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CAROTENE BIOSYNTHESIS BY A CELL EXTRACT OF *ASPERGILLUS GIGANTEUS* MUT *ALBA*

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Key Word Index—*Aspergillus giganteus*; Ascomycetes; carotenoids; biosynthesis; cell-free system; photoregulation.

Abstract—A cell extract prepared from lyophilized mycelia of light-grown cultures of *Aspergillus giganteus* mut *alba* converted [2-^{14}C]mevalonic acid into phytoene, lycopene, β-carotene and squalene, but from similar preparations from dark grown cultures formed only squalene. The carotenogenic activities of the cell extracts varied with the age of the cultures. Phytoene synthetase was located in the cytosolic fraction, whereas the dehydrogenation and cyclisation steps were catalysed by membrane-bound enzymes. Dithiothreitol, ATP, Mn^{2+}, Mg^{2+}, NAD and NADP were essential for the formation of carotenes from mevalonic acid, whilst FAD was required for phytoene metabolism. Oxygen enhanced the conversion of phytoene into other carotenes.

INTRODUCTION

Carotenoid biosynthesis in a number of non-photosynthetic microorganisms has been shown to be photoregulated [1]. On the basis of these studies it has been proposed that light induces the *de novo* synthesis of carotenogenic enzymes. The most direct approach for establishing if this is correct and in determining which enzymes are photoinducible, is to compare *in vitro* activities of cell extracts from light- and dark-grown cultures. Unfortunately, the *in vitro* activities of the dehydrogenase and cyclase enzymes of carotenoid biosynthesis are rather low in cell-free systems from most of the organisms concerned [2]. Therefore, our current understanding of which carotenogenic enzymes are photoinducible mainly concerns the steps leading up to phytoene biosynthesis. For example, prephytoene pyrophosphate synthetase is totally photoinduced in a *Mycobacterium* sp. and the formation of geranylgeranyl pyrophosphate in this organism is several-fold higher in light-grown cultures [3]. Phytoene synthesizing-activity is increased nine-fold on illumination of dark-grown *Neurospora crassa* with blue light [4], and HMG-CoA reductase activity is higher in *Rhodotorula minuta* grown under continuous illumination rather than in darkness [5].

We have recently demonstrated that carotene biosynthesis in *Aspergillus giganteus* mut. *alba* is photoregulated by blue light at the transcriptional level (El-Jack, M., Bramley, P. M. and Mackenzie, A., unpublished results). In order to ascertain which enzymes are photoinduced, we have now developed a cell-free system of *A. giganteus* which exhibits high carotenogenic activities in light-grown cultures, but no such conversions are observed in a cell extract from dark-grown cells. In addition, some of the properties of the enzymes associated with carotene biosynthesis have been elucidated.

Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DTT, dithiothreitol; phytoene, 7, 8, 11, 12, 7′, 8′, 11′, 12′-octahydro-ψ,ψ-carotene; lycopene, ψ,ψ-carotene; β-carotoene, β,β-carotene; HMG-CoA, 3-hydroxy-3-methylglutaryl Coenzyme A; HMG CoA reductase, E.C. 1.1.1.34.

RESULTS AND DISCUSSION

A cell extract from light-grown cultures of *A. giganteus* converted [2-^{14}C]MVA into phytoene, lycopene, β-carotene and squalene (Table 1, Fig. 1). Incorporation of radioactivity into lycopene and β-carotene was linear up to a protein concentration of at least 5 mg per incubation (Fig. 1A) and for a period of up to 60 min (Fig. 1B). The specific enzyme activity of the extract (typically *ca* 1700 dpm into β-carotene/mg protein) is considerably higher than those reported for other photoregulated fungi, e.g. *N. crassa* (170 dpm into coloured carotenoids/mg protein) [6] and *Phycomyces blakesleeanus* (444 dpm into β-carotene/mg protein) [7].

