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IN VITRO AND *IN VIVO* BIOSYNTHESIS OF XANTHOPHYLLS BY THE CYANOBACTERIUM *APHANOCAPSA*

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Abstract—Of the six carotenoids identified in the cyanobacterium *Aphanocapsa*, β-carotene, zeaxanthin, echinenone and myxoxanthophyll are the major pigments, whilst β-cryptoxanthin and 3-hydroxy-4-keto-β-carotene are present only in trace amounts. With the exception of zeaxanthin, the other xanthophylls could be formed *in vitro* from [^{14}C]phytoene in high yields, especially β-cryptoxanthin and 3-hydroxy-4-keto-β-carotene. In a time course experiment of xanthopyll biosynthesis the flow of radioactivity from [^{14}C]phytoene was followed through the pools of phytofluene, lycopene, and β-carotene. The reaction sequence from phytoene to xanthophylls is sensitive *in vitro* to both difunone, an inhibitor of carotene desaturation, and CPTA, an inhibitor of cyclization.

INTRODUCTION

Cyanobacteria are photosynthetic organisms which contain β-carotene (β,β-carotene) and various xanthophylls in their photosynthetic membranes [1, 2]. In addition, xanthophylls such as zeaxanthin (β,β-carotene-3,3′-diol) and echinenone (β,β-carotene-4-one) have been found to be associated with isolated cytoplasmic membranes [2–5].

Many cyanobacteria have been studied for their carotenoid composition (see refs [6, 7]). In most of the species β-carotene, echinenone, zeaxanthin and myxoxanthophyll [2-(β-L-rhamnopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β-ψ-carotene-3,1′-diol] are the predominating carotenoids. Cell extracts of *Aphanocapsa* have been used to perform *in vitro* carotenogenesis [8, 9]. It is the only organism with oxygenic photosynthesis so far which has yielded a cell-free system capable of converting geranylgeranyl pyrophosphate [8] or phytoene (1,8,11,12,7′,8′,11′,12′-octohydro-ψ,ψ-carotene) [10] into β-carotene. Nevertheless, little is known about the carotenoids of this unicellular cyanobacterium. Therefore, in this communication we report on the identification and the quantification of its carotenoids. In addition, we have employed a photosynthetic membrane preparation from *Aphanocapsa* to demonstrate the *in vitro* conversion of phytoene into both carotenes and xanthophylls.

Abbreviations—CPTA, 2-(4-chlorophenylthio)triethylamine hydrochloride; difunone, 5-(dimethyl-aminoethylene)-2 oxo-4-phenyl-2,5-dihydrofurane-carbonitrile.

RESULTS AND DISCUSSION

Carotenoid content of Aphanocapsa

Individual carotenoids, purified by two TLC systems, were identified by their spectral properties. Their absorption maxima in ethanol (Table 1) closely correspond with the values in the literature [11]. The carotenoid pattern in

Table 1. Identification of carotenoids from *Aphanocapsa*

	Spectroscopic parameters	
	EtOH λ_{max} (nm)	MS (*m/z*)
β-Carotene	(426), 450, 476	—
Zeaxanthin	(428), 451, 478	568 $^{-}$[M]$^{+}$, [M − 18]$^{+}$, [M − 92]$^{+}$, [M − 106]$^{+}$
Myxoxanthophyll*	449, 474, 505	566 [M]$^{+}$ (aglycone), [M − 16]$^{+}$, [M − 18]$^{+}$, [M − 41]$^{+}$, [M − 122]$^{+}$, [M − 147]$^{+}$, [M − 239]$^{+}$
Echinenone	—, 461, —	550 [M]$^{+}$, [M − 92]$^{+}$, [M − 106]$^{+}$
β-Cryptoxanthin	(426), 449, 478	552 [M]$^{+}$, [M − 18]$^{+}$, [M − 92]$^{+}$, [M − 106]$^{+}$
3-Hydroxy-4-keto-β-carotene	—, 460, —	566 [M]$^{+}$, [M − 2]$^{+}$, [M − 16]$^{+}$, [M − 18]$^{+}$, [M − 79]$^{+}$, [M − 92]$^{+}$, [M − 106]$^{+}$, [M − 154]$^{+}$, [M − 158]$^{+}$, [M − 217]$^{+}$ 137, 203

*Rhamnose identified as the sugar moiety by TLC using the method of ref. [25].

