzyme in the pathway, phytoene desaturase, was found only in the chloroplast (Grünewald, K., Eckert, M., Hirschberg, J., and Hagen, C. (2000) Plant Physiol. 122, 1261–1268), a transport of intermediates from the site of early biosynthetic steps in the chloroplast to the site of oxygenation and accumulation in cytoplasmic lipid vesicles is proposed.

Carotenoids play major roles in oxygenic photosynthesis where they function in light harvesting and protect the photosynthetic apparatus from excess light by energy dissipation (1). Carotenoids that fulfill these processes are commonly referred to as primary carotenoids, because they are essential for the basic metabolism of the organism. In contrast, secondary carotenoids (SC)† are defined functionally as carotenoids that are not obligatory for photosynthesis and are not localized in the thylakoid membranes of the chloroplast (2). SC function in specific stages of development (e.g. flower, fruit), mainly for coloration or under extreme environmental conditions. In plants, SC are often accumulated in special structures, for instance in plastoglobuli of chromoplasts. In some green algae, however, SC accumulate outside the plastid in cytoplasmic lipid vesicles. One typical example is the unicellular microalga Haematococcus pluvialis, well known for its massive accumulation of ketocarotenoids, mainly astaxanthin and its acylesters, in response to various stress conditions, e.g. nutrient deprivation or high irradiation (3). Different functions of SC in H. pluvialis such as acting as a sunshade (4), protecting from photodynamic damage (5), or minimizing the oxidation of storage lipids (6) have been proposed. There is growing commercial interest in the biotechnological production of astaxanthin because of its antioxidative properties and the increasing amounts needed as supplement in the aquaculture of salmonoids and other seafood (7). H. pluvialis is one of the preferred microorganisms for this purpose because it accumulates SC at up to 4% of its dry mass (3).

The pathway of astaxanthin biosynthesis in H. pluvialis was elucidated by inhibitor studies (8), and most of the involved genes are cloned (6, 9, 10). In higher plants and green algae, the carotenoid precursor, isopentenylpyrophosphate (IPP) is derived from the DOXYP pathway (synonyms are nonenalolane or MEP pathway, Ref. 11). For SC synthesis in H. pluvialis this was confirmed with inhibitor studies (12). The first specific steps in carotenogenesis lead to the formation of the tetraterpenic phytoene. Following desaturation and β-cyclization, β-carotene is formed. The subsequent steps in the pathway leading to astaxanthin in H. pluvialis are catalyzed by β-carotene hydroxylase (10) and β-carotene oxygenase (CORTO, synonym is β-carotene ketolase, BKT; for a recent review see Cunningham and Gantt, Ref. 13 and Fig. 1).

Little is known about the regulation of SC synthesis in vivo in response to stress. The gene for CORTO, the enzyme studied in this paper, was cloned from two different strains of H. pluvialis by Lotan and Hirschberg (14) and Kajiwara et al. (15). A series of β-carotene oxygenases (among them one from H. pluvialis), and bacterial β-carotene hydroxylases were characterized in vitro with respect to substrate specificity and cofactor requirements (16, 17). Moreover, conversion of β-carotene by cell extracts of H. pluvialis was reported (18). Recently, we have studied regulation and compartmentation of phytoene desaturase (PDS), an early enzyme of the carotenoid biosynthesis pathway; LHC, light harvesting complex; MEP, 2-methyl-3-erythritol-4-phosphate; MGDG, monogalactosyldiacylglycerol; NF, norflurazon; PC, phosphatidylcholine; PDS, phytoene desaturase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; TAG, triacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

* Preliminary in vivo data were presented at International Congress XI on Photosynthesis in Budapest, 1998. This study was supported in part by Grant B301-69013 from the Thuringer Ministerium fur Forschung, Wissenschaft und Kultur. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Dedicated to the occasion of the 65th birthday of Prof. Dr. Wolfram Braune.

