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THE SUBCELLULAR DISTRIBUTION OF CAROTENOIDS IN *NEUROSPORA CRASSA*

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Abstract—The intracellular localization of carotenoids in the fungus *Neurospora crassa* was examined after completion of photoinduced biosynthesis of these pigments. Differential centrifugation of cell homogenates yielded subcellular fractions which were characterized by activities of several marker enzymes for cell constituents and in part purified by subsequent sucrose density gradient centrifugation. Most (*ca* 58%) of the carotenoids were found to be localized in lipid globules, but substantial amounts are also associated with two membrane fractions that were rich in membranes of the endoplasmic reticulum as indicated by high activities of NADPH- and NADH–cytochrome *c* reductase. These results, along with the coincidence in the distribution of both carotenoids and activities of specific marker enzymes in the sucrose density gradients, led to the conclusion that apart from lipid globules, carotenoids are also localized in membranes of the endoplasmic reticulum.

INTRODUCTION

In many plants and micro-organisms biosynthesis of carotenoids is stimulated by light. However, a strict photocontrol has been reported only for a few fungal and bacterial species [1, 2]. For the elucidation of the photoregulatory mechanism a knowledge of both carotenogenesis and subcellular localization of pigments is necessary. The pathways of carotenoid biosynthesis are well known in a variety of fungi [3, 4], whereas less information is available on the site of synthesis and the intracellular localization of these pigments. Investigations on the subcellular distribution of carotenoids in fungi have yielded conflicting results. Carotenoids have been found either exclusively in mitochondria [5, 6] or mainly in lipid globules [7, 8], but they are also present in other organelles.

A more detailed study by Riley and Bramley [9] with a mutant of *Phycomyces* revealed that the bulk of carotenoid was located in lipid globules and a very small amount was associated with a membrane fraction. In higher plant tissue carotenogenesis has been suggested to occur in the thylakoid membranes of plastids [10]. Since in fungi cellular membranes such as the endoplasmic reticulum or mitochondrial membranes are also good candidates for lipid biosyntheses, the occurrence of carotenoids in endomembranes could provide a clue to the understanding of the mechanism of photoregulated carotenoid synthesis.

We therefore examined the subcellular distribution of carotenoids in *Neurospora crassa*, whose carotenogenesis is strictly photoinduced [11] and paid special attention to membrane fractions that might carry pigments.

RESULTS

Differential centrifugation of crude extracts

The biosynthesis of carotenoids was induced by illumination of dark-grown mycelia. After completion of pigment production the mycelia were homogenized and the crude extract was fractionated by differential centrifugation, yielding pellets that were either enriched in mitochondria or represented microsomal-membrane fractions. The supernatant obtained by the last centrifugation step was referred to as soluble fraction and there was a lipid layer on top. The distribution of protein, some typical marker enzyme activities and carotenoids among the different cell fractions are shown in Table 1. The presence in the supernatant of enzyme activities that are commonly assumed to be markers for particulate cell fractions indicates that cell organelles were damaged by the homogenization procedure. However, the data clearly demonstrate that the specific activities of the membrane-bound mitochondrial marker enzymes, cytochrome *c* oxidase and succinate–cytochrome *c* reductase [12, 13] were increased in the 13 000 g pellet (P_{13}). A similar pattern of distribution was also obtained for fumarase activity (data not shown). NADPH–cytochrome *c* reductase, a marker for membranes of the endoplasmic reticulum [14, 15], showed maximum specific activity in the P_{21}- and P_{115}-fraction. In contrast, activity of the antimycin-insensitive NADH–cytochrome *c* reductase that is presumed to be associated with both mitochondria and the microsomal fraction [12, 15, 16], was distributed predominantly among these fractions. All enzymes tested, except for

Table 1. Distribution of protein, marker enzyme activities and carotenoids among cell fractions obtained by differential centrifugation

