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THE EFFECT OF DIPHENYLAMINE ON CAROTENOGENESIS IN *PHYCOMYCES BLAKESLEEANUS*

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Abstract—The presence of diphenylamine (DPA) during growth of mutant strains of *Phycomyces blakesleeanus* caused the expected inhibition of the formation of unsaturated carotenes, and the accumulation of phytoene in all cases. Cell extracts from DPA-grown cultures incubated with [2-^{14}C]mevalonic acid, also exhibited these effects. Inhibition of *in vitro* carotenogenesis was similarly shown by extracts from normally-grown mycelia, incubated with DPA. Removal of DPA from mycelia or from cell extracts, resulted in the formation of unsaturated carotenes. The ratio of 15-*cis*–all-*trans*-phytoenes from mycelia grown ± DPA was only marginally altered, but, *in vitro*, the presence of DPA caused a significant increase in the formation of the all-*trans* isomer in the C5 strain. These results indicate that DPA acts by post-translational regulation of enzymic activities.

INTRODUCTION

Diphenylamine (DPA) has been widely used for many years to inhibit carotenoid biosynthesis in fungi and bacteria (for review, see ref. [1]). It is also an inhibitor of algal carotenogenesis [2]. Despite its widespread use *in vivo*, the precise mode of action of DPA is still unknown. It has been suggested that it binds to the 'carotene dehydrogenase' thereby inhibiting carotene formation [3], or alternatively that it inhibits mRNA production [4].

Most fungi, including wild type [5] and mutant strains [6] of *Phycomyces blakesleeanus*, when cultured with DPA, accumulate massive amounts of phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) instead of their normal complement of unsaturated carotenoids. Since the quantity of phytoene is greater than the amounts of the normal carotenoids, and also phytoene formation is reported to be increased by DPA in a *carB* (phytoene-accumulating) strain of *Phycomyces* [6], it is thought that DPA stimulates phytoene biosynthesis as well as inhibiting its desaturation. It has also been shown to increase the percentage of all-*trans*-phytoene in both *Rhodospirillum rubrum*, and a lycopene (ψ,ψ-carotene)-accumulating strain of *P. blakesleeanus* [7], at the expense of the normally predominant 15-*cis* isomer.

With these considerations in mind, we have investigated the action of DPA on carotenogenesis in a range of *car* mutants of *P. blakesleeanus*, using *in vivo* and *in vitro* systems. Our results show that DPA affects carotene biosynthesis by altering the activities of enzymes rather than controlling protein synthesis.

RESULTS AND DISCUSSION

The formation of unsaturated carotenes in shake cultures of several mutants of *Phycomyces* was totally inhibited by 70 μM DPA (Table 1). In the presence of this compound phytoene and, to a lesser extent, phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) were accumulated at the expense of lycopene (C9) or β-carotene (β,β-carotene; C2, M1 and C115). A similar effect by DPA has been demonstrated with the wild type strain [5, 6, 8].

The amount of phytoene accumulated by C2 in the presence of DPA is more than 10-times higher than the amount of β-carotene in the control. This is probably due to the relaxation of the negative feedback control, by the end product, known to operate in this pathway [9]. This explanation is supported by the lack of a similar effect on both *carR* and *carB* mutants (C9 and C5, respectively). In these strains control by the end product should be derepressed, even in the absence of DPA, because of the lack of β-carotene. Similarly, constitutive derepression should affect C115 and M1, since both carry mutations in the *carS* gene, which is involved in control by β-carotene [10]. As expected, the accumulation of phytoene by C115, in the presence of DPA, is only slightly higher than the amount of β-carotene in control cultures (Table 1). A more significant difference was found with the M1 strain. This might be explained by assuming a leaky nature of the *carS* mutation in this strain. A stimulatory effect by DPA itself, independent of end product regulation, cannot be completely ruled out, but if this is the case it should manifest itself with the C5, C9 and C115 strains.

Lycopene (ψ,ψ-carotene) and β-carotene formation were also inhibited by DPA, *in vitro* (Table 2). Incorporation of [2-^{14}C]MVA into phytoene, under these conditions, was significantly increased in all four cell extracts. No consistent trend for *in vitro* squalene formation was found. The inhibition of phytoene desaturation *in vitro* suggests that DPA inhibits enzymic activity, rather than protein synthesis. This is confirmed by the carotenogenic activities of cell extracts from C115 mycelia, grown

Table 1. The effect of 70 μM DPA on the carotene content (μg/g dry wt) of shake cultures of *Phycomyces* strains C115, C9, C5, C2 and M1

		C115		C9		C5		C2		M1	
		−DPA	+DPA	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA
Total phytoene		N.D.*	2638	610	1390	1944	2192	N.D.	56	N.D.	3222
Phytofluene		N.D.	99	Tr†	59	Tr	N.D.	N.D.	N.D.	N.D.	Tr
Lycopene		N.D.	N.D.	1940	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
β-Carotene		2000	N.D.	Tr	N.D.	N.D.	N.D.	4	N.D.	1936	N.D.
Carotene content (% of control)		100	132	100	57	100	113	100	1400	100	166
% isomeric phytoenes	15-*cis*-	—	93.7	94.0	91.1	96.0	93.3	—	87.0	—	94.0
	All-*trans*-	—	6.3	6.0	8.9	4.0	6.9	—	13.0	—	6.0

*N.D., not detected (< 0.1 μg/g dry wt).
†Tr, trace (0.1–0.5 μg/g dry wt).