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† The abbreviations used are: SC, secondary carotenoids; BKT or CRTO, β-carotene oxygenase; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-erythritymethylhomoserine; DOXP, 1-deoxy-x-xylulose-5-phosphate; DPA, diphenylamine; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography; IPP, isopentenylpyrophosphate; LHC, light harvesting complex; MEP, 2-methyl-3-erythritol-4-phosphate; MGDG, monogalactosyldiacylglycerol; NF, norflurazon; PC, phosphatidylcholine; PDS, phytoene desaturase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; TAG, triacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

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The enzymes catalyzing the late enzymatic steps, namely β-carotene oxygenase (CRTO), β-carotene ketolase, BKT, presented in this paper, and the β-carotene hydroxylase (CRTR-B) are indicated.

H. pluvialis is distinguished in that it accumulates large amounts of carotenoids in lipid vesicles outside the plastid (3, 20). This has given rise to speculation about the possible existence of a biosynthetic pathway specific for secondary carotenogenesis that is localized in the cytoplasm, as was supported by the existence of two different IPP isomerases in H. pluvialis (6).

However, no extra pathway specific for SC biosynthesis in the cytosol of H. pluvialis was found at the level of PDS (19). It was therefore hypothesized that carotenoids are transported from the site of biosynthesis (plastid) to the site of accumulation (cytoplasmic lipid vesicles). Here, we present a study of the origin of these lipid vesicles as well as regulation and compartmentation of the SC biosynthetic-specific ketolase CRTO in flagellates of H. pluvialis using immunolocalization and cell fractionation techniques. Our results indicate that the last oxygenation steps in the astaxanthin biosynthesis pathway take place outside the plastid in the cytoplasmic lipid vesicles and is discussed relative to the role of this sequestering structure in SC accumulation.

MATERIALS AND METHODS

Cell Growth Conditions—H. pluvialis Flotow (No.192.90, culture collection of the University of Göttingen, Germany; synonym: _Haematococcus lacustris_ (Girod) Rostafinski) was grown autotrophically in a two-step batch cultivation system as described (21). Following precultration for 5 days at 25 μmol of photons m⁻² s⁻¹ of white fluorescent light (Osem L36/W25, Berlin, Germany), flagellates in the logarithmic growth phase were exposed to SC inducing conditions (nitrate-deprived medium and 150 μmol of photons m⁻² s⁻¹ of continuous white light) leading to accumulation of SC in the lightadapted developmental state of _H. pluvialis_ (21). These flagellates surrounded by a thin extracellular membrane are more accessible to biochemical and ultrastructural analysis than the thick-walled and resistant aplanospore state.

Photor flux densities were measured using a LI-189 photometer (LI-COR, Lincoln, NE), and cell number was determined using a Cell Counter CaSy 1 (Schräfe Systems, Reutlingen, Germany). At the time points specified, sample aliquots corresponding to a defined cell number were collected by centrifugation at 1,400 × g for 2 min.

Preparation of Cell Fractions—Cell fractions were prepared by gentle filtration rupture that produced less contamination of the lipid vesicle fraction by light harvesting complexes (LHCl) and chlorophylls than sonication. Aliquots of cells were harvested by centrifugation at 10,000 × g for 2 min and resuspended in buffer consisting of 0.1 M Tris-HCl, pH 6.8, 5 mM MgCl₂, 10 mM NaCl, 10 mM KCl, 5 mM Na₂EDTA, 0.3 mM sorbitol, 1 mM aminobenzoamide, 1 mM aminohexanacid, and 0.1 mM phenylmethylsulfonyl fluoride. The hyperosmotically shocked cells were broken by passage through a 10-μm isopore polycarbonate filter (Millipore, Eschborn, Germany). The filtrate was centrifuged at 10,000 × g for 10 min at 4 °C to yield a chloroplast and cell debris pellet. The supernatant was transferred to a fresh tube and centrifuged again at 10,000 × g for 10 min at 4 °C. The suspension below the lipid vesicle fraction floating on top was transferred to a fresh tube and centrifuged at 76,000 × g for 2 h at 4 °C. The resulting microsome pellet was separated from the supernatant fraction. All fractions were stored at −20 °C.