Aphanocapsa includes the most abundant combination in cyanobacteria, i.e. β-carotene, zeaxanthin, echinenone and myxoxanthophyll [6, 7, 12]. The three xanthophylls were further identified by the corresponding molecular ions in their mass spectra (Table 1). Furthermore, the characteristic losses of 92, 106 or 158 mass units from the polyene chains and of water $[M-18]^+$ from hydroxy carotenoids were observed. Myxoxanthophyll showed the characteristic mass peak for myxol [13]. However, the molecular ion was not obtained. Therefore, the sugar moiety of myxoxanthophyll was identified as rhamnose by co-chromatography on TLC.

In addition to these carotenoids, β-cryptoxanthin (β,β-carotene-3-ol) was also detected, and identified by its molecular ion and the same mass fragmentation peaks as zeaxanthin. This xanthophyll has been found previously only in a few other cyanobacteria from various groups [7].

The presence of a hydroxy-keto-β-carotene was indicated by a molecular ion of 566. The mass peak of $[M-154]^+$ resulting from the cleavage of the 6,7-bond which strongly suggests that the oxo and the hydroxy group are both located on the same ionone ring [14] was very small. However, the mass peaks at 137 and 203 resulting from an end group carrying a keto as well as a hydroxy function as outlined in ref. [15] give substantial evidence that the biosynthesis of the 3-hydroxy-4-keto isomer rather than the 3′ or 4′ hydroxy derivative can be assumed. The absorption maximum of 472 nm instead of 466 nm in benzene [11] is also an indication that the 3-hydroxy-4-keto-β-carotene is present in *Aphanocapsa*. The other mass peaks observed for this xanthophyll (Table 1) were the same as those obtained from fragmentation of astaxanthin which contains β-ionone rings with identical substitutions [16]. There are only two previous reports of 3-hydroxy-4-keto-β-carotene occuring in cyanobacteria, i.e. *Spirulina platensis* [7] and *Chlorogleopsis fritschii* [2]. The detection of 3-hydroxy-4-keto-β-carotene in *Aphanocapsa* is the first report of its presence in a unicellular species of the order *Chroococcales*. Another xanthophyll found in trace amounts (about 0.1%) with a polarity between myxoxanthophyll and zeaxanthin showed the same absorption spectrum as myxoxanthophyll and is most likely to be myxol (myxoxanthophyll aglycone). Myxol was found recently in *Chlorogleopsis* [2].

In *Aphanocapsa* carotenoids (3.4 mg/g dry wt) account for about 0.3% of the total dry weight. The dominant carotenoid is β-carotene which represents 45% of the carotenoids. Major xanthophylls are zeaxanthin (22%), myxoxanthophyll (19%), and echinenone (12%). β-Cryptoxanthin and 3-hydroxy-4-keto-β-carotene accounted for only 1–2% of the carotenoids. Due to its very low concentration in the cells, β-cryptoxanthin may be a metabolic pool for the conversion of β-carotene into zeaxanthin rather than a functional component of *Aphanocapsa*. In the case of 3-hydroxy-4-keto-β-carotene, it can be speculated that either the hydroxylation reaction of β-carotene to β-cryptoxanthin is not totally specific and also uses small amounts of echinenone as a substrate or conversely that the insertion of the oxo group into β-carotene is also accompanied by a similar reaction with β-cryptoxanthin. The quantitative distribution of carotenoids in *Aphanocapsa* closely corresponds to the amounts of major carotenoids found in thylakoids [5] and in cells of the very closely related species *Microcystis* and *Merismopedia* [6].

In vitro *biosynthesis of carotenoids*

Cell-free carotenoid biosynthesis with *Aphanocapsa* was carried out with [^{14}C]phytoene as the substrate. [^{14}C]Phytoene was generated from [2-^{14}C]mevalonic acid *in situ* by a cell free system from the phytoene desaturase-deficient mutant of *Phycomyces blakesleeanus* [C5 carB10(−)]. The phytoene generated in this way was employed as a substrate by *Aphanocapsa* membranes which were obtained by lysozyme digestion of the cell wall and subsequent osmotic shock. A time-dependent metabolism of [^{14}C]phytoene was observed throughout the incubation period as it was converted into other carotenes (Fig. 1A). The flow of radioactivity through the carotene pathway was followed, showing maximum incorporation of radioactivity into phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) after 60 min, then into lycopene (ψ,ψ-carotene) after 90 min, and finally into β-carotene after 120 min of incubation (Fig. 1B). During this period, xanthophylls were steadily formed. The highest incorporation of radioactivity from [^{14}C]phytoene was found in β-cryptoxanthin and 3-hydroxy-4-keto-β-carotene (Fig. 1C). Echinenone and myxoxanthophyll were labelled to a smaller, but nevertheless substantial, extent. In a recent study with broken *Aphanocapsa* membranes prepared by homogenisation of whole cells with glass beads, cell-free formation of β-cryptoxanthin could not be observed [10]. Instead, radioactivity from phytoene was found in high yields in myxoxanthophyll. This allocation in the net flow of radioactivity into xanthophylls is presumably an