Fraction	Protein		Cytochrome c oxidase		Succinate–cyt. c red.		NADH–cyt. c reductase		NADPH–cyt. c reductase		Carotenoids	
	Total (mg)	%	Sp. act. (*)	% act.	Sp. act. (†)	% act.	Sp. act. (†)	% act.	Sp. act. (†)	% act.	Sp. cont.	%
RE	823	—	4.8	—	0.73	—	0.49	—	0.27	—	3.33	—
$P_{2.3}$	59	7.3	9.9	10.7	1.53	1.8	0.34	12.4	0.08	1.4	1.28	4.5
P_{13}	63	7.7	18.5	63.4	5.22	86.9	0.53	23.3	0.09	6.9	1.16	4.2
P_{21}	29	3.6	6.9	12.7	0.78	5.3	0.57	9.8	0.27	20.4	4.45	9.0
P_{115}	99	12.2	3.4	7.3	0.37	6.0	0.54	28.1	0.56	25.6	3.47	17.9
S_{115}	420	51.7	0.6	4.8	0.01	0.9	0.18	23.9	0.19	35.5	0.34	6.6
Lipid layer	142	17.5	0.5	1.1	0	0	0.11	2.5	0.18	9.2	6.65	57.8

Cell fractions were isolated as described in the Experimental. Units of enzyme activity are μkat (*) or nkat (†); specific activities ('sp. act.') are units/mg protein. For carotenoids specific content ('sp. cont.') is given as μg carotenoids × 10/mg protein. '% act.' represents per cent of overall total enzyme activity recovered from the fractions. '%' is expressed as percentage of the recovered amounts of protein and carotenoids, respectively.

catalase considered as a marker for microbodies [17] (data not shown), had only very low activity in the lipid layer. The enzyme activities recovered from the P_{13}-pellet suggest that this fraction was rich in mitochondria, while both the P_{21}- and P_{115}-fraction appeared to be membrane fractions. Due to its high ATPase activity the P_{21}-fraction of *N. crassa* has previously [18] been termed a 'presumptive plasma membrane fraction'. The pattern of distribution of the carotenoids demonstrates quite clearly that the major portion (*ca* 58%) was recovered from the lipid layer, but substantial amounts were also found in the two membrane fractions.

Purification of two fractions by discontinuous sucrose density gradient centrifugation

The data listed in Table 1 indicate that a small fraction of the total amount of the carotenoids might be associated with the mitochondrial fraction (P_{13}) as reported previously for *N. crassa* and *Phycomyces* [5, 6]. However, contamination of the particulate fractions by other cell structures was unequivocally shown by the distribution of the different marker enzyme activities. Also, in the course of cell fractionation carotenoid-rich lipid globules are likely to adhere to other cell organelles, yielding an erroneous pigment distribution. Therefore, to examine the fractions for possible contamination both the P_{13}- and P_{115}-pellet that contained carotenoids along with distinct cell structures were further purified by centrifugation in discontinuous sucrose gradients. This gradient permitted the resolution of a sizeable amount of carotenoid-bearing material.

Resolution of a P_{115}-fraction by discontinuous sucrose density gradient centrifugation resulted in the pattern of distribution of protein, carotenoids and marker enzyme activities illustrated by Fig. 1. Due to the rather small amounts of carotenoids per gradient fraction several fractions were combined prior to pigment extraction. The data show that the bulk of protein sedimented at a density of 1.076 kg/l. The peaks of activity of the ER marker enzymes NADPH- and NADH–cytochrome c reductase coincided with

