Astaxanthin accumulation in *Haematococcus* requires a cytochrome P450 hydroxylase and an active synthesis of fatty acids

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Abstract Astaxanthin accumulation by green microalgae is a natural phenomenon known as red snows and blood rains. The fact that astaxanthin synthesis requires oxygen, NADPH and Fe^{2+} led Cunningham and Gantt [Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 557–583] to propose that a cytochrome P450-dependent enzyme might be involved in the transformation of β-carotene to astaxanthin. In *Haematococcus* only esterified astaxanthin molecules accumulate, but it is not determined whether a fatty acid synthesis should occur simultaneously to allow pigment accumulation. The aim of this contribution was to answer these two questions using specific inhibitors of β-carotene (norflurazon) and fatty acid (cerulenin) synthesis, and of cytochrome P450 enzyme activity (ellipticine). © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Secondary carotenoid synthesis; High-light stress; Ketocarotenoid; Green alga *Haematococcus pluvialis*

1. Introduction

Under high-light stress *Haematococcus pluvialis* synthesizes the secondary carotenoid astaxanthin [2,3]. In vivo measurements suggest that two different pathways for astaxanthin biosynthesis are functioning: the first one would start by oxidation of β-carotene and would have echinenone, canthaxanthin and adonirubin as intermediates, whereas the second one would begin by β-carotene hydroxylation and would have β-cryptoxanthin, zeaxanthin and adonixanthin as intermediates (reviewed in [1,3–5]). Although the genes encoding the enzymes – β-carotene-C-4 oxygenase and β-carotene hydroxylase – involved in these pathways have been cloned [6–10], the nature of the enzymes remains partially undetermined. The fact that astaxanthin synthesis requires oxygen [11,12], NADPH [12] and Fe^{2+} [6] has led Cunningham and Gantt [1] to propose that a cytochrome P450-dependent enzyme might be involved in the transformation of β-carotene to astaxanthin. Regardless of the astaxanthin biosynthetic pathway, the chromophore is esterified by one or two fatty acids [13]. Usually, only the esterified forms are accumulated by *H. pluvialis* [14,15]. However, one of us (B.S.) isolated other genera, which accumulate only non-esterified astaxanthin [15]. Therefore, it is interesting to determine if *Haematococcus*, which usually produces esterified molecules of astaxanthin, can, under certain conditions, accumulate non-esterified molecules. Alternatively, the availability of fatty acids could constitute a sine qua non for secondary carotenoid accumulation, like in *Dunaliella* [16], where the fatty acids participate in the formation of β-carotene droplets, but, of course, not for any esterification.

The aim of this contribution is to answer these questions. To reach this goal, we tested the effects of specific inhibitors of cytochrome P450 activity (ellipticine [17]) and of fatty acid synthesis (cerulenin [18]) for their ability to abolish the astaxanthin production in high-light stressed *H. pluvialis*.

2. Materials and methods

2.1. Growth conditions

H. pluvialis (Flotow) cells were grown in an air-lift bioreactor (2.5 l), in which the N-enriched (9 mM $NaNO_3$) modified Bristol medium [19] is continuously renewed (rate: 1.25 ml/min). The photon flux at the surface of the bioreactor was approximately 50 μmol/m^2/s PAR. In these conditions, the culture was maintained at a high and constant division rate. Only green and motile cells were used for the experiments.

To trigger astaxanthin accumulation, the cells were illuminated by two lamps (Mazda MTIL-F, 250 W) delivering approximately 1100 μmol/m^2/s PAR of polychromatic light. The temperature of the culture was kept constant at 20 ± 1°C by water circulation. Aliquots of the cell suspension were regularly taken during the stress treatment and used for pigment analysis.

Prior to high-light stress, the green cells were incubated in the dark for 30 min, in the presence of cerulenin (0.76 μg/ml; Sigma), ellipticine (25.00 μg/ml; Sigma) or norflurazon (20.00 μg/ml; Merck).

2.2. Pigment extraction and analysis

The cells were broken using a French press (SLM Aminco; pressure: 1100 psi). The broken cells were pelleted and resuspended with methanol. The procedure was repeated until a colorless pellet was obtained. The pigments were analyzed by reversed-phase HPLC equipped with a diode array detector, according to Lemoine et al. [20]. Program: solvent A (acetonitrile/methanol 70:30 v:v) was delivered isocratically from 0 to 8 min followed by a linear gradient of 0–40% of solvent B (methylene chloride). This solvent mixture was maintained isocratically until 25 min. The column was re-equilibrated between two analyses for a minimum of 20 min with solvent A. All the runs were performed at room temperature. The flow rate was 1 ml/min. HPLC-grade methanol, methylene chloride and acetonitrile were purchased by Merck (Darmstadt, Germany).

