CAROTENE BIOSYNTHESIS WITH ISOLATED PHOTOSYNTHETIC MEMBRANES

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1. Introduction

There are several cell-free systems from eukaryotic organisms capable of in vitro carotenogenesis. The system from the fungus *Phycomyces*, converting mevalonic acid (MVA) into β-carotene has been extensively investigated [1,2]. A homogenate from *Neurospora* can synthesize phytoene from MVA and other prenyl pyrophosphates [3,4]. Isolated tomato [5], *Narcissus*-flower [6], and *Capsicum*-fruit [7] chromoplasts are reported to convert isopentenyl pyrophosphate (IPP) into β-carotene and be able to catalyze other reactions along this pathway. Recently, an *Amphidinium* homogenate has been found to synthesize xanthophylls and β-carotene from MVA [8].

We report here on a cell-free carotenogenic system from the blue-green alga *Aphanocapsa:* the first such system from an autotrophic prokaryote. The system is photosynthetically active and converts geranyl-geranyl pyrophosphate (GGPP) into β-carotene and xanthophylls. Data are presented showing the effects of various cofactors and 2-phenylpyridazinone analogs [9] on the carotenogenic ability of this system.

2. Materials and methods

Aphanocapsa (ATCC 6714) was cultivated for 4 days in 1-l Fernbach flasks at 35°C under 15 W/m^2 fluorescent light. Cultures were harvested with chlorophyll contents of 15 μg/ml. Growth medium and general conditions are described in [10]. Spheroplasts were prepared by lysozyme treatment (8 mg/ml; Sigma, Munich) according to [11] for 2 h at 35°C and then suspended in 10 mM tricine–NaOH buffer (pH 7.8), [*N*-tris-(hydroxymethyl)-methyl glycine] with 10 mM $MgCl_2$, 2.5 mM Na_2HPO_4, 2.5 mM K_2HPO_4, and 0.5 M sucrose. The untreated suspension was diluted 10-fold and washed with either water or 0.4 M Tris–HCl (pH 7.8), [tris-(hydroxymethyl)-amino methane]. Washed suspensions were spun down and the pellet resuspended in 0.4 M Tris–MCl (pH 7.8), to a chlorophyll concentration of 500 μg/ml (washed thylakoid suspension).

The incubation medium was essentially that used with *Phycomyces* extracts [12] in a final volume of 500 μl. Radioactive substrates were equivalent to 0.5 μCi of either GGPP, IPP or MVA (specific activities 4.22, 55 and 10 mCi/mmol, respectively). Unless stated otherwise, extracts having a pH of 7.5 were incubated for 90 min, at 20°C, under 35 W/m^2 white light, and contained 100 μg of chlorophyll. Reaction was stopped by adding methanol (3.5 ml) and 0.5% acetone (v/v) in petrol, b.p. 50–70°C (9 ml). The tubes were shaken vigorously and the contents poured into a mixture of petrol (10 ml) and saturated NaCl solution (50 ml). The petrol phase was collected and the solvent evaporated under nitrogen. The residue was redissolved in 50 μl of petrol, placed on silica-gel plates (Merck, Darmstadt) and developed in 7% (v/v) benzene in petrol. The yellow band was removed, the carotenes eluted with diethyl ether, reduced to 200 μl in volume under N_2, and applied to activated Al_2O_3 plates (fluorescent type: Merck, Darmstadt) together with phytoene marker. These were firstly developed for 5 min with 0.6% acetone (v/v) in petrol and subsequently in a chamber containing only petrol. The β-carotene and phytoene bands were scraped off and transferred into scintillation vials filled with 3 ml of Unisolve I (Zinsser, Frankfurt) and assayed for radioactivity by liquid scintillation. Data are means of four determinations: phytoene and β-carotene were mea-

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sured by two independent incubations each done in duplicate.

[2-^{14}C]Geranylgeranyl pyrophosphate was obtained from [2-^{14}C]geranylgeraniol which was chemically prepared from [2-^{14}C]ethylbromacetate (Amersham-Buchler, Braunschweig) and all-trans farnesyl acetone according to [13]. [2-^{14}C]mevalonic acid lactone and [1-^{14}C]isopentenyl pyrophosphate were purchased from the same company.

3. Results and discussion

Table 1 shows how carotenogenic activity of this system varies with preparation. Untreated spheroplast preparations (no. 1) convert less GGPP but to a lower phytoene:β-carotene incorporation ratio than do resuspended ones (no. 2–4). The increased phytoene incorporation in resuspended preparations could be due to phytoene synthase dissociating from the dehydrogenase complex. No phytoene accumulation is found in intact cells of *Aphanocapsa.* As yet, only carotenogenic systems from chromoplasts yield such an efficient phytoene conversion in vitro with a ^{14}C-ratio of phytoene:β-carotene incorporation of 0.5 [6,7]. Fungal systems show comparatively poor phytoene desaturation in vitro [2,4,12].

A time-course experiment (fig.1) showed significant incorporation into phytoene and β-carotene from labeled GGPP after only 10 min incubation. Incorporation into phytoene increased only slightly during the 135 min incubation with no significant deflection as was reported for the *Narcissus* chromoplast time course [6]. We suggest that in *Aphanocapsa,* a tight link exists between phytoene synthesizing and metabolizing enzymes allowing rapid phytoene dehydrogenation. β-Carotene formation showed a sigmoidal time course while saturation was not obtained within 2 h. It should be noted that this system also synthesizes xanthophylls, but this is the subject of another publication.

