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# Independence of the carotene and sterol pathways of *Phycomyces*

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Light, chemicals, and mutations that affect the carotene content of the fungus *Phycomyces blakesleeanus* had practically no effect on the ergosterol content. Lovastatin, a specific inhibitor of hydroxymethylglutaryl coenzyme A reductase, blocked growth at 1 μM; sodium DL-mevalonate (10 mM) fully reversed this inhibition. In the presence of [$^{14}$C]mevalonate, a *carS* mutant accumulated 16 times more β-carotene than the wild-type with a specific radioactivity five times lower. The specific radioactivity of ergosterol was different from that of β-carotene, even when calculated in terms of the constituent isoprene units, and unaffected by the *carS* mutation. The carotene and sterol pathways of *Phycomyces* are independently regulated and physically separated in different subcellular compartments.

Mevalonate; Lovastatin; β-Carotene; Ergosterol; Hydroxymethylglutaryl coenzyme A reductase; *car* mutant

## 1. INTRODUCTION

Carotenes and sterols are terpenoid compounds derived from hydroxymethylglutaryl coenzyme A. The first step in their synthesis is the reduction of this precursor to mevalonate; a long series of reactions leads to the production of the C15 compound farnesyl pyrophosphate and then to the C20 compound geranylgeranyl pyrophosphate. The condensation of two molecules of farnesyl pyrophosphate is the first specific reaction of sterol biosynthesis, and the condensation of two molecules of geranylgeranyl pyrophosphate, that of the carotenes [1]. In the fungus *Phycomyces blakesleeanus* [2], ergosterol is the main sterol [3] and β-carotene the main carotene [4,5].

The accumulation of β-carotene by wild-type *Phycomyces* is considerably increased by blue light [5,6] and by many chemicals, including dimethyl phthalate [7,8]. The *car* mutants differ from the wild-type in their carotene content [9]. The various genetic and environmental factors in the production of β-carotene have been integrated into a general theory of the regulation of the pathway [8,10].

To elucidate the relationship between the sterol and the carotene pathways we have studied the effects of light, dimethyl phthalate, and *car* mutations on the production of ergosterol and the radioactive labelling of carotene and ergosterol with exogenous mevalonate.

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## 2. MATERIALS AND METHODS

Strain NRRL1555, the standard wild-type of *Phycomyces blakesleeanus* Bgff., and various mutants with altered carotenogenesis [9] were grown from heat-activated spores (50–200 spores/ml medium) on plastic plates containing 25 ml or 10 ml of minimal agar incubated at 22°C in the dark for four days [10].

Mycelial extracts [11], were fractionated in a neutral $Al_2O_3$ (CAMAG brought to Brockmann grade III) column (3 cm long, 1 cm wide) and eluted with petrol (boiling range 50–70°C) containing increasing concentrations of ethyl ether: none (elutes successively phytoene and β-carotene), 0.5% (ergosterol esters), 6% (lycopene), and 12% (free ergosterol). Carotenes were quantitatively determined from their extinction coefficients [12]. For ergosterol, the extinction coefficient at 296 nm (10 mg·ml$^{-1}$ ethanol, path=1 cm) was taken as 166. The ergosterol fraction may be contaminated with episterol, the second most important sterol in *Phycomyces* [3], which does not absorb at 296 nm; the specific radioactivity of ergosterol may thus be overestimated. The same applies to ergosterol esters and episterol esters.

Dried DL-2-$^{14}$C mevalonic acid lactone ($2.0 \times 10^{12}$ Bq·mol$^{-1}$, (Amersham International, Amersham, Bucks., UK) and non-radioactive mevalonic acid lactone (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 25 mM NaOH, incubated at 50°C for 2 h to produce the sodium salt, and added to the autoclaved minimal agar medium. In labelling experiments *Phycomyces* was grown on a dialysis membrane placed on the surface of 10 ml minimal agar with labelled sodium mevalonate (10 mM, 0.4 Bq·nmol$^{-1}$; this last value was not precisely measured). β-Carotene and ergosterol were extracted from the mycelium, purified, and dissolved in toluene with 5 mg·ml$^{-1}$ diphenyloxazol and the radioactivity was measured with a Beckman LS2800 scintillation counter.

Lovastatin (mevinolin), a gift of Merck Research Laboratories, Rahway, NJ, USA, was treated as mevalonate to convert it to the sodium salt, dissolved at 1 mM in dimethyl sulfoxide and added to the autoclaved minimal agar medium.