The different pigments were quantified, using the method of external standards, on the basis of their elution peak area recorded at 430 nm (chlorophyll (Chl) *a*), 437 nm (violaxanthin), 450 nm (antheraxanthin, β-carotene), 458 nm (Chl *b*), 467 nm (canthaxanthin, echinenone, hydroxyechinenone) and at 480 nm (astaxanthin).

$A^{\%}_{1\,cm}$ values cited by Britton et al. [21] and Lichtenthaler [22] were

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used to determine the amounts of the individual pigments used for calibration of the response at these wavelengths in the elution mixture.

Each experiment was repeated at least three times. To keep the figures clear, only the mean values are presented. Typically, the standard deviation was lower than 10%.

3. Results and discussion

3.1. Accumulation of astaxanthin in the absence of inhibitors and in the presence of cerulenin, a specific inhibitor of fatty acid synthesis

It is a common feature that microalgae synthesize secondary carotenoids when they are stressed. Usually several stresses are combined to increase the secondary carotenoid production (e.g. [15,23]). Consequently, it is difficult to link particular aspects of this synthesis to any of the stresses applied. In this work, we used only high-light irradiation to stress green and motile cells of *H. pluvialis*. Therefore, the observations reported in this article are only the consequence of the high-light stress.

On the basis of the variations of individual xanthophyll amounts during a high-light stress in *H. pluvialis*, Donkin [24] gave evidence for the existence of two phases in the astaxanthin synthesis by this alga: (1) synthesis using the preexisting β-carotene and (2) astaxanthin synthesis through a de novo β-carotene synthesis. However, these measurements were not frequent enough to draw kinetics of the synthesis of the different pools of astaxanthin. In order to establish the time course of β-carotene utilization and secondary carotenoid accumulation, we repeated this experiment with a higher sampling rate (one to two samples every hour) and analyzed the pigments by HPLC. The kinetics of the variations of β-carotene concentration was used as a master (Fig. 1). Three phases were observed:

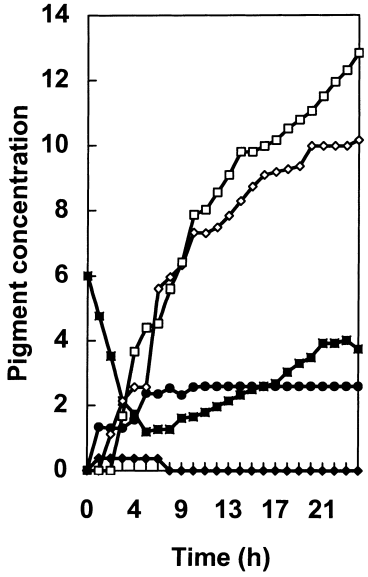

Fig. 1. Variations of the concentration of β-carotene (■) and of secondary carotenoids: (♦) echinenone, (●) non-esterified astaxanthin, (◇) astaxanthin monoester, (□) astaxanthin diester, during a 24 h high-light stress in the absence of inhibitors.

1. *During the first 5 h of illumination, the β-carotene amounts decreased*. During the first hour of stress, only non-esterified astaxanthin and low echinenone amounts were accumulated. They saturated after approximately 1 and 5 h of illumination, respectively. Detection of mono- and diesters of astaxanthin was delayed by 1 and 2 h, respectively. This observation might mean that astaxanthin esterification requires a fatty acid synthesis.
   During the first 2 h of stress, the consumed amount of β-carotene was equivalent to the sum of the different accumulated secondary carotenoids. Later, the total amount of astaxanthin produced became larger than the amount of β-carotene consumed (Table 1), confirming Donkin's conclusion that β-carotene has to be de novo synthesized to maintain astaxanthin production. This was fully confirmed by the use of norflurazon, an inhibitor of β-carotene synthesis at the level of phytoene desaturase (reviewed in [25]). The accumulation of astaxanthin stopped after 6 h of stress (data not shown). The total amount of accumulated secondary carotenoids was equivalent to the amount of β-carotene consumed (Table 1). The only detected intermediate was echinenone.
2. *Between the fifth and the seventh hour of illumination, the β-carotene amounts reached a transient minimal level*. Since the different esterified astaxanthin molecules were still accumulated during this phase, we conclude that de novo β-carotene synthesis was exactly compensated by its conversion to secondary carotenoids.
3. *After the seventh hour of stress, the β-carotene content increased progressively together with that of the esterified astaxanthin molecule forms, while the free astaxanthin amount remained unchanged*. At the end of the studied period the saturation phase was not yet reached (Fig. 1). Preferential accumulation of astaxanthin esters was already reported in *H. pluvialis* [13,14,23] and also in other algae [15].