Lipid Analysis—Cell aliquots or lipid vesicle preparations were extracted essentially as described (22). Lipids were then separated on HPTLC plates (Merk, Darmstadt, Germany), developed for two-thirds of the plate in chloroform/methanol/acetic acid, 73:25:2:4 (v/v/v/v) to separate the neutral lipids from the pigments. Lipids were identified by cochromatography of standard substances and by color reaction with different spray reagents (ninhydrin for free amines of phosphatidylethanolamine (PE) and phosphatidylserine (PS); α-naphthol for glyco- and sulfolipids; molybdenium blue for phospholipids; Dragendorff’s reagent for quaternary amines, phosphatidylcholine (PC) and dicylglyceroltrimethylhomoserine (DGTS)). Quantification of individual lipids was performed densitometrically after visualization by Godin’s spray reagent (23) and calibration with standard substances. For quantification of DGTS and PS, calibration data of PE and PC were used, respectively.

Treatment with Inhibitors of Carotenogenesis—At the onset of SC inducing conditions, diphenylamine (DPA, Sigma) was added to a final concentration of 30 μM. After 3 days, cells were washed three times, resuspended in fresh nitrate-deprived medium, and incubated for 2 more days under SC inductive conditions with 5 μM norflurazon (NF; SAN 9879; Sandoz Basel, Switzerland), 30 μM DPA or 30 μM DPA plus 5 μM NF, respectively.

Protein Preparation—The 17-amino acid peptide LP/HRRLSGR/GFLPAPA (corresponding to the C terminus of residues 304–329) in the predicted sequence of BKT (15) and residues 315–329 (with the last three amino acids missing) in the predicted sequence of CRTO (14), was chemically synthesized and purified (Alpha Diagnostics International, San Antonio, TX). The peptide was coupled to thyroglobulin by means of glutaraldehyde and used for immunization of rabbits to raise polyclonal antibodies as described (24). The raw serum was deployed without further purification.

Protein Analysis—Cell pellets were thawed on ice, suspended in break buffer, and broken by sonication for 1 min on ice (Vibra-Cell 72405 sonication processor, Sonics & Materials, Danbury, CT; pulse mode, 0.75 s on, 1 s off, 60 watts output). Break buffer with SDS was added to yield a final concentration of 2% SDS (w/v). Solubilization, especially of hydrophobic proteins like CRTO, was carried out upon shaking at 2,000 rpm for 2 h at 20 °C. Samples were centrifuged to remove unsolubilized material, and sample loading buffer to a final concentration of 50 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), and 0.01% bromphenol blue was added. Cell fractions were thawed on ice and resuspended in break buffer with 2% SDS (w/v), and solubilization was performed as described for total cell extracts. Before loading cell aliquots, samples were boiled for 5 min. Proteins were separated on 12% SDS-polyacrylamide gels essentially as described (25). For Western blot analysis, the gels were electrophoretically transferred semidry onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and treated with Ponceau S for staining the protein ladder transiently. Membranes were blocked in blocking buffer containing 5% w/v nonfat dry milk, 1% Tween 20 (w/v), 150 mM NaCl, and 25 mM Tris-HCl, pH 7.6 at 4 °C overnight. The blots were incubated with anti-CRTO antibodies in blocking buffer at 1:2,500 dilution for 2 h at 4 °C and thereafter with secondary antibody alkaline phosphatase conjugates (Bio-Rad, Munich, Germany) used at 1:500 dilution. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT), the labeling was quantified using densitometry (Scanpack 3.0, Biometra, Göttingen, Germany).
Pigment Extraction and HPLC Analysis—Cell pellets were extracted quantitatively in 100% acetone at 4 °C, and the pigment content was determined spectrophotometrically according to Lichtenenthaler (27). Fractions were freeze-dried, and carotenoids were extracted with 200 μl of acetone (the chloroplast fraction was extracted with 500 μl of acetone) at 4 °C. In vitro incubations were freeze-dried, and pigments were extracted with acetone, at 4 °C. Prior to HPLC analysis, samples were filtered and 20% water (v/v) was added. HPLC analysis was performed as described (21).

RESULTS

Origin of the SC-accumulating Lipid Vesicles—Lipid profiles of total extracts from cells drawn after 4 days of exposure to conditions inductive for SC synthesis revealed massive accumulation of TAG during SC synthesis (Fig. 2). Concomitantly, the amount of most membrane lipids, especially of MGDG, decreased whereas that of DGDG and DGTS increased slightly (Table I). Analysis of the lipid vesicles formed under inductive conditions revealed triglycerides as their predominant lipid class. Membrane lipids accounted for less than 5% (w/w) of total lipids in this fraction. No MGDG was detectable, and DGTS was made up of more than half of the membrane lipids in this fraction besides significant amounts of PC and of PE.