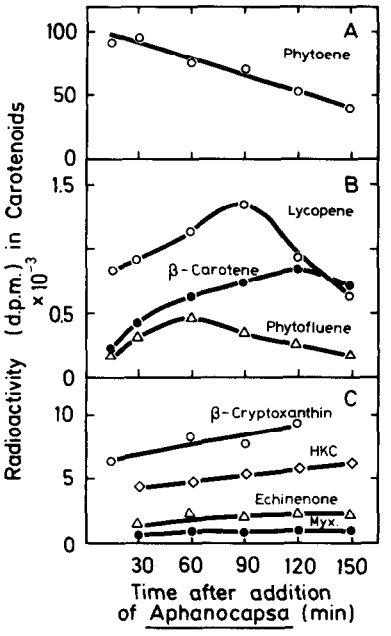

Fig. 1. Time course of cell-free [^{14}C]phytoene conversion into carotenoids. [^{14}C]Phytoene was generated over 2 hr at 35° by a cell extract of *Phycomyces* C5 carB10(−), incubated with 0.5 μCi DL-[2-^{14}C]mevalonic acid. Then osmotically shocked spheroplasts were added, equivalent to 225 μg chlorophyll. A, Phytoene metabolism; B, flow of radioactivity through carotenes; C, formation of xanthophylls. HKC, 3-hydroxy-4-keto-β-carotene; Myx, myxoxanthophyll.

artefact caused by the difference in preparation of the membranes. Apparently, homogenization disrupts the membranes responsible for the formation of bicyclic hydroxy carotenoids and thus inhibits their formation. The biosynthesis of myxoxanthophyll is not affected, and so radioactivity is channeled towards this xanthophyll only. Nevertheless, as shown in Fig. 1, intact *Aphanocapsa* membranes are capable of an efficient conversion of carotenes into various xanthophylls. *In vitro* hydroxylation reactions, the introduction of an oxo group, and the formation of a glycocarotenoid can be achieved. However, it is not possible to find substantial radioactivity in zeaxanthin. The reason for this is unclear, but may reflect inhibition of the enzyme responsible for introduction of a hydroxyl group at C-3′ of β-cryptoxanthin.

There are only a few other reports of cell-free systems capable of xanthophyll biosynthesis. One has been developed from a high zeaxanthin-producing strain of *Flavobacterium*. Homogenates from this species can use mevalonic acid as substrate and, apart from various carotene intermediates, the formation of zeaxanthin but not of its precursor β-cryptoxanthin, has been observed [17, 18]. β-Cryptoxanthin formation from isopentenyl pyrophosphate has been achieved with isolated *Capsicum* chromoplasts [19]. Incorporation into carotenes is quite high, but only 3–5% of the β-carotene is converted into β-cryptoxanthin, which itself shows the highest incorporation of all the xanthophylls.

The biosynthetic conversion of phytoene to β-cryptoxanthin in *Aphanocapsa* can be inhibited by the carotene desaturase inhibitor difunone and the cyclase inhibitor CPTA (Table 2). These compounds interfere with the conversion of phytoene to phytofluene [9] or lycopene to β-carotene (β,β-carotene) [20] by increasing the accumulation of radioactivity in phytoene and lycopene, respectively. Subsequently, a lack of precursors for the formation of β-carotene and β-cryptoxanthin is evident. This experiment demonstrates that β-carotene is involved in the biosynthesis of β-cryptoxanthin from phytoene in the cell-free system.

In conclusion, we have identified and quantified the carotenoids of *Aphanocapsa* and obtained a membrane preparation from this organism which synthesizes all the endogenous xanthophylls, apart from zeaxanthin, from [^{14}C]phytoene *in vitro*. This cell-free system can be used to investigate various aspects of the metabolic pathway as well as specific reactions in xanthophyll biosynthesis. This system might also be helpful in elucidating whether cytoplasmic membranes are capable of synthesizing some of the xanthophylls. If so, it would be an indication against the possibility that xanthophylls associated with cytoplasmic membranes are an artefact of the preparation procedure.