In order to definitively confirm that astaxanthin accumulation in *Haematococcus* requires an active synthesis of fatty acids, the cells were incubated with a specific inhibitor of this pathway, i.e. cerulenin [26]. Cerulenin inhibited astaxanthin accumulation. In fact, very few non-esterified and monoesterified astaxanthin molecules were found to accumulate. In contrast, β-carotene de novo synthesis was not repressed (Fig. 2, Table 1). This result demonstrates that an active synthesis

Table 1
Effect of the different inhibitors on the consumption of β-carotene and violaxanthin and on the synthesis of secondary carotenoids, and antheraxanthin and zeaxanthin during a high-light stress of 6 h

Inhibitor	β-Carotene consumed	Secondary carotenoid accumulated	Violaxanthin consumed	Antheraxanthin+zeaxanthin accumulated
None	4.80 ± 0.35	9.30 ± 0.80	1.97 ± 0.20	1.72 ± 0.15
Norflurazon	3.91 ± 0.25	3.98 ± 0.35	1.23 ± 0.11	1.09 ± 0.09
Cerulenin	−11.88 ± 1.05	0.46 ± 0.03	N.D.	N.D.
Ellipticine	1.61 ± 0.15	1.60 ± 0.15	1.81 ± 0.15	1.67 ± 0.15

Numbers in fmol/cell. N.D.: not determined ($n = 3$).

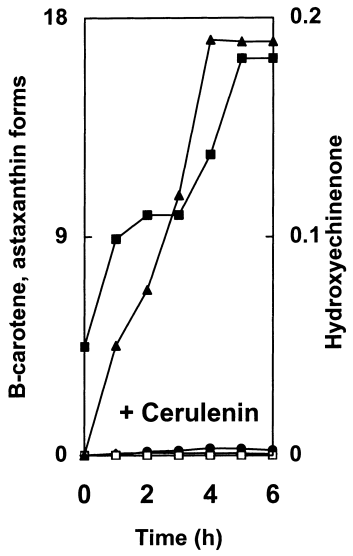

Fig. 2. Variations of concentration in β-carotene (■) and in secondary carotenoids in the presence of cerulenin: (▲) hydroxyechinenone, (●) non-esterified astaxanthin, (□) astaxanthin monoester and (◇) astaxanthin diester. Concentrations are in fmol/cell.

of fatty acids is coordinated to astaxanthin production. The only intermediate which was observed was hydroxyechinenone.

3.2. Accumulation of astaxanthin in the presence of ellipticine, a specific inhibitor of cytochrome P450 enzymes

It has been shown that astaxanthin synthesis requires oxygen [11,12], NADPH [12] and Fe^{2+} [6]. Since these three compounds constitute typical cofactors of cytochrome P450-dependent enzymes, one may postulate that such an enzyme is involved in astaxanthin biosynthesis. In order to verify this hypothesis, we tested the effect of ellipticine, a specific inhibitor of cytochrome P450-dependent enzymes [17], on astaxanthin accumulation. In the presence of this xenobiotic, the production of astaxanthin is completely abolished. In fact, approximately one third of the pre-existing pool of β-carotene

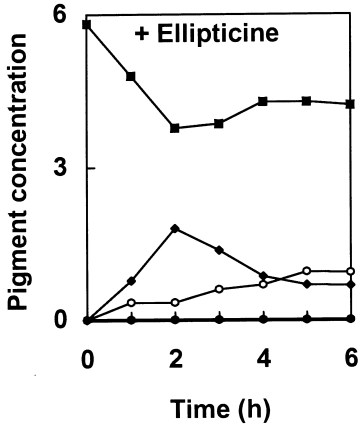

Fig. 3. Variations of the concentration in β-carotene (■) and in secondary carotenoids in the presence of ellipticine: (♦) echinenone, (○) canthaxanthin, (●) non-esterified astaxanthin, (◇) astaxanthin monoester and (□) astaxanthin diester. Concentrations are in fmol/cell.