Coupled In Vivo Inhibitor Treatments—Application of low concentrations of DPA under conditions inductive for synthesis of SC led to accumulation of β-carotene instead of ketocarotenoids in H. pluvialis (8, 21, 28). Lipid vesicles in the cytoplasm of treated cells appeared yellow instead of red in control samples, suggesting that β-carotene accumulated in the cytoplasm (8). To substantiate this observation, we determined the pigment composition in different cellular compartments. Results revealed a predominant accumulation of β-carotene inside the lipid vesicles of DPA-treated cells (Fig. 3). To test if this extraplastidic β-carotene can be converted to astaxanthin, DPA was removed concomitantly with the addition of NF to inhibit carotenoid de novo synthesis at the level of phytoene desaturase. Beside the known bleaching effect of NF leading to a reduced amount in total carotenoids, a significant decrease in the ratio of β-carotene to ketocarotenoids occurred inside the lipid vesicles (Fig. 3). The pattern of SC in this fraction did not differ significantly from untreated samples consisting mostly of mono- and diesters of astaxanthin.

Generation of Antibodies against CRTO—Compartmentation studies and regulation analysis of the late steps in SC biosynthesis in H. pluvialis require specific antibodies against the enzymes involved. Attempts to obtain antibodies against the His-tagged C terminus of CRTO, encompassing two-thirds of the polypeptide overexpressed in Escherichia coli, were unsuccessful. Despite poor expression and isolation difficulties because of pronounced hydrophobic behavior of the protein, necessary amounts of the antigen were recovered by Ni²⁺-affinity chromatography and subsequent purification steps. The generated polyclonal antibodies recognized a series of proteins on Western blots and did not meet the needs for localization experiments, even after parallel immunization experiments and

Table I

| Time after onset of induction | Extract | Lipidsa |
|------------------------------|---------|---------|
|                              |         | TAG     | MGDG    | DGTS    | SQDG    | DGTS    | PE      | PC      | PG      | PS      |
|                              |         | pg per cell | % of total lipid mass | |
|                              | day     | n.d.b   | 4.97    | 4.82    | 6.68    | 7.40    | 7.79    | 3.65    | 2.29    | 1.21    |
| 0                            | Total cells | n.d.b   | 12.20   | 5.81    | 6.20    | 8.40    | 6.79    | 2.11    | 1.51    | 1.32    |
| 4                            | Total cells | 76.09   | 12.20   | 5.81    | 6.20    | 8.40    | 6.79    | 2.11    | 1.51    | 1.32    |
| 4                            | Lipid vesicles | 95.46   | 1.07    | 0.54    | 1.19    | 0.66    | 0.68    | 0.14    | 0.26    |

a Abbreviations of lipids are as in Abbreviations footnote.

b n.d., not detectable.

SE (n = 4 to 8) did not exceed 10%.

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Germany). Total protein content was determined by means of the detergent compatible protein assay kit (Bio-Rad, Munich, Germany).

Electron Microscopy and Immunolocalization—For ultrastructural examination, algal cells were harvested at 550 × g for 3 min and then fixed with 0.7% glutaraldehyde, 0.8% paraformaldehyde, and 1% OsO₄ simultaneously in growth medium for 25 min at 4 °C. After several washes in distilled water the specimens were dehydrated in graded ethanol series. The 70% ethanol step was performed in the presence of 3% uranylacetate for 10 min. Cells were embedded in LR Gold (London Resin, London) according to the manufacturer’s instructions. Before immunogold labeling, ultrathin sections were cut as described (19) and etched to unmask antigenic determinants (26). Etching was done by floating grids section side down on 2% H₂O₂ for 2 min at room temperature followed by three washes on distilled water. The grids were exposed to anti-CRTO antibody at 1:100 dilution, and immunogold labeling was performed as described (19). Subsequent to poststaining with 3% aqueous uranylacetate (w/v) for 5 min and 1% aqueous lead citrate (w/v) for 20 s, immunogold-labeled sections were examined in a Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany) at 80 kV.