Table 1. The effect of oxygen on carotenogenesis by cell extracts of *A. giganteus*

Compound	Incorporation (dpm/mg protein)		Ratio
	$+O_2$	$-O_2$	$+O_2:-O_2$
Total terpenoids	33917	33143	1.02
Phytoene	2130	6330	0.34
Lycopene	1009	260	3.88
β-Carotene	1659	165	10.1
Squalene	4210	4122	1.02

Cell extracts were incubated with 0.25 μCi (3*R*)-[2-^{14}C]MVA, under aerobic and anaerobic conditions. Protein concentration 4.2 mg/incubation. Total terpenoids refers to the incorporation of MVA into the lipid extracts of the incubations.

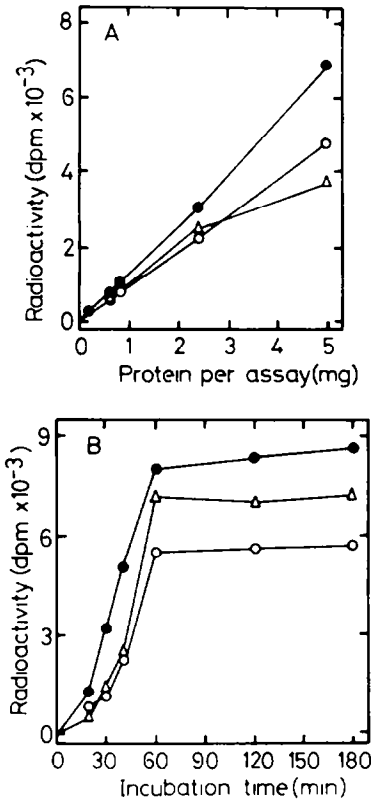

Fig. 1. The formation of phytoene (Δ), lycopene (O) and β-carotene (●) by a cell extract of *A. giganteus* with respect to protein (A) and time (B). All incubations were at 35° and contained 0.25 μCi (3R)-[2-^{14}C]MVA. The protein content in B was 4.9 mg/incubation.

The levels of carotenogenic activities of the cell-free system were dependent upon several factors. Extracts prepared from different ages of mycelia exhibited significantly different specific activities when incubated aerobically with [2-^{14}C]MVA (Fig. 2). Maximum incorporations of radioactivity were found with cultures grown for 88 hr; *in vitro* preparations of older cultures exhibited much lower enzymic activities, with stationary phase cultures (5 days) containing only some 5% of the maximum activities. A similar age-related phenomenon has been reported for the C9 (lycopene-accumulating) strain of *Phycomyces* [8], although the *in vitro* formation of phytoene and β-carotene in this fungus has two peaks of activity, one at late exponential and another at late stationary phase [8].

Although the incorporation of [2-^{14}C]MVA into both total terpenoids and squalene is virtually unaffected by *in vitro* anaerobiosis, the pattern of carotene formation is noticeably altered (Table 1). The presence of oxygen enhances the conversion of phytoene into lycopene and β-carotene. The same effect has been noted in *Phycomyces* [8] and in *Aphanocapsa* [9], but the formation of unsaturated C_{30}-carotenoids by a cell extract of *Streptococcus faecium* shows no such requirement for oxygen [10], and phytoene biosynthesis is not increased under anaerobic conditions into a cell-free preparation of *Flavobacterium* R1519 [11]. It has been suggested that oxygen is required for *in vitro* phytoene metabolism in

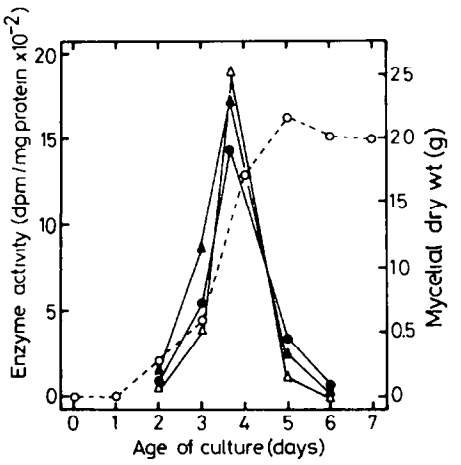

Fig. 2. Carotenogenic activities of cell extracts of *Aspergillus* prepared from different ages of culture, as estimated by the incorporation of (3R)-[2-^{14}C]MVA (0.25 μCi) into phytoene (▲), lycopene (●) and β-carotene (Δ). The growth curve (- - O - -) is shown.