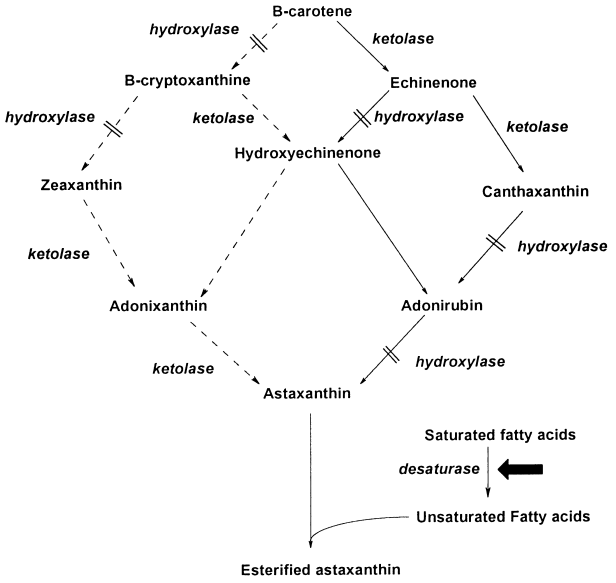

Fig. 4. Scheme of the conversion of β-carotene to astaxanthin by β-carotene-C-4 oxygenase (ketolase) and β-carotene hydroxylase (hydroxylase) enzymes in *H. pluvialis*. The interrupted arrows indicates pathways that were not observed. The signs '⫴' and '→' indicate the enzymatic reactions that ellipticine and cerulenin inhibit, respectively.

is transformed to echinenone, which is in turn converted to canthaxanthin (Fig. 3 and Table 1). The prolongation of the high-light stress, up to 24 h, did not modify these pigment contents (data not shown). The results suggest that canthaxanthin and/or echinenone accumulation depresses the enzyme β-carotene oxygenase activity through a feedback mechanism. The fact that only oxidation products of β-carotene (i.e. echinenone and canthaxanthin) are detected constitutes a strong clue in favor of the involvement of a cytochrome P450-dependent enzyme in the hydroxylation steps of astaxanthin synthesis in vivo. This conclusion is in line with the finding that cytochrome P450-dependent enzymes are mainly involved in hydroxylation activities (reviewed in [27–29]).

3.3. Effects of the inhibitors on zeaxanthin synthesis

In the absence of inhibitors, the level of zeaxanthin+antheraxanthin was higher after the stress than before. This increase was compensated by the decrease of violaxanthin revealing the operation of the xanthophyll cycle and its photoprotective role under high-light stress (Table 1). Ellipticine had no effect on the xanthophyll cycle (Table 1). In this condition, no β-carotene accumulation was observed. As the xanthophyll cycle pool of pigments remained constant, these observations suggest that zeaxanthin is likely not an intermediate of the astaxanthin biosynthesis. Thus, the astaxanthin biosynthesis appears to begin by addition of one keto-group leading to the production of echinenone, which is already detected after 1 h of stress. This conclusion is in line with the finding that β-C-4-oxidase can only accept an unsubstituted β-ionone ring as substrate [10,11].

4. Conclusions

Astaxanthin synthesis in *H. pluvialis* first occurs at the ex-

pense of the pre-existing β-carotene. Two pathways have been identified: the first one involves hydroxyechinenone (hydroxylation+oxidation), whereas the second involves canthaxanthin (with two oxidation steps; see Fig. 4). The hydroxylase activity appears to be cytochrome P450-dependent. When hydroxylase activity is inhibited by ellipticine, only canthaxanthin accumulates. The fact that in the presence of the inhibitors the increase of the zeaxanthin level during the high-light stress always corresponds to the decrease of the violaxanthin level strongly suggests that zeaxanthin is not an intermediate of the astaxanthin pathway in our conditions since it is only formed through the operation of the xanthophyll cycle which is triggered in high-light stress conditions.

In *H. pluvialis*, astaxanthin synthesis requires an active synthesis of fatty acids. Therefore, the strains isolated by Kopecky et al. [15], which accumulate only non-esterified or only monoesterified or diesterified astaxanthin molecules, appear an interesting material for further studies on the coordination of these two pathways, especially to determine whether the fatty acid synthesis is required for the esterification of the astaxanthin molecules and/or for the formation of the astaxanthin droplets.

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