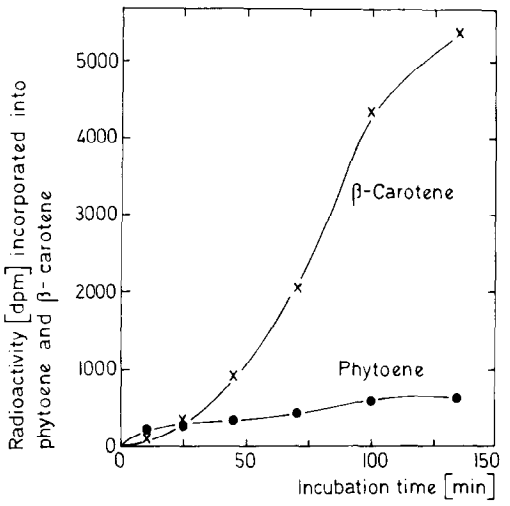


Fig.1. Time course of formation of phytoene and β-carotene from [^{14}C]geranylgeranyl pyrophosphate.

Cofactor requirements for carotenogenesis were assayed with water-washed spheroplasts. It is evident from the data (table 2, no. 2) that ATP stimulates phytoene synthesis from GGPP. A similar stimulating effect of ATP on the conversion of GGPP into phyto-

Table 1
Incorporation of radioactivity (dpm) from [^{14}C]substrates equivalent to 0.5 μCi by thylakoids from *Aphanocapsa* equivalent to 100 μg of chlorophyll

No.	Spheroplast treatment, substrates added	Radioactivity incorporated into			^{14}C Ratio of synthesized phytoene/β-carotene
		phytoene	β-carotene	phytoene + β-carotene	
(1)	Untreated + MVA[a]	0	0	0	–
	Untreated + IPP[a]	0	0	0	–
	Untreated + GGPP	50	1098	1148	0.05
(2)	Resuspended in Tris-buffer + GGPP (0.4 M, pH 7.8)	693	6953	7646	0.10
(3)	Washed with buffer, then resuspended + GGPP	873	6759	7632	0.13
(4)	Washed with water, then resuspended + GGPP	1033	7060	8093	0.15

[a] Incorporation of radioactivity into petrol phase (see section 2) from MVA was 1794 dpm and from IPP 1999 dpm

Table 2
Requirement of cofactors, oxygen, and light for the conversion of geranylgeranyl pyrophosphate into phytoene and β-carotene

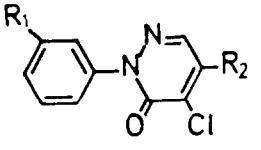
Assay composition and conditions	Radioactivity (dpm) incorporated into			^{14}C Ratio of synthesized phytoene/β-carotene
	phytoene	β-carotene	phytoene + β-carotene	
(A) All incubations aerobic and illuminated				
(1) All nucleotides (ATP, NAD, NADP and FAD) or ATP and NAD/NADP only or ATP and FAD only	1033	4465	5498	0.23
(2) All nucleotides except ATP	226	1942	2168	0.12
(3) ATP only	631	3121	3752	0.20
(4) No nucleotides	Backgrd. (75)	288	–	–
(B) All incubations contain all nucleotides				
(5) Aerobic and dark	832	4569	5401	0.18
(6) Anaerobic and light	1162	4306	5468	0.27
(7) Anaerobic and dark	5324	467	5791	11.4

Aphanocapsa spheroplasts washed with water (100 μg chlorophyll) were incubated with GGPP equivalent to 0.5 μCi over 90 min. For complete medium see section 2. Expt. no. 7,8 were done in Thunberg tubes which were evacuated by a water-jet pump until foaming had stopped

ene was reported for a tomato-plastid preparation [14]. Our studies suggest that *Aphanocapsa* requires only ATP plus either NAD, NADP, or FAD for carotenogenesis from GGPP (table 2, no. 1–4). A similar nucleotide requirement is found with gel-filtered cell-free extracts from the C115 strain of *Phycomyces* (I. E. C. et al., unpublished). The nucleotide requirement in tomato chromoplasts is more specific: phytoene dehydrogenation requires NADP whilst lycopene cyclization requires FAD [15].

Light had no effect on total carotenogenesis in aerobic and anaerobic preparations (table 2, no. 5–7).

Table 3
Influence of 2-phenylpyridazinone derivatives on the conversion of geranylgeranyl pyrophosphate into phytoene and β-carotene by a cell-free system from *Aphanocapsa*

Additions (1 μM)	Radioactivity (dpm) incorporated into		^{14}C Ratio of synthesized phytoene/β-carotene
	phytoene	β-carotene	
			
No. R_1 R_2			
(1) CF_3 $NHCH_3$	9907	1834	5.40
(2) CF_3 OCH_3	2256	3679	0.62
(3) H OCH_3	727	3824	0.19
(4) Control, no addition	624	4033	0.16

Spheroplasts washed with buffer (100 μg chlorophyll) were incubated with GGPP equivalent to 0.5 μCi for 90 min

As washed thylakoid preparations photosynthetically produce oxygen in the light, without électron donors or acceptors added (80–100 μmol/mg chlorophyll × h), it is suggested that phytoene desaturation is oxygen-dependent. In anaerobic dark samples, conversion of phytoene to β-carotene was drastically inhibited (no. 7).

In higher plants and green algae, certain 2-phenyl-pyridazinones inhibit phytoene desaturation [9,16]. The effect of such compounds on carotenogenesis in our cell-free system is presented in table 3. It is seen that norflurazon (compound no. 1), BAS 44521 (no. 2), and BAS 13761 (no. 3) exhibit a strong, intermediate and weak effect on phytoene desaturation, respectively. These results conform with whole-cell studies using *Scenedesmus* [17] and *Aphanocapsa* cultures. A good correlation between in vivo and in vitro studies in response to herbicides makes the *Aphanocapsa* cell-free system ideal for testing potencies of other bleaching herbicides on photosynthetic tissue.

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