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INHIBITION OF PHYTOENE DEHYDROGENATION AND ACTIVATION OF CAROTENOGENESIS IN *PHYCOMYCES*

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Key Word Index—*Phycomyces blakesleeanus*; Mucorales; carotenes; cinnamic alcohol, thymol, diphenylamine; dimethyl phthalate.

Abstract—Cinnamic alcohol, thymol, and diphenylamine inhibited phytoene dehydrogenation in *Phycomyces blakesleeanus*. At the same time the overall activity of the pathway, measured by the total accumulation of carotenes, increased. The activation of the pathway by diphenylamine is a mere consequence of end-product shortage. This activation was not seen in the mutants lacking end-product regulation. The mutant range of cinnamic alcohol coincided with that of dimethyl phthalate and veratrol, two potent activators of carotenogenesis. Cinnamic alcohol was thus probably a member of the phenol group of activators of carotenogenesis, with separate activating and inhibiting actions.

INTRODUCTION

The brilliant colours of the carotenoids are due to their conjugated double bonds. The colourless 40-carbon precursor, phytoene, is repeatedly desaturated to produce compounds with strong absorption of visible light. The four dehydrogenations that convert phytoene into lycopene in the fungus *Phycomyces blakesleeanus* are carried out by an aggregate of four molecules of a single enzyme, the product of gene *carB* [1–4]. In a similar way, the five dehydrogenations in the fungus *Gibberella fujikuroi* appear to be catalysed by the same enzyme [5].

Diphenylamine [6, 7] is a universal inhibitor of phytoene desaturation. In *Phycomyces* it causes a drastic decrease in the concentration of β-carotene, the final product of the pathway, and an accumulation of desaturation intermediates (phytoene, phytofluene, ζ-carotene and neurosporene, listed in order of decreasing abundance) [8–11]. The effect occurs *in vitro*, showing that the inhibitor acts on the function of the enzymes, and disappears upon removal of diphenylamine [11].

The concentrations of intermediates in the presence of diphenylamine exceed the concentration of β-carotene in its absence. This overall activation of the pathway is a consequence of end-product regulation [12, 13]. End-product regulation does not occur in mutants without an essential mediator (*carS* mutants) or mutants without end product (*carB* mutants, defective in phytoene dehydrogenation; *carR* mutants, defective in lycopene cyclization).

Many external agents stimulate carotenogenesis in *Phycomyces*. They fall into at least four groups with different mechanisms of action, exemplified by light, retinol, trisporates, and dimethyl phthalate [13]. Some phenols, such as dimethyl phthalate, 1,2-dimethoxi-4-propenylbenzene, veratrol, cinnamic alcohol, cinnamic aldehyde, and thymol, when added to the culture medium of *Phycomyces*, cause an increased accumulation of β-carotene and of desaturation intermediates; the relation of intermediates to final product varies from one phenol to another [14].

We have investigated the actions of cinnamic alcohol and thymol on carotenogenesis, compared them with those of diphenylamine and observed their synergism with light. In order to understand these actions we have used mutants with different blocks in carotenogenesis or its regulation.

RESULTS

Effects of the chemicals on the wild type

Phycomyces mycelia are light yellow when grown under standard conditions in the dark, greenish yellow in the presence of cinnamic alcohol or thymol, and whitish in the presence of diphenylamine. A strong green fluorescence under a long-UV lamp (Sylvania Blacklite Blue P20T/2) is observed in the mycelia grown with any of these chemicals. The three chemicals are toxic and the inhibition of mycelial growth sets upper limits to the concentrations that may be used in the experiments. In comparison with the untreated controls (Table 1), mycelia exposed to the chemicals (Table 2) contain considerable amounts of the intermediates of phytoene dehydrogenation and higher total carotene concentrations. The abundance of the precursors correlates with their degree of saturation The concentrations of the final product, β-carotene, decrease in the presence of diphenylamine and increase in the presence of cinnamic alcohol.