In Vitro Incubations—Incubations were carried out in a total volume of 600 μl under conditions essentially as reported (16, 17). Cell fraction aliquots of 10⁶ cells were suspended in break buffer (0.1 M Tris-HCl, pH 6.8, 0.3 M sorbitol, 1 mM amminobenzamide, 1 mM aminohexanacid, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) in a total volume of 300 μl. Following the addition of 295 μl of cofactor buffer (5 mM ascorbic acid, 1 mM dithiothreitol, 0.5 mM FeSO₄, 0.1% deoxycholate (w/v), 0.5 mM 2-oxoglutarate) and brief mixing, the reaction was initiated by addition of 5 μl of a 1% β-carotene stock solution (w/v) in chloroform. In parallel samples, 100 μM DPA were added to inhibit β-carotene oxygenase. Incubation was performed under continuous stirring for 2 h in the dark at 30 °C. Reactions were terminated by freezing the samples in liquid nitrogen.

Fig. 2. Changes in the lipid pattern of H. pluvialis. Lipid extracts of flagellates before (lane 2) and after 4 days (lane 3) of exposure to conditions inductive for SC biosynthesis and SC-containing lipid vesicles from the latter stage (lane 4) were analyzed by HPTLC and were visualized by Godin’s spray reagent (lane 1, standard substances).

The relative position of the lipids is indicated on the left; lipids are defined in the abbreviations footnote. pigm., pigments; S.E.T, likely sterol esters.
various purification approaches by affinity chromatography. Interestingly, we noticed an increasing oligomerization tendency of the overexpressed antigen up to the octamer, even under denaturing SDS-polyacrylamide gel electrophoresis conditions, dependent on storage time. Finally, we immunized rabbits with a 17-mer synthetic oligopeptide corresponding to the C-terminal part of the predicted structure of CRTO. Database searches revealed no counterparts of this peptide among plant amino acid sequences.

Abundance of CRTO during SC Accumulation—The ability of the antibodies to recognize less than 30 ng of CRTO in Western blots was verified with the E. coli-overexpressed C-terminal part of the enzyme (data not shown). Abundance of CRTO was examined in total cell extracts of start samples and of samples taken 1, 2, 3, 4, and 7 days after inducing SC synthesis in the flagellates of H. pluvialis by intense illumination and nitrate deprivation (Fig. 4A). No CRTO was observed before the second day after induction. After this time, the amount of a 34-kDa protein increased rapidly in parallel to SC accumulation (Fig. 4B). The apparent molecular mass of the recognized protein was ~3 kDa smaller than predicted from the cDNA sequence of CRTO (14). The preimmune serum did not detect this polypeptide (not shown).

Immunogold Localization of CRTO—The antibodies against the C-terminal 17-mer of CRTO were tested on LR Gold sections of H. pluvialis flagellates that have previously been shown to present the best combination for structural preservation and maintenance of antigenic structures (19). To prevent the extraction of cellular lipids during dehydration steps and to ensure full preservation, high pressure cryofixation in combination with cryodehydration was applied. However, despite a number of modifications of the preparation protocol, the structure of lipid vesicles could not be improved. Thus, an etching technique was chosen as described (26). During the ethanol dehydration process before embedding, the lipid vesicles remained intact because of lipid cross-linking by OsO₄. Probing the sections with polyclonal antibodies against different photosynthetic proteins (19) did not reveal any signals because of masking of antigenic determinants by the fixative. To unmask antigenic determinants after etching was confirmed with anti-LHC and anti-PDS antibodies, which detected the corresponding polypeptides as reported previously (19). After challenging the sections with the polyclonal antibodies raised against the CRTO C-terminal 17-mer, two cell compartments became specifically immunogold-labeled in the course of SC synthesis, namely the chloroplast and, becoming dominant, the lipid vesicles (19). The only notable signal in the cytosol was obtained not restricted to the periphery, but was scattered throughout the vesicles. The only notable signal in the cytosol was obtained after 2 days of inductive conditions (15%) and was localized in close contact to the Golgi cisternae (not shown). No specific labeling was observed when sections were probed with preimmune serum (not shown).