Phycomyces in order to maintain a suitable level of oxidised pyridine nucleotides and FAD [8], since NADPH inhibits the formation of phytoene in tomato fruit plastids [12, 13]. Whether this is also true for the fungal enzymes must await the outcome of studies with purified enzyme systems. It is noteworthy, however, that carotene formation by *Aspergillus* requires NAD(P), whilst lycopene and β-carotene are also dependent upon FAD (Table 2).

In common with many other carotenogenic systems [2] the conversion of MVA or IPP into carotenes necessitates the presence of ATP, a divalent metal ion and DTT (Table 2). However, the metal ion requirement differs with the two substrates. With MVA both Mn^{2+} and Mg^{2+} are essential, but only Mn^{2+} is required when IPP is the substrate. Presumably the phosphorylation reactions from MVA to IPP require Mg^{2+}, and the subsequent isomerization and prenylation steps are dependent upon Mn^{2+}. Although ATP is not required as a phosphate donor for the conversion of IPP into carotenes, it clearly stimulates these steps *in vitro* (Table 2). Similar results with cell extracts of *Aphanocapsa* [9], *Streptococcus* [10], tomato fruits [12, 13] and *Phycomyces* [14] have been reported, but the mechanism of activation is unknown. It has been suggested that this apparent stimulation is a secondary effect, whereby the ATP drives a parallel pathway necessary for terpenoid biosynthesis [10], or else it is an allosteric effector [15]. Elucidation of this phenomenon must await studies on the purified enzyme.

Subcellular fractionation of the *Aspergillus* extract into cytosolic (S_{105}) and microsomal (P_{105}) fractions and subsequent determination of carotenogenic activities, revealed that only phytoene and squalene formation, from [2-^{14}C]MVA, occurred in the S_{105} fraction (Table 3). Reconstitution of the crude extract, by mixing S_{105} with P_{105} restored full carotenogenic activities, indicating that lycopene and β-carotene biosynthesis is catalysed by microsomal enzymes. This was confirmed by the use of [^{14}C]phytoene as the substrate, prepared *in situ* from [2-^{14}C]MVA using a coupled assay with the C5 (phytoene-accumulating) strain of *Phycomyces*. Under

Table 2. Cofactor requirements for carotenoid biosynthesis from [2-^{14}C]MVA and [1-^{14}C]IPP in cell extracts of *A. giganteus*

Cofactor	% Incorporation from [2-^{14}C]MVA*		
	Phytoene	Lycopene	β-Carotene
Complete	100	100	100
None	1.2	1.1	1.0
−DTT	0.0	0.9	2.2
−FAD	78.7	13.9	1.8
−NADP	0.5	3.2	1.6
−NAD	0.9	0.8	0.2
−ATP	0	0.1	0.5
$-Mn^{2+}$	2.8	0.8	0.4
$-Mg^{2+}$	0.6	0.7	0.9
	% Incorporation from [1-^{14}C]IPP†		
Cofactor	Phytoene	Lycopene	β-Carotene
Complete	100	100	100
−ATP	56.8	58.9	53.9
$-Mn^{2+}$	80.5	89.4	80.5
$-Mg^{2+}$	0.0	00,	1.0

Cell extracts were desalted by passage through Sephadex G25, and then incubated with 0.25 μCi(3*R*)-[2-^{14}]MVA or 0.25 μCi [1-^{14}C]IPP.

*Incorporations from 0.25 μCi(3*R*)-[2-^{14}C]MVA were 4173, 2875 and 4201 dpm into phytoene, lycopene and β-carotene, respectively. Protein concentration, 0.8 mg/incubation.