Effects of the chemicals on the car *mutants*

The accumulation of desaturation intermediates in the presence of cinnamic alcohol or diphenylamine occurs in

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Table 1 Strains used in this work and their carotene content

Strain	Relevant genotype	Main carotene* Name	Amount†	N‡
NRRL1555	Wild type	β-Carotene	62±12	8
C2	*carA5*	β-Carotene	4±2	4
C5	*carB10*	Phytoene	2856±225	4
C9	*carR21*	Lycopene	2578±345	4
C115	*carS42*	β-Carotene	3200±412	4
S119	*carA113*	β-Carotene	24±6	4
S144	*carI131*	β-Carotene	43±10	4

*The main carotene represents at least 90% of the total carotene content of the strain

†The values are averages and standard deviations (μg per g dry weight)

‡Number of independent experiments

Table 2 Carotene analyses* of wild-type mycelia grown four days in the dark in the presence of cinnamic alcohol (2 mM), thymol (0 2 mM), or diphenylamine (0 1 mM)

Carotene	Cinnamic alcohol ppm	%	Thymol ppm	%	Diphenyl-amine ppm	%
Phytoene	2806	85	2051	83	2325	91
Phytofluene	231	7	215	9	164	6
ζ-Carotene	127	4	106	4	44	2
Neurosporene	48	1	43	2	18	1
β-Carotene	111	3	61	2	6	0
Total	3323	100	2476	100	2557	100

*The values (ppm = μg per g dry weight) are averages of three independent experiments

all strains (Table 3). There is, of course, the trivial exception of strain C5, a *carB* mutant defective in phytoene dehydrogenase

The total carotene content of strain C2, a *carA* mutant, which is usually negligible, becomes appreciable in the presence of either diphenylamine or cinnamic alcohol. The total carotene content of strains C5, C9 and C115 doubles in the presence of cinnamic alcohol but remains the same in the presence of diphenylamine Neither compound causes a large increase in the total carotene content of strains S119 and S144. These were isolated by Dr F. J. Murillo in Sevilla as chemoinsensitive mutants: their β-carotene content is not very different from that of the wild type, but is not increased by either retinol, dimethyl phthalate or veratrol [13]

Synergisms between light and chemicals

Neither diphenylamine nor cinnamic alcohol preclude the stimulation of carotenogenesis by light (Table 4), but there are some unexpected interactions The combination of light and cinnamic alcohol is unexpectedly synergic. The increase in the total carotene content vastly exceeds the sum of the increases due to each agent, the dehydrogenations are almost completely inhibited and no β-carotene is formed. Photoinduction in the presence of cinnamic alcohol is the largest in *Phycomyces*, with over 4 mg carotenes per g dry weight.

The combination of light and diphenylamine leads to a more modest increase in total carotene content and to a reduced inhibition of dehydrogenation, with an increase in the β-carotene content

The relation of phytofluene to ζ-carotene in illuminated cultures is lower than in dark cultures This could be expected, because phytofluene is more photolabile than ζ-carotene [15]. Another striking case of synergism is that of light, dimethyl phthalate, and genetic inability to desaturate phytoene (Table 5).

DISCUSSION

The four successive dehydrogenations in the *Phycomyces* carotene pathway are inhibited by cinnamic alcohol, thymol, and diphenylamine At each step, part of the precursor flow accumulates unaltered in the cells and the rest is metabolized further The inhibition occurs not only in the wild type, but in all mutants with active dehydrogenations, independently of other changes in the pathway or its regulation.

Since the four reactions are carried out by identical enzymes, it is not surprising that they are all sensitive to the inhibitors The results do not allow us to obtain the inhibition constants, K_i, of each dehydrogenation by each inhibitor. The accumulation of intermediates is influenced by other factors, such as the stability of the

Table 3 Carotene analyses* of mycelia of six *car* mutants grown for four days in the dark in the presence of diphenylamine (DPA, 0 1 mM) or cinnamic alcohol (CA, 2 mM)*

Carotene	C2 DPA	C2 CA	C5 DPA	C5 CA	C9 DPA	C9 CA	C115 DPA	C115 CA	S119 DPA	S119 CA	S144 DPA	S144 CA
Phytoene	280	1057	2584	3989	1850	2133	2315	2422	120	338	104	149
Phytofluene	4	103	–	–	120	252	190	275	17	11	5	12
ζ-Carotene	–	39	–	–	18	123	28	130	5	12	–	4
Neurosporene	–	24	–	–	10	114	10	99	–	4	–	–
Lycopene	–	–	–	–	28	2043	–	–	–	–	–	–
β-Carotene	–	36	–	–	–	24	48	1886	–	6	–	5
Total	284	1259	2584	3989	2026	4689	2591	4812	142	371	109	170

*The values (μg per g dry weight) are averages of two independent experiments, – indicates less than 1 μg per g dry weight