Detection of CRTO in Subcellular Fractions—To ascertain the results from the immunogold localization experiments, four cellular fractions were obtained: (i) a pellet containing mainly the chloroplast, (ii) a supernatant fraction, (iii) microsomes and cytoplasmic membranes, and (iv) the lipid vesicles (19). The polypeptides in each fraction were analyzed by Western blots using the anti-CRTO antibodies. A 34-kDa polypeptide was observed in the chloroplast membrane fraction (Fig. 6), in the lipid vesicle fraction and, to a small extend, in the microsome fraction. Fractions shown here were derived from flagellates 7 days after start of SC induction, thus representing the maximum of CRTO protein in total extracts (Fig. 4).

In Vitro Metabolism of β-Carotene in Cell Fractions—To provide additional support for astaxanthin synthesis inside the lipid vesicles, we investigated the ability of various subcellular fractions to metabolize β-carotene in vitro. Cofactors and reaction conditions were essentially as reported recently for recombinant β-carotene oxygenases from different organisms (16, 17). Cell fractions were prepared from flagellates exposed for 3 days to SC inductive conditions, which contained relatively low
initial amount of ketocarotenoids. We observed a conversion of β-carotene to ketocarotenoids in the lipid vesicle fraction, but not in the chloroplast fraction (Fig. 7). The SC product pattern included mainly mono- and diesters of astaxanthin. The pool sizes, i.e. the total of β-carotene and ketocarotenoids, remained constant in all samples. Control experiments with heat-denatured extracts (16) were not feasible because of concomitant degradation of fraction pigments. Therefore, DPA was applied to inhibit β-carotene oxygenase but did not completely prevent the conversion of β-carotene in the lipid vesicle fraction. This is consistent with results from in vivo experiments (Fig. 3, Ref. 21).

**DISCUSSION**

Carotenoid accumulation in plant cells requires specialized accumulation structures (29). Changes in the lipid composition during the period of induction of SC synthesis in *H. pluvialis*, namely prominent TAG accumulation and remarkable reduction of the chloroplast-specific lipid MGDG, reflect the microscopically visible formation of lipid vesicles (20, 30) and corresponding changes in the photosynthetic apparatus (30, 31), respectively. To elucidate whether the SC-accumulating lipid vesicles of *H. pluvialis* are derived from the plastid or from cytoplasmic compartments, we analyzed their lipid composition separately. As expected, TAG made up the main part of lipids in this fraction (95%). Of the membrane lipids, the plastidic MGDG was totally absent, whereas PE as a typical nonplastidial lipid was found. DGT, the major membrane forming lipid in the SC-containing vesicles, which are surrounded by a half-membrane (20), is known to be primarily localized in nonplastidial membranes (32). The second abundant lipid DGDG was recently shown to be synthesized in the cytoplasm under nutrient starvation conditions (33). Altogether the results point to a cytoplasmic origin of the lipid vesicles presenting an oleosome-like structure (34).

Low concentrations of DPA inhibit ketocarotenoid biosynthesis by preventing the introduction of oxygen functions and, thus, β-carotene accumulates instead of ketocarotenoids (8, 21, 28). Furthermore, Harker & Young (8) observed in cells pretreated with DPA that in the presence of norflurazone, a known inhibitor of phytoene desaturase, SC were formed at the expense of β-carotene. We repeated this experiment with our cultivation scheme where SC accumulate in the flagellated state of *H. pluvialis*, thus allowing pigment analysis of the cytoplasmic lipid vesicles after cell fractionation. Surprisingly, the β-carotene that accumulated inside these lipid vesicles was oxygenated to ketocarotenoids. This implies that the cytoplasmic-located lipid vesicles play a role in the synthesis of SC in addition to their function as storage structure for these compounds.