†Incorporations from 0.25 μCi [1-^{14}C]IPP were 9351, 4464 and 10911 dpm into phytoene, lycopene and β-carotene, respectively. Protein concentration 0.8 mg/incubation.

these incubation conditions the P_{105} fraction converted some 48% of the [^{14}C]phytoene into lycopene and β-carotene, whereas the cytosol did not metabolize this substrate (Table 3). The membrane-bound nature of phytoene-metabolizing enzymes has been found in all organisms studied to date, e.g. *Phycomyces* [16], *Neurospora* [6], *Capsicum* [17] and *Narcissus* [18], with the exception of the halophile *Halobacterium cutirubum*

Table 3. Subcellular distribution of carotenogenic enzymes in *A. giganteus*

Compound	Incorporation (dpm/mg protein)				
	From(3*R*)-[2-^{14}C]MVA*			From [^{14}C] phytoene†	
	S_{10}	S_{105}	$S_{105}+P_{105}$	S_{105}	P_{105}
Phytoene	3234	4636	3554	14510	8783
Lycopene	1643	0	1701	0	1836
β-Carotene	3093	0	2395	0	3202
Squalene	6630	5455	3425	10497‡	14999‡

*From 0.25 μCi(3*R*)-[2-^{14}C]MVA. Protein concentrations: S_{10}, 3.47; S_{105}, 2.7; $S_{105}+P_{105}$, 3.7 mg/incubation.

†40296 dpm [^{14}C]phytoene, obtained from *Phycomyces* C5 *carB*10(−). Protein concentrations: S_{105}, 3.3; P_{105}, 2.5 mg/incubation.

‡36601 dpm [^{14}C] squalene also present from C5 *carB*10(−).

[19], but the location of 'phytoene synthetase' may be either as a peripheral membrane protein, which is readily dislodged during the preparation of a cell extract [16, 18] or a genuinely soluble, cytosolic enzyme [20]. It is possible that the high osmolarity buffer used in the present study has removed phytoene synthetase from its loose association with a membrane during the extraction procedure.

A comparison of the incorporation of [2-^{14}C]MVA into the terpenoids of cell extracts from light- and dark-grown cultures showed major differences. Total incorporation dropped some 80% in the dark-grown extract; there was no synthesis of carotenes and squalene formation fell to 21% of the light-grown value (Table 4). In order to eliminate the possibility of an inhibitor being present in the dark-grown cells, a mixture of cell extracts from light- and dark-grown cultures was incubated with [2-^{14}C]MVA. The specific enzyme activities in this case were approximately 50% of those in the light-grown extract (Table 4). These data show that the loss of activity in the dark-grown extract is not due to the presence of an inhibitor, and therefore must be a result of the absence of the appropriate enzymes. The precise number and type of

Table 4. Carotene and squalene formation by cell extracts of light- and dark-grown cultures of *A. giganteus*

Compound	Incorporation (dpm/mg protein)*		
	Light-grown	Dark-grown	Light- + dark-grown (1:1 v/v)
Total terpenoids	38341	7870	19100
Phytoene	1664	0	807
Lycopene	921	0	515
β-Carotene	1712	0	992
Squalene	3344	711	2143

*From 0.25 μCi(3*R*)-[2-^{14}C]MVA. Protein concentrations: light-grown, 4.1; dark-grown, 4.6; mixture of light- and dark-grown, 4.3 mg/incubation. Total terpenoids refers to the incorporation of MVA into the lipid extracts of the incubations.

enzymes which are only present in light-grown cells cannot be defined from these results, since MVA is not an immediate precursor of phytoene and the other carotenes. However, the *in vitro* synthesis of squalene, albeit at a significantly reduced level, indicates that the enzymes up to FPP formation are present, probably in reduced amounts. Therefore the protein(s) which are only present in illuminated cultures of *Aspergillus* must be one or all of those catalysing the conversions of GGPP to β-carotene. We are currently developing assays for each of these enzymes in order to establish which enzymes are totally photoinducible.

EXPERIMENTAL

Organisms. Aspergillus giganteus mut. *alba*, strain 101.64, was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The C5*carB*10(−) strain of *Phycomyces blakesleeanus* was kindly provided by the Departmento de Genêtica, Universidad de Sevilla, Spain.

Radiochemicals. (3*R*)-[2-^{14}C]MVA lactone, (53 mCi/mmol) and [1-^{14}C]IPP, ammonium salt (53 mCi/mmol) were obtained from Amersham International plc, Amersham, HP7 9LK, U.K. The MVA lactone was converted to the Na salt of its acid prior to use [8].