Table 4. Synergism of light and the inhibitors, carotene content* of wild-type NRRL1555 mycelia grown either four days in the dark or two days in the dark and two days under white light (15 W m^{-2}), always in the presence of either cinnamic alcohol (2 mM) or diphenylamine (0.1 mM)

Carotene	Cinnamic alcohol dark	Cinnamic alcohol dark/light	Diphenylamine dark	Diphenylamine dark/light
Phytoene	1815	6885	2767	3656
Phytofluene	105	1	211	147
ζ-Carotene	46	7	74	92
Neurosporene	10	—	5	15
β-Carotene	43	—	2	67
Total	2019	6893	3059	3977

*The values (μg per g dry weight) are averages of two independent experiments, – indicates less than 1 μg per g dry weight. Control mycelia grown in the absence of the chemicals contain β-carotene only, 66 ± 21 μg per g dry weight in the dark, 546 ± 84 in dark/light (averages and standard deviation of six independent analyses)

Table 5. Synergism of light and dimethyl phthalate, β-carotene content* of wild-type NRRL1555 mycelia and phytoene content* of C5 mycelia grown either four days in the dark or two days in the dark and two days under white light (15 W/m^2), in the presence or the absence of dimethyl phthalate

Strain	Illumination	Dimethyl phthalate 0	Dimethyl phthalate 2 mM
NRRL1555	dark	68	987
	dark/light	482	2524
C5	dark	2813	6958
	dark/light	4355	8966

*The values (μg per g dry weight) are averages of two independent experiments.

intermediates and their chances of repeated access to the dehydrogenases after failing at any of them.

The inhibition of the dehydrogenations is always accompanied by an overall activation of the pathway, manifest in the increased total carotene accumulations. The concept of the 'mutant range' of the action of a chemical [13] can be used to decide whether cinnamic alcohol and diphenylamine activate the pathway by the same or different mechanisms. Cinnamic alcohol causes large increases in the total carotene content of the wild type and the mutants C2, C5, C9, and C115, each affected at a different gene; diphenylamine, only in the wild type and the C2 mutant. The actions of the two chemicals are thus mediated by different gene products and thus have different mechanisms.

The activation of the pathway by diphenylamine is a mere consequence of the reduction in β-carotene production. End-product regulation does not operate in strains C5, C9, and C115, because of lack of β-carotene (strains C5 and C9) or loss of the *carS* gene function which mediates end-product regulation (strain C115). Diphenylamine fails to increase the total carotene content in these strains.

The activation of the pathway by cinnamic alcohol is not a mere consequence of β-carotene shortage. The 'mutant range' of cinnamic alcohol coincides with that of dimethyl phthalate and veratrol [13]. This implies that cinnamic alcohol is itself a member of the class of phenol activators. The activating and inhibiting abilities of the phenols do not maintain a constant relation [14]; dimethyl phthalate is a strong activator and a poor inhibitor, cinnamic alcohol and thymol are strong inhibitors.

We do not know why the combination of light and cinnamic alcohol almost completely inhibits phytoene dehydrogenation. A photoproduct of cinnamic alcohol may be responsible. In any case, the production of β-carotene ceases. The consequence is a triple stimulation of the pathway: by cinnamic alcohol (as an activator of the phenol group), by light, and by lack of end product.

The same coincidence of a good phenol activator, photoinduction, and lack of end-product regulation occurs more clearly when a *carB* mutant, which makes no β-carotene, is simultaneously exposed to dimethyl phthalate and light. The resulting phytoene accumulation, *ca* 9 mg per g dry weight, is the highest known in *Phycomyces*. These results suggest that light, phenols, and lack of end product are separate activators of carotenogenesis.

EXPERIMENTAL

Strain NRRL1555 is the standard wild type of the fungus *Phycomyces blakesleeanus* Burgeff. The mutants were isolated by various authors after exposure of the spores of NRRL1555 to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [16] Cultures were grown at 22° on minimal agar for 4 days in the dark, unless otherwise stated [3].

EtOH solns of cinnamic alcohol, diphenylamine, thymol, and dimetyl phthalate were added to the medium before pouring the plates. The final concn of EtOH in the medium was equal in test and control plates and always under 8 ml/l

Carotenes were extracted [17], separated by CC [18], identified by their chromatographic behaviour and their absorption spectra, and quantified from their extinction coefficients [19]

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