Table 2. The effect of 100 μM DPA on the incorporation of [2-^{14}C]MVA (10^{-3} dpm/mg protein) into terpenoids of *Phycomyces* cell extract of strains C115, C9, C5 and C2*

	C115		C9		C5		C2	
	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA
Total lipids	101	123	71	74	62	56	70	49
Phytoene	6.8	12	14	36	5.7	11	1.5	6.9
Lycopene	0.52	0†	0.6	0.05	0	0	0	0
β-Carotene	1.5	0.15	0	0	0	0	0.06	0
Squalene	51	40	24	31	16	19	14	14
Effect on incorporation (% of controls)								
Phytoene	100	174	100	263	100	189	100	471
Lycopene	100	0	100	9.3	—	—	—	—
β-Carotene	100	9.7	—	—	—	—	100	0
Squalene	100	78.2	100	129	100	118	100	103

*Mean values of duplicates, using 1 μCi DL-[2-^{14}C]MVA. Protein concentrations: C115, 2.9 mg; C9, 3.0 mg; C5, 4.3 mg; C2, 1.4 mg per 1 ml incubation.

† < 40 dpm/fraction.

in the presence or absence of DPA. Cell-free preparations of DPA-grown mycelia were unable to metabolize [2-^{14}C]MVA into β-carotene but showed a significant stimulation of phytoene formation, *in vitro* (Table 3). Analysis of these cell extracts showed that DPA was present at a concentration of some 0.8 mM, but that it could be removed by passage through a Sephadex G25 column. Subsequent incubation of the filtered extract with [2-^{14}C]MVA revealed incorporation into both β-carotene and phytoene, in a ratio similar to that of the control incubation (Table 3).

Attempts to reverse the inhibitory effect of DPA on *in vivo* carotenogenesis were only partially successful. Removal of DPA from C115 mycelia and subsequent resuspension in buffer containing cycloheximide, showed only a limited regeneration of β-carotene at the expense of unsaturated precursors (Table 4). This agrees with studies on the C9 [7] and wild type [5] strains, and is presumably due to the removal of the accumulated polyenes from the carotenogenic enzyme complex into the lipid droplets found in *Phycomyces* [11]. More significantly, however, the *in vitro* carotenogenic activities of DPA-grown mycelia were regenerated to those of the control mycelia on removal of DPA (Table 4). This is further support for the hypothesis that the inhibitory effect of DPA is post-translational, since the presence of cycloheximide prevents protein synthesis in *Phycomyces* [12, 13].

Previous investigations have implicated all-*trans* phytoene as an intermediate in carotene biosynthesis in *Phycomyces* [7, 14, 15], although normally it is present in only minor quantities compared to the 15-*cis* isomer [16]. Consequently, this isomer might be expected to be accumulated in DPA-grown cultures. However, the ratios of *cis*–*trans*-phytoene were not significantly altered in the presence of DPA, and the relative amounts of the isomers in those strains which do not normally accumulate phytoene (M1, C2 and C115) were similar to those of the phytoene-containing strains (C9 and C5) under these conditions (Table 1). In contrast, the formation of the two isomers *in vitro* from [2-^{14}C]MVA was significantly altered on addition of DPA to the incubation: an increase in the amount of all-*trans*-phytoene was observed (Table 5).

The differences between *in vivo* and *in vitro* results with

Table 3. Incorporation of DL-[2-^{14}C]MVA (10^{-3} dpm/mg protein) into terpenoids of cell extracts of C115 *carS* 42 *mad* 107(−), grown ± 70 μM DPA*

Cell extract	Control (−DPA)	Grown +DPA	Grown +DPA; DPA removed by gel filtration†
Total lipids	87	88	37
Phytoene	15	39	2.7
β-Carotene	1.6	0‡	0.44
Squalene	36	20	11
Ratios			
P:β-C	9.06	—	6.09
P:S	0.41	1.93	0.25

†Extract passed through Sephadex G25 prior to incubation.

*From 1 μCi DL-[2-^{14}C]MVA.

‡ < 40 dpm/fraction.

Protein concentrations: DPA-grown, unfiltered, 4.5 mg; control, 6.0 mg; DPA-grown, filtered, 1.4 mg, per 1 ml incubation.