Media and culture conditions. A. giganteus was maintained on Wickerhams MYPG medium, containing 2% agar, according to ref. [21]. Liquid cultures (300 ml) were grown in the same medium, without agar, in 2 l conical flasks at 24° in an orbital shaker at 160 rpm. Cultures grown in darkness were completely wrapped in 2 layers of black polythene, whilst light-grown cultures were grown under white fluorescent light (type F 30T12/WW/RS, GTE, Sylvanian Ltd., Charlestown, Shipley, West Yorkshire, U.K.) with an intensity of 50 W/m^2, as measured at the surface of the cultures. In all cases, except for the experiments shown in Fig. 2, liquid cultures were grown for 88 hr. *Phycomyces* was grown and maintained as described previously [22].

Preparation of cell extracts. Aspergillus mycelia were harvested, washed with distilled water and squeezed to hand dryness. After freezing at −70°, the samples were lyophilized. The dried mycelia were either stored at −70° under desiccation or rubbed through a sieve (mesh size 355 μm) at room temp. to produce a fine powder. This (typically 1 g) was mixed with 0.4 M Tris–HCl buffer pH 8.0 containing 5 mM DTT (1:8 w/v, except in Fig. 1A) and the resultant paste centrifuged at 10000 *g* for 20 min at 4°. The supernatant (S_{10}) was used as the cell-free preparation. When necessary this fraction was recentrifuged at 105000 *g* for 75 min at 4° to yield the cytosolic (S_{105}) and microsomal (P_{105}) fractions. In experiments to elucidate cofactor requirements (see Table 2) the S_{10} fraction was desalted by passage through a Sephadex G25 column (5 × 1 cm) pre-equilibrated with 0.4 M Tris–HCl buffer, pH 8.0. The cell extract of *Phycomyces* C5 *carB*10(−) was prepared as described in a previous publication [7]. Protein concentrations of the cell extracts were determined by the method of ref. [23], using BSA as a standard.

Enzyme assays. The activities of carotenogenic enzymes of *Aspergillus* were estimated by determining the incorporation of radioactivity from [2-^{14}C]MVA, [1-^{14}C]IPP or [^{14}C]phytoene into carotenes. Typically, each incubation (500 μl) was for 2 hr at 35° and contained: (3*R*)-[2-^{14}C]MVA or [1-^{14}C]IPP, 0.25 μCi (4.7 nmol) or [^{14}C]phytoene, 40296 dpm; ATP, 5 μmol; NAD, NADP, FAD, 1 μmol each; $MgCl_2 \cdot 6H_2O$, 2 μmol; $MnCl_2 \cdot 4H_2O$, 3 μmol; *Aspergillus* cell extract, 200 μl, 0.4 M Tris–HCl buffer, pH 8.0 containing 5 mM DTT, to 500 μl. [^{14}C]Phytoene was prepared *in situ* using the cell extract (200 μl) of the C5 (phytoene-accumulating) strain of *Phycomyces*. In this case, the C5 extract was incubated with the above cofactors for 2 hr at 35°, and then unlabelled MVA (470 nmol) added to the incubation mixture, prior to the addition of the *Aspergillus* cell-free system. This mixture was incubated for 2 hr at 35°, during which time the [^{14}C]phytoene was metabolized by the *Aspergillus* carotenogenic enzymes. This sequential coupling assay has been used previously with C5 and *Aphanocapsa* preparations [24]. Anaerobic incubations were carried out in Thunberg tubes, as described previously [8]. The [^{14}C]carotenes and squalene were extracted from the incubations with petrol (bp 40–60°) and purified to constant specific radioactivity by a combination of TLC systems. The lipid extract was first chromatographed on silica gel, using 15% toluene–petrol (bp 60–80°) as the developing solvent. The carotenes were eluted and re-chromatographed on activated Al_2O_3 plates, and developed with 3% toluene–petrol (bp 60–80°), as described previously, [24, 25]. The purified terpenoids were scraped off the thin layers and radioassayed by liquid scintillation counting [26].

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