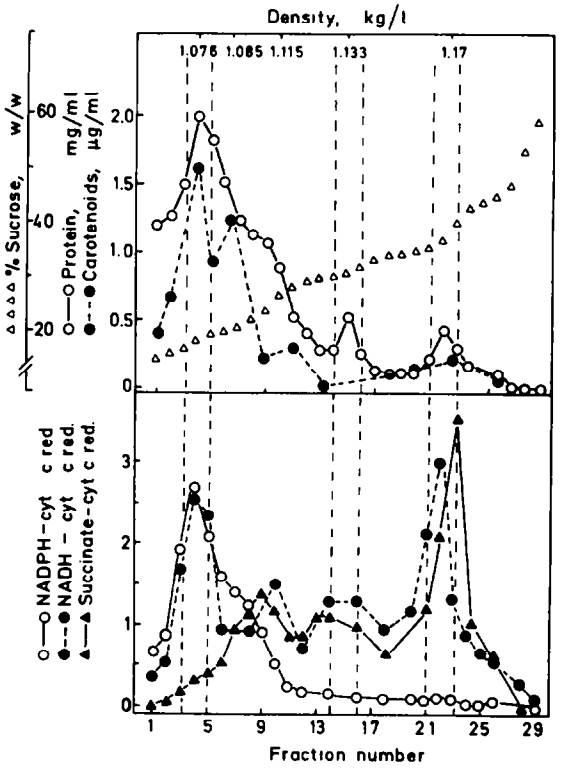

Fig. 1. Distribution of protein, enzyme activities and carotenoids after separation of a P_{115}-fraction in a discontinuous sucrose density gradient. The gradient was centrifuged for 135 min at 60 100 g. Units of enzyme activity are nkat/ml gradient fraction.

the major protein peak. A small protein band at a density of 1.17 kg/l. revealed maximum activities of the mitochondrial marker enzymes, cytochrome c oxidase and succinate–cytochrome c reductase, indicating that the P_{115}-fraction was contaminated to some extent by mitochondria. Marker enzyme activities of both, mitochondria and ER were present at a density about 1.133 kg/l.

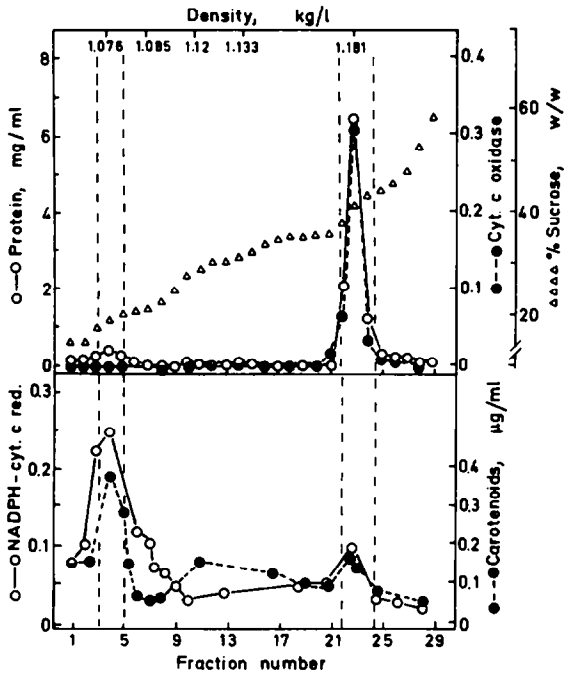

Fig. 2. Distribution of protein, enzyme activities and carotenoids after separation of a mitochondrial fraction (P_{13}) in a discontinuous sucrose density gradient. Centrifugation time of the gradients: 135 min at 60 100 g. Units of enzyme activity (per ml gradient fraction) are: Cyt. *c* oxidase: mkat; NADPH-cyt. *c* red.: nkat.

The profile of carotenoid distribution demonstrated that *ca* 85% of the total amount of carotenoids of the P_{115}-fraction sedimented with the particulate fraction containing ER membranes. Only a minor portion of the pigments banded together with the peak of the mitochondrial marker enzyme activity which also exhibited NADH–cytochrome *c* reductase activity. This indicated that the microsomal membrane fractions were indeed contaminated by mitochondrial membranes which, however, accounted for a negligible portion of the membrane associated carotenoids. Only a small amount of carotenoids was detected in the supernatant fluid of the gradient.

Resolution of the P_{13}-pellet by sucrose density gradient centrifugation was performed in the same way and the results are shown in Fig. 2. Essentially all of the protein sedimented with a distinct band at a density of 1.181 kg/l. from which also maximum activities of cytochrome *c* oxidase (Fig. 2), as well as fumarase and succinate–cytochrome *c* reductase (data not shown) were recovered, confirming that the majority of mitochondria banded there. A small protein band with a density of 1.076 kg/l. coincided with the distribution of NADPH–cytochrome *c* reductase activity and thus resembled the major protein band observed in gradients loaded with microsomal cell fractions, although the enzyme activity was much lower.