Table 4. Changes in the mycelial carotene content, and *in vitro* carotenogenic activities, of C115 *carS* 42 *mad* 107(−), grown with 70 μM DPA, following resuspension in 50 mM phosphate buffer, pH 7.0, containing cycloheximide

	Carotene concentration of mycelia (μg/g dry wt)						Incorporation of [2-^{14}C]MVA into cell extracts (10^{-2} dpm/mg protein)*					
			Time after resuspension (hr)						Time after resuspension (hr)			
Fraction	Control	At harvest	0	1	2	4	Control	At harvest	0	1	2	4
Phytoene	0	5984	4096	2304	3424	3104	4.75	198.7	53.1	50.6	42.8	40
Phytofluene	0	98	89	396	652	296	0	0.9	0	0	0.8	0
Lycopene	0	0	36	176	264	342	3.6	0.5	4.0	4.5	5.3	2.8
β-Carotene	2000	0	40	328	452	484	9.2	1.4	13.1	10.6	10.3	5.6

Following resuspension in 50 mM phosphate buffer, pH 7.0, containing cycloheximide (100 μg/ml), samples of DPA-grown mycelia were removed at timed intervals and analysed for carotenes. Other samples, taken over the same time period, were assayed for *in vitro* carotenogenic activities. 0, 0.1 μg/g dry wt or 40 dpm/fraction.

*Incorporations of [2-^{14}C]MVA are from 1 μCi of the DL racemate. Protein concentrations of cell extracts: control, 3.2 mg; at harvest, 3.6 mg; t_0, 3.4 mg; t_1, 2.9 mg; t_2, 2.4 mg; t_4, 2.5 mg, per 1 ml incubation.

Table 5. The effect of 100 μM DPA on the *in vitro* incorporation of [2-^{14}C]MVA(10^{-3} dpm) into 15-*cis*- and all-*trans*-phytoenes of the C5 strain

Fraction	Control	+ 100 μM DPA
15-*Cis*-phytoene	1.2	0.44
All-*trans*-phytoene	3.2	6.6
Ratio *cis*–*trans*	0.37	0.07

respect to the stimulation of total phytoene formation in C9 and C5 (Tables 1 and 2) and the preferential accumulation of the all-*trans* isomer (Tables 1 and 5) may be a result of changes in equilibria, associated with the complex sequence of reactions between MVA and the carotenes, caused by the preparation of cell extracts. Lyophilization of *Phycomyces* is known to disrupt cell membranes [11, 17] and also cell extracts contain insufficient quantities of endogenous carotenes to trigger the postulated feedback control mechanisms associated with this pathway [14].

In conclusion, our results indicate that DPA exerts both its effects, i.e. inhibition of phytoene desaturation, and stimulation of phytoene biosynthesis, by post-translational modifications of enzyme activities. The precise mechanisms of these interactions must await the purification of the appropriate enzymes.

EXPERIMENTAL

Organisms. The five strains of *P. blakesleeanus*: M1 *carS* 43(+), C115 *carS* 42 *mad* 107(−), C9 *carR* 21(−), C5 *carB* 10(−) and C2 *carA* 5(−) were obtained from the culture collection of the Departmento de Genética, Universidad de Sevilla, Spain. Their nomenclature [18], growth and maintenance conditions [16] have been described previously.

Radiochemical. DL-[2-^{14}C]Mevalonic acid lactone (18 mCi/mmol) was obtained from Amersham International Ltd., Amersham, U.K. It was converted to the Na salt prior to use [19].

Addition of DPA. This was added in MeOH soln, either at inoculation of cultures or to incubations before the addition of cell extracts. The final concn of MeOH was $\leqslant 1\%$ in either case.

Removal of DPA from mycelia and cell extracts. Mycelia, grown with DPA, were harvested as described previously [19], and then washed repeatedly with 50 mM KPi buffer, pH 7.0, containing cycloheximide (100 μg/ml until no more DPA could be detected spectrophotometrically in the washings (λ_{max} 258 nm). Mycelia were finally resuspended in the washing buffer to the same vol. as the original culture. The DPA was removed from cell extracts of DPA-grown cells by passage through a Sephadex G25 column (20 × 1.5 cm) (flow rate 0.6 ml/min) with 0.2 M Tris–HCl buffer, pH 8.0, containing cycloheximide (100 μg/ml). The void vol. contained enzyme activity, but no DPA.

Preparation of cell extracts, incubation conditions, extraction and purification of radioactive polyenes, extraction of carotenoids from mycelia and quantitative determination of carotenes. These have all been described in detail in earlier publications [19, 20].

Separation of isomeric phytoenes. 15-*Cis*- and all-*trans*-phytoenes from mycelial extracts were separated on columns of grade I Al_2O_3 [16]. The radioactive isomers from incubations were purified on $AgNO_3$–Si gel (1:10) TLC, developed with petrol–EtOAc–di-*iso*-propylether (2:1:1) [21].

Radioassay and detection of radioactive bands on TLC. ^{14}C-labelled terpenoids were detected on thin-layers with a radiochromatogram spark chamber (Birchover Instruments Ltd., Letchworth, U.K.). Terpenoids were eluted from thin-layers with Et_2O and aliquots assayed by liquid scintillation counting [22].

Protein determination. Proteins in cell extracts were estimated with Folin reagent [23], using bovine serum albumin as a standard.

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