A crucial point in understanding the regulation of secondary carotenogenesis in *H. pluvialis* is the localization of the enzymes involved. Therefore, immunolocalization using antibodies against SC-specific enzymes was chosen to gain corresponding data. The problems that occurred during overexpression of the SC-specific CRTO and its subsequent purification brought us in contact with its special properties, particularly the very hydrophobic behavior of the enzyme. Probably because of sequence similarity of CRTO to fatty acid desaturases (13) and the relatively high antigenicity of the conserved di-iron binding regions containing histidine residues (35), the polyclonal antibodies generated from the overexpressed antigen showed cross-reactivity with many other proteins. In contrast, antibodies generated against a 17-mer synthetic oligopeptide representing the C-terminal part of the predicted structure of CRTO, still recognizing the CRTO polypeptide expressed in *E. coli*, reacted specifically with a 34-kDa polypeptide in the protein extract of *H. pluvialis* cells. The slight decrease in the apparent molecular mass as compared with the size predicted (14, 15) might indicate processing of a N-terminal transit signal peptide. From the highly conserved structure of the β-carotene oxygenases from two different strains of *H. pluvialis* with respect to the oligopeptide used for immunization and from the polyclonal nature of the antibodies, it can be concluded that isoenzymes of CRTO should have been recognized in our cytoimmunochemical experiments. Additionally the similar size of CRTO observed in the chloroplast and in the lipid vesicles did not support the existence of isoenzymes or different β-carotene oxygenases in *H. pluvialis* as was speculated from the two existing sequences (6). More likely this reflects strain differences.

The observed pattern of CRTO induction in parallel to carotenoid accumulation denotes the essential role of the enzyme in SC biosynthesis. β-carotene oxygenase mRNA levels have been shown to exhibit similar kinetics of induction during the first 4 days of our cultivation scheme, but thereafter they declined to

**Table II**

| Time of exposure | Mean of gold particles per cell | Distribution of immunogold labeling in various compartments<sup>a,b</sup> | Residue |
|------------------|--------------------------------|-------------------------------------------------|---------|
|                  |                                 | Chloroplast | Nucleus | Cytosol | Lipid vesicles |       |
| **day**          |                                 | %          | %       | %       | %            | %      |
| Start            | 3.2 ± 2.2                       | 31.6 ± 60.1 | 5.3 ± 13.4 | 65.8 ± 17.4 | 0.0 ± 0.0 | -2.6 ± 9.2 |
| 2                | 36.8 ± 2.6                      | 64.6 ± 5.4 | 0.0 ± 0.0 | 14.7 ± 2.3 | 15.0 ± 2.1 | 5.7 ± 1.3 |
| 4                | 79.8 ± 1.0                      | 55.1 ± 11.5 | 0.7 ± 0.5 | 5.1 ± 0.7 | 37.1 ± 3.4 | 2.0 ± 0.3 |
| 7                | 29.8 ± 2.9                      | 30.7 ± 7.4 | -4.2 ± 3.0 | -3.0 ± 1.9 | 74.7 ± 4.6 | 1.8 ± 1.9 |

<sup>a</sup> After subtraction of the corresponding count found in preimmune labeled sections.

<sup>b</sup> S.E. of day 4 and day 0; days 2 and 7 are given for n = 24 and n = 12, respectively.

**Fig. 5.** Immunogold labeling of β-carotene oxygenase in flagellates of *H. pluvialis* exposed for 4 days to SC-inducing conditions. Section of a flagellate showing the peripheral part of a cell is presented. Bar represents 1 μm. Ch, chloroplast; Ex, extracellular matrix; Li, lipid vesicles; St, starch; Va, vacuole.
50% of the maximum (19). This behavior was different for the earlier carotenogenic enzyme PDS that showed a parallel change in the amounts of mRNA and of the protein (19). Thus, beside regulation at the mRNA level, post-translational mechanisms seem to be involved in CRTO induction.

Immunogold labeling of ultrathin sections revealed that in contrast to PDS, which is localized in the chloroplast only, CRTO is present both in the chloroplast and inside the SC-containing lipid vesicles. Interestingly, the signals were not restricted to the vesicle boundary but were distributed throughout the whole lumen of the vesicles, consistent with the observed hydrophobic behavior of CRTO. Because this location was confirmed by Western blot experiments in cell fractions, we conclude that CRTO occurs in both compartments. Colocalization of proteins and carotenoids in sequestering structures was reported for the carotene globule protein (CGP) in Dunaliella bardawil, a close relative of H. pluvialis (36). This protein is restricted to the periphery of the globules and was suggested to function in stabilizing the β-carotene globule structure within the chloroplast. A similar function beside ketolase activity is unlikely for CRTO, because of its low abundance. The discrepancy between the exclusive chloroplast localization of PDS that is up-regulated during SC biosynthesis (19) and the in vivo and (exclusive) in vitro CRTO activity in the lipid vesicles implies a transport of carotenoid precursors, possibly of β-carotene, across the chloroplast envelope into the cytoplasm where they are sequestered in the lipid vesicles. However, electron microscopic investigation did not reveal any structure for such a transport, at least not at the level of membrane-enclosed vesicles (30).