## 3. RESULTS

The carotene content of *Phycomyces* mycelia varies enormously as a result of variations in the culture con-

Table I

Carotene and ergosterol contents[a] of 4-day-old *Phycomyces* mycelia

| Strain, genotype, conditions | Carotene[b] | Ergosterol | | |
|---|---|---|---|---|
| | | Free | Esterified | Total |
| NRRL1555(standard wild-type | | | | |
| In the dark | 0.08 | 2.3 | 0.4 | 2.7 |
| In the light[c] | 0.52 | 2.5 | 0.9 | 3.4 |
| With ethanol[d] | 0.07 | 2.5 | 0.4 | 2.9 |
| With dimethyl phthalate[e] | 2.20 | 2.3 | 0.5 | 2.8 |
| Mutants in the dark[f] | | | | |
| S102 (wild-type) | 0.09 | 2.3 | 0.6 | 2.9 |
| C2 (*carA5*) | 0.003 | 2.1 | 0.5 | 2.6 |
| S119 (*carA113*) | 0.020 | 2.6 | 0.4 | 3.0 |
| S283 (*carA5 carS42*) | 0.48 | 2.5 | 0.2 | 2.7 |
| C5 (*carB10*) | 2.70 | 3.6 | 0.8 | 4.4 |
| A98 (*carC652*) | 0.07 | 3.0 | 0.7 | 3.7 |
| S144 (*carI131*) | 0.025 | 2.7 | 0.6 | 3.3 |
| C9 (*carR21*) | 2.5 | 3.1 | nd | |
| C144 (*carRA3*) | 0.05 | 2.8 | 0.3 | 3.1 |
| C115 (*carS42*) | 2.1 | 2.5 | 0.7 | 3.2 |

[a] The data (mg·g$^{-1}$ dry weight) are averages of two independent experiments. In repetitions of experiments, the analyses of β-carotene show an average relative error of 3%, those of esterified ergosterol, 18%, and those of free ergosterol, 7%.
[b] Phytoene in strain C5, lycopene in strains C9 and C144, and β-carotene in the others.
[c] Continous illumination with 15 W·m$^{-2}$ white light.
[d] 1% (v/v).
[e] 3 mM (added as an ethanol solution; final concentration of ethanol in the medium, 1% v/v).
[f] The genotype relevant for carotenogenesis is given in parenthesis after the name of the mutants.

ditions or the presence of mutations in various *car* genes (Table I). Thus, either the β-carotene content of the wild-type in the presence of dimethyl phthalate or the phytoene content of strain C5 are more than a thousand times the β-carotene content of strain C2. At the same time, the content of ergosterol, whether esterified or free, remains essentially constant. Thus, the 13 analyses of the total ergosterol content in Table I average 3.1 mg/g dry weight; their average variation coefficient is 16%, not much larger than the variation coefficient for repetitions of the analyses of a strain under a given set of conditions. The largest deviation is that of strain C5, with 1.4 times the average. Moreover, the variations in ergosterol content do not correlate with the variations in carotene content. We conclude that light, dimethyl phthalate, and *car* mutations do not affect ergosterol content.

Carotenes have been radioactively labelled by growing *Phycomyces* on [$^{14}$C]mevalonic acid lactone [13], but the specific radioactivity was low. Lovastatin, a specific inhibitor of hydroxymethylglutaryl coenzyme A reductase [14], may be used to determine the conditions under which mevalonate can be taken up efficiently. *Phycomyces* did not grow on medium with 1 μM lovastatin. This inhibition was countered by the presence of mevalonate in the medium. To achieve normal growth sodium DL-mevalonate had to be added at 10 mM, DL-mevalonic acid lactone at 100 mM; no growth was observed with either 1 mM sodium DL-mevalonate or 10 mM DL-mevalonic acid lactone.

A superproducer of β-carotene (strain C115, a *carS* mutant) and the wild-type were labelled in parallel experiments with radioactive sodium mevalonate (Table II). The *carS* mutation does not modify either the concentration or the specific radioactivity of ergosterol, whether free or esterified. The *carS* mutant accumulated 16 times more β-carotene than the wild-type with a specific radioactivity 5 times lower. Thus ergosterol and β-carotene are made from different mevalonate pools and subject to separate regulation. This requires that the two pathways be physically separated in different subcellular compartments. Free and esterified ergosterol appear to be made from a single precursor pool, because they have the same specific radioactivity of about 0.12 Bq·nmol$^{-1}$ of isoprene units. This corresponds to a mixture of exogenous and endogenous mevalonate. The β-carotene in the wild-type (0.28 Bq·nmol$^{-1}$ isoprene units) is made essentially from exogenous mevalonate. The β-carotene in the superproducing mutant (0.055 Bq·nmol$^{-1}$ isoprene units) comes mostly from endogenous mevalonate, presumably because not enough exogenous mevalonate arrives at the appropriate location at the rate required by the increased demand.

The 'pulse and chase' experiment (Table III) shows that β-carotene and ergosterol are very stable in *Phycomyces*: the radioactive label incorporated into them in the first 4 days of growth remained in the same compounds for at least 2 days after the mycelia were transferred from labelled to unlabelled media. From the fourth to the sixth day there were no major changes in the dry weight of the mycelia or in the carotene and ergosterol contents. Thus, degradation and bioconversion do not play any important roles in maintaining the concentrations of these chemicals.