EXPERIMENTAL

Organisms. The C5 carB10(−) strain of *Phycomyces blakesleeanus* was obtained from the culture collection of the Departamento de Genética, Universidad de Sevilla, Spain. Growth and maintenance conditions were according to ref. [21]. *Aphanocapsa*, strain PCC 6714, was cultivated as described previously [22].

Radiochemical. DL-[2-^{14}C]Mevalonic acid lactone (53 nCi/nmol) was obtained from Amersham Buchler, Braunschweig, West Germany. It was converted to the Na salt prior to use [23].

Extraction, identification and quantification of carotenoids. Carotenoids were extracted from *Aphanocapsa* cells with MeOH containing 6% KOH at 65° for 20 min in darkness. After partition into Et_2O, the carotenoids were separated and purified by a combination of TLC systems. The bands from a silica gel G plate developed with toluene–EtOAc–MeOH (15:4:1) [11] were scraped off and eluted with Et_2O–MeOH (5:1) and rechromatographed on freshly activated Al_2O_3 plates with varying amounts of Me_2CO in petrol (bp 60–80°). Echinenone (R_f 0.66) was chromatographed with 15% Me_2CO and 3-hydroxy-4-keto-β-carotene (R_f 0.37), zeaxanthin (R_f 0.24) as well as β-cryptoxanthin (R_f 0.49) with 35% Me_2CO. For myxoxanthophyll (R_f 0.04) 5% MeOH in Me_2CO was used. Absorption and mass spectra were recorded on these samples. The extinction coefficients for quantitation were taken from ref. [11]. Identification of the sugar moiety of myxoxanthophyll was achieved by acid hydrolysis of the xanthophyll [24] followed by TLC of the sugar on silica gel [25], together with standard sugars including rhamnose, glucose, ribose and galactose. Mass spectra were recorded on an AE1 MS902 or VG 707OE spectrometer. Fragmentation patterns were compared to lit. values [13–16].

Preparation of cell extracts and incubation conditions. For cell-free assays with *Aphanocapsa* intact membranes were prepared by lysozyme digestion of the cell wall and osmotic shock [21]. The preparation of the cell-homogenates from *Phycomyces* was as described in ref. [23]. The coupled incubations were carried out either by adding *Aphanocapsa* membranes and *Phycomyces* homogenates simultaneously (Table 2) or sesquentially (Fig. 1). In the latter case, the *Phycomyces* extracts are allowed to form [^{14}C]phytoene from [2-^{14}C]mevalonic acid over a 2 hr period before the *Aphanocapsa* membranes are added in order to convert the accumulated [^{14}C]phytoene. Full details of the incubation conditions are described elsewhere [10].

Table 2. Inhibition of β-cryptoxanthin formation from [^{14}C]phytoene* by *Aphanocapsa* membranes† by desaturase and cyclase inhibitors

	Radioactivity (dpm × 10^{-3})				
	Phytoene	Phytofluene	Lycopene	β-Carotene	β-Cryptoxanthin
Control	8.0	0.6	4.5	6.3	10.8
+ Difunone (1 μM)	16.0	4.0	1.6	2.7	0.9
+ CPTA (100 μM)	8.5	0.6	5.4	0.6	0.3

*Phytoene generated from 0.5 μCi DL-[2-^{14}C]MVA by C5 extract.

†Equivalent to 287 μg chlorophyll/incubation; 2 hr; 35°; simultaneous incubation of C5 and *Aphanocapsa* cell extracts.

Purification of radioactive carotenoids. The procedures for isolation and purification of [^{14}C]carotenes have been described in detail in previous publications [8, 10]. For purification of xanthophylls the origins of the silica gel G thin layers, used in the purification of the carotenes, were eluted with Et_2O–MeOH (5:1) and rechromatographed on two subsequent TLC systems in the same way as the non-radioactive xanthophylls in order to achieve constant specific activity. Prior to TLC, β-cryptoxanthin and 3-hydroxy-4-keto-β-carotene (25 μg) were added as markers. Finally, all the carotenoids were scraped off the thin layers and subjected to liquid scintillation counting [26].

Other estimations. Chlorophyll content of *Aphanocapsa* membranes was determined in 80% Me_2CO [27]. Protein was quantitated with the Bio-Rad protein assay reagent, using BSA as a standard.

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