The bulk of the carotenoids present in the mitochondrial fraction again sedimented with the membrane fraction regarded to be the ER fraction by virtue of its enzyme activity. Only a minor portion of the pigments was associated with the mitochondrial peak, containing only 0.7% of the total carotenoids recovered from the P_{13}-pellet (Table 1). Moreover, since there was still some activity of NADPH–cytochrome *c* reductase present in the mitochondrial band the presence of ER membranes cannot be excluded.

DISCUSSION

In agreement with results obtained for other fungi [8, 9] the major portion of carotenoids of *N. crassa* was recovered from a supernatant lipid layer when cell homogenates were fractionated by differential centrifugation. This fraction is essentially comprised of lipid globules [8, 9] or spherosomes [19]. However, in contrast to findings previously reported for *N. crassa* [5] and other fungi [6, 8] the present data show that carotenoids are in addition associated with two membrane fractions (Table 1), which on the basis of high activities of NADPH- and NADH–cytochrome *c* reductase are suggested to be rich in membranes of the endoplasmic reticulum [14–16]. A more definite attachment of pelletable, membrane-bound carotenoids could be demonstrated for the microsomal cell fraction. Carotenoids sedimented in sucrose density gradients (Fig. 1) together with the activity of NADPH–cytochrome *c* reductase at a rather low density of *ca* 1.08 kg/l. A similarly low equilibrium density was also found for a particulate carotenoid-containing cell fraction of *Phycomyces* [9] and for a membrane fraction of the ER of higher plant cells [20, 21]. A minor fraction of the carotenoids was also associated with other cell fractions that sedimented with densities of 1.12 and 1.133 kg/l. and that may also represent membranes of the endoplasmic reticulum [15, 21–23].

Since in addition all subcellular fractions contained low amounts of carotenoids one might argue that the pigments become artificially bound to particulate fractions during the cell homogenization and differential centrifugation procedures [9]. However, this objection may be dismissed for several reasons. Our data clearly show that during gradient centrifugation the organelles banded at the same densities (Figs. 1,2) as those reported in the literature, despite the preceding differential centrifugation, thus indicating no substantial irreversible aggregation of organelles with lipids. Furthermore, when a mitochondrial fraction was resolved by sucrose density gradient centrifugation the major portion of the carotenoids consistently sedimented with the contaminating ER fraction identified by its NADPH–cytochrome *c* reductase activity, which clearly indicates that the pigments were associated with the ER. The presence in a distinct mitochondrial fraction of both pigments and activity of an ER marker enzyme could be also explained by connection of the outer membrane of mitochondria with the ER as was previously observed in a slime mutant of *N. crassa* [19]. Similarly, such connections could simulate the occurrence of carotenoids in the outer membrane of mitochondria of *N. crassa* [5].

Our data provide evidence that carotenoids are located in lipid globules and additionally in membrane fractions, presumed to represent membranes of the ER. How far carotenoids are located and synthesized at the same subcellular site remains to be answered.

EXPERIMENTAL

Biological material and growth conditions. Neurospora crassa, strain ATCC 10816 (C.B.S. Baarn, the Netherlands)

was cultivated as described previously [24, 25]. For submerged cultures liquid media were inoculated with conidial suspension (5×10^6 conidia/ml medium) and aerobically incubated on a shaker in the dark for 22 hr at 27°. The mycelia were harvested and re-suspended in an incubation medium (16 mM KH_2PO_4; 50 mM glucose) as described elsewhere [26]. Experimental conditions for the illumination with white light as described previously [26]. Time of illumination was 3 hr and photoinduced carotenoid synthesis was completed in the dark after a subsequent period of *ca* 6 hr.