Two enzymes are involved in the biosynthesis of astaxanthin from β-carotene, β-carotene C-4 oxygenase (ketolase) and β-ring hydroxylase. The in vivo and in vitro conversion of β-carotene to astaxanthin in the cytoplasmic lipid vesicles also predicts the occurrence and activity of a β-ring hydroxylase in this compartment. This is of particular interest, because a β-carotene hydroxylase exists in the chloroplast too, as is evident from the formation of zeaxanthin. As the closest relatives of CRTO, fatty acid desaturases are localized in a number of different compartments, among them the chloroplast and microsomes (13, 37). These enzymes act on very hydrophobic substrates. That could be the origin of the ability of CRTO to act in the extraordinary environment of lipid vesicles. On the other hand, this hydrophobic environment might provide an explanation for the sustained increase of the CRTO protein despite the decreasing mRNA level, by protecting protein from protein-protein attacks. A possible explanation for the mode of action of CRTO in this compartment is that the enzyme, albeit its presence throughout the lipid vesicle matrix, is active only at the periphery, allowing access to cofactors needed because of the spatial closeness to ER structures and Golgi vesicles. This hypothesis is strengthened by electron microscopic observation on this prominent colocalization (20).

Evolutionarily, one could imagine a desaturase engaged in chloroplast fatty acid desaturation that was excreted into the cytoplasmic lipid vesicles and subsequently acquired the competence to oxygenate β-carotene. Although a search for transit signal peptides did not yield a clear result, the nuclear-encoded CRTO could be transported first into the chloroplast as proposed for all plant carotenoid biosynthesis enzymes (11), including the β-carotene hydroxylase (13). β-carotene oxygenase might then be exported from the chloroplast into the cytoplasm, possibly together with the substrate β-carotene, accumulating in the oselos-like lipid vesicles. The tendency of the almost complete CRTO antigen to form multimers could play an important role, causing a changed secondary structure of the complex favorable for transport outside the chloroplast. Additionally, the monomer might be the active form of the enzyme that can be established only in a very hydrophobic environment. That speculation is supported by the fact that in vivo CRTO activity is increased by the presence of a strong detergent, deoxycholate, in the cofactor buffer (17). Import studies that could test our hypotheses cannot be performed with H. pluvialis due to the reticulate structure of the chloroplast and the difficulties associated with isolating this compartment intact.

Further studies will focus on the carotenoid transport from the chloroplast and the role of the lipid vesicles as storage structures in the regulation of SC accumulation. The latter fact was brought recently into consideration. Rabbani et al. (38) showed that in the unicellular alga D. bardawil, the secondary β-carotene accumulation in intraplasmatic lipid droplets is controlled by the formation of this sequestering structure. Our hypothesis on the origin of CRTO would also imply that β-carotene accumulation in D. bardawil represents the phylogenetically older type of SC accumulation in plants, conserved in chromoplasts of higher plants.

Acknowledgments—We thank Prof. W. Braune who had suggested and encouraged us to work on Haematococcus. We are grateful to M. Fiedler for technical assistance, V. Mann, M.-A. Rebhan, and M. Utting for practical support and helpful discussions. We also acknowledge M. Eckert (Jena, Dept. of Zoology) for advice regarding the antibody generation. M. Melzer (IPK Gatersleben) for helping us with cryofixation and cryoehydration, Dr. M. Ramm (Jena, Dept. of Pharmaceutical Biology) for valuable advice on thin layer chromatography and L. Johanningeimer (University of Halle) for kindly providing anti-spinach-LHC and anti-spinach-rubisco small subunit antibodies.

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J. Biol. Chem. 2001, 276:6023-6029.
doi: 10.1074/jbc.M006400200 originally published online November 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006400200

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