## 4. DISCUSSION

We have found that the environmental and genetic factors that regulate carotenogenesis (light, dimethyl phthalate, various *car* mutations) have no effect on the ergosterol content of the mycelia. We conclude that the carotene and sterol pathways of *Phycomyces* are independently regulated. Light stimulates carotenogenesis in many microorganisms [15,16], but little is known about its effects on other terpenoids; it does not affect the ergosterol content of *Rhodotorula* [17] or the gibberellin production of *Gibberella* [18].

*Phycomyces* can synthesize its terpenoids from exogenous mevalonate, as shown by the normal growth

Table II

Labelling of carotene and ergosterol in mycelia of the wild-type and a *carS* mutant (strain C115) grown for 4 days in the presence of labelled mevalonate[a]

| | β-Carotene | Ergosterol | | |
|---|---|---|---|---|
| | | Free | Esterified | Total |
| Wild-type | | | | |
| Content ($mg \cdot g^{-1}$ dry weight) | 0.10 | 2.9 | 0.8 | 3.7 |
| Specific radioactivity | | | | |
| $Bq \cdot nmol^{-1}$ | 2.25 | 0.66 | 0.54 | 0.63 |
| $Bq \cdot nmol^{-1}$ isoprene units[b] | 0.28 | 0.13 | 0.11 | 0.13 |
| *carS* mutant | | | | |
| Content ($mg \cdot g^{-1}$ dry weight) | 1.65 | 3.3 | 0.8 | 4.1 |
| Specific radioactivity | | | | |
| $Bq \cdot nmol^{-1}$ | 0.44 | 0.60 | 0.69 | 0.62 |
| $Bq \cdot nmol^{-1}$ isoprene units[b] | 0.055 | 0.12 | 0.14 | 0.12 |

[a] The values are the average of at least two analyses from independent experiments.

[b] For β-carotene, 1/8 of the preceding line: each molecule of β-carotene is made up of 8 isoprene units, each derived from a mevalonate molecule. For ergosterol, 1/5 of the preceding line: each molecule of ergosterol is made up of 6 isoprene units, each derived from a mevalonate molecule, but one of the carbons derived from the labelled 2-C carbon of mevalonate is secondarily lost.

rate in the presence of lovastatin and mevalonate. A high concentration of sodium mevalonate is required, about 10 times the amount needed to synthesize the ergosterol present in the cells. The lactone form of mevalonate is even less effective.

After in vivo labelling with radioactive sodium mevalonate, the specific radioactivity of β-carotene is different from that of ergosterol. A large increase in the production of β-carotene (in the *carS* mutant) is accompanied by a lowered specific radioactivity of the product. The carotene and sterol pathways are not only independently regulated, but physically separated. This separation does not begin with the first specific reactions of each, but with the synthesis of mevalonate or earlier, since there are separate mevalonate pools for different pathways. The usual view of a terpenoid 'tree', or branched pathway, is inappropriate, at least for the carotene and sterol pathways of *Phycomyces*.

In plants, carotenoids are synthesized in the plastids and sterols in the cytoplasm [19]. In *Phycomyces* the biosynthesis of carotenes appears to occur in special protein-coated lipid droplets [20,21]. The different specific radioactivity of ergosterol and β-carotene can be explained as a consequence of the compartmentation of the respective biosyntheses. Once inside the cell, the exogenous mevalonate could be diverted to one or other pathway according to the accessibility and activity of the respective compartments.

In the 'tree' model, the carotene pathway is a small side branch of the sterol pathway; the critical element in the regulation must be the fate of the last common precursor, farnesyl pyrophosphate, to form either squalene or geranylgeranyl pyrophosphate. The independence of the sterol and carotene pathways implies that the critical elements are the construction and the overall activity of the separate sterol and carotene compartments. These may be composed, to a large extent, of common elements.

Carotenes and sterols are subject to degradation and bioconversion. Thus, β-carotene is the precursor [22] of trisporic acids, the sexual pheromones that *Phycomyces* and other Mucorales diffuse to the medium. The 'pulse and chase' experiment shows that catabolism does not play an essential role in establishing the concentrations of carotene and sterol in the cells. This was already known for β-carotene [13]. The very high β-carotene content of the *carS* and other mutants cannot be attributed to decreased degradation, but to increased synthesis.

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Table III

Catabolism of carotene and ergosterol in the wild-type and a *carS* mutant (strain C115)

| Strain | Dry weight | β-Carotene | | Ergosterol | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Free | | Esterified | | Total | |
| Sample | (mg) | mg | Bq | mg | Bq | mg | Bq | mg | Bq |
| Wild-type | | | | | | | | | |
| 4 days | 107 | 11 | 48 | 314 | 523 | 88 | 120 | 402 | 643 |
| 6 days | 92 | 14 | 38 | 285 | 437 | 67 | 124 | 352 | 561 |
| *carS* mutant | | | | | | | | | |
| 4 days | 104 | 157 | 129 | 311 | 473 | 75 | 132 | 387 | 604 |
| 6 days | 93 | 180 | 128 | 326 | 472 | 99 | 172 | 425 | 644 |

After 4 days on minimal agar with labelled sodium mevalonate, cultures were transferred for 2 more days to minimal agar. Values are averages for the material found in four 10-ml plates.

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