Homogenization. Pigmented mycelia were harvested by filtration in a Büchner funnel, rinsed carefully with deionized H_2O and buffer, squeezed to remove excess H_2O and weighed (wet wt). Subsequently the mycelial pads (30–60 g wet wt) were resuspended in twice the vol. of ice-cold homogenization buffer (containing 0.44 M sucrose; 2 mM EDTA; 20 mM DTT; 10 mM Tris–HCl, pH 7.4) and homogenized by grinding with sand in a mortar for 8 min. All procedures were carried out at 0–4° in dim light in order to minimize photodestruction of carotenoids.

Cell fractionation. After removing the cell debris by centrifugation at 500 g for 10 min the supernatant crude extract ('RE') was fractionated by differential centrifugation yielding pellets that were enriched in mitochondria and microsomal membranes. The centrifugation steps were sucessively 2300 g, 10 min; 13 000 g, 12 min; 21 000 g, 12 min; 115 000 g, 60 min. After each centrifugation the supernatant fluids were carefully removed and the corresponding pellets were washed and resuspended in homogenization buffer yielding the particulate fractions $P_{2.3}$, P_{13}, P_{21} and P_{115}. The supernatant soln obtained by the last centrifugation step was regarded as the soluble cell fraction (S_{115}) with a lipid layer on top.

Density gradient fractionation. To assess the fractions for homogeneity, fractions P_{13} and P_{115} were purified in discontinuous sucrose density gradients. The gradients were composed of increasing sucrose concns (w/w) in 2 mM EDTA and 10 mM Tris–HCl, pH 7.4: 2 ml 60%, 5 ml 45%, 7.5 ml 35%, 6 ml 30%, 6 ml 21% sucrose. The particulate fraction (3–4 ml) was layered on the top of the gradients and centrifuged for 135 min at 60 100 g (average) in a Beckman SW 27 rotor or a WKF SW rotor. The gradients were fractionated into 1 ml samples immediately after centrifugation using an ISCO Density Gradient Fractionator. Enzyme activities were determined within 3 days after cell disruption.

Determination of enzyme activities. All assays were performed at room temp. by methods essentially as described in the literature: catalase (EC 1.11.1.6) by the method of ref. [27], for extinction coefficient see ref. [28]; fumarase (EC 4.2.1.2) see ref. [29]. Cytochrome c oxidase (EC 1.9.3.1) was measured by a slight modification of the method of ref. [30]; in a final vol. of 1.55 ml the assay mixture contained 100 mM K–Pi buffer, pH 7.0, 0.06% cytochrome c reduced, 0.01% Tween 85 and enzyme preparation. Samples were incubated with 0.01% Tween 85 for 1 min, the reaction started by addition of cytochrome and the oxidation of cytochrome immediately recorded at 550 nm. The KCN-sensitivity of the reaction (0.45 mM KCN) was tested without pre-incubation and after 1 min blank reaction. Cytochrome c oxidase is calculated from the first order velocity constant. The absorption coefficients used were $\varepsilon_{red} = 28.4 \times 10^3$ cm²/mol and $\varepsilon_{ox} = 8.1 \times 10^3$ cm²/mol. Antimycin-insensitive NADH–cytochrome c reductase (EC 1.6.99.3) and also succinate–cytochrome c reductase (EC 1.3.99.1) were determined by the method of ref. [31]; after blank reaction (1.5 min) antimycin-insensitive enzyme activities were tested by addition of 20 μg antimycin. The microsomal NADPH–cytochrome c reductase (EC 1.6.2.a) was recorded according to ref. [14]; the reaction was started by enzyme addition. The absorption coefficients used for the last three enzymes were $\varepsilon_{550} = 18.5 \times 10^6$ cm²/mol for cytochrome c reduction.

Determination of carotenoids. Extraction and quantitative determination of carotenoids was slightly modified according to ref. [26]. From the aq. samples lipids were extracted with MeOH–Me_2CO (1 : 1) and solubilized in petrol (30–50°). The amount of carotenoids was estimated using an $E^{1\%}_{1\,cm}$ -value of 2000, a value based upon taking into account the composition of the carotenoid mixture of *N. crassa* [25].

Other determinations. Protein content was determined by the method of Lowry *et al.* [32] with bovine serum albumin as standard. Sucrose concns were estimated from refractive indices, determined with an ATAGO refractometer.

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