Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

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Effect of oxygen on the development of mitochondria in squash hypocotyls during the early stages of growth

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **56** (1974), n.5, p. 814–821.

Accademia Nazionale dei Lincei

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Fisiologia vegetale. — *Effect of oxygen on the development of mitochondria in squash hypocotyls during the early stages of growth* (*). Nota di FRANCO ROLLO, presentata (**) dal Corrisp. E. MARRÈ.

RIASSUNTO. — L'effetto di diverse concentrazioni di ossigeno (3% e 20%) sullo sviluppo del sistema mitocondriale è stato studiato in ipocotili di zucca nelle prime fasi di crescita. 1) Una minor disponibilità di ossigeno riduce lo sviluppo delle attività enzimatiche mitocondriali in misura pressoché proporzionale alla riduzione dell'aumento delle proteine totali del tessuto. 2) Un'analisi di frazioni mitocondriali purificate su gradiente ha indicato che, in condizioni di ridotta disponibilità di O_2 (3%), compaiono mitocondri in cui le attività specifiche (attività enzimatica/proteine mitocondriali) della fumarasi e della succinico deidrogenasi sono pressoché uguali a quelle di mitocondri estratti da ipocotili cresciuti con O_2 al 20%, mentre l'attività specifica della citocromo ossidasi è sensibilmente inferiore. I risultati vengono confrontati con le differenti risposte che altri tipi di organismi presentano in condizioni di carenza di ossigeno.

INTRODUCTION

Conditions of reduced availability of oxygen are often encountered by both plants and animals. Data collected in a wide range of different organisms have shown that the mitochondrial apparatus can react to different conditions affecting the organism by operating changes in both the morphology and the enzymatic activities of the mitochondria. In rats kept under conditions of reduced oxygen (10%) for periods of 24 hours to one week, the ratios cytocrome oxidase/succinate dehydrogenase and cytocrome oxidase/NAD-cyt.c reductase, measured in several tissues (spleen, liver, cardiac tissue) were found to decrease the longer the animal was maintained under conditions of low oxygen tension [1]. In regenerating rat liver cells the ratio cytochrome oxidase/succinate dehydrogenase is double the ratio shown by normal cells [1]. In facultative anaerobes, such as *Saccharomyces cerevisiae* [1], lowering the pO_2 below a given threshold induces the inhibition of the mitochondrial apparatus and the appearance of a glycolytic metabolism as a response to anaerobiosis. In strictly aerobic yeasts, two types of responses have been observed. In some cases as in *Candida* [2] a lower oxygen tension causes the inhibition of synthetic activities in both cytoplasm and mitochondria, thus showing the interdependence between these two cell components: the overall effect is that the growth rate of the organism is uniformly slowed down. In at least one other case, *Rhodotorula gracilis*, a clearly adaptive response has been observed: a lower oxygen tension in this case affects the synthesis of mitochondrial structures to a lesser degree than it inhibits the accumulation of

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(**) Nella seduta del 28 maggio 1974.

total proteins. As a result, the ratio mitochondria/cytoplasm increases, thus allowing a better utilization of oxygen when this is available only in highly limiting amounts [3].

Little information is available on possible regulatory effects by oxygen on biosynthetic activities in higher plants. It is however well known that during the life cycle of these organisms several circumstances arise (e.g. during germination of the seed) which may be accompanied by a condition of limited availability of oxygen.

Squash seeds, for example, deprived of the seed coat, are still enveloped by a thin membrane which is almost impermeable to the air. The removal of this membrane accelerates the rate of germination and induces a remarkable increase in the rate of respiration and in the development of some mitochondrial and cytoplasmic enzymes [4]. The inhibiting effect shown by the pellicle on the development of cytoplasmic and mitochondrial enzymes can be reproduced by placing the seeds, deprived of the pellicle, to germinate in 3 % oxygen. This material appears therefore to lend itself remarkably well to the study of a possible effect of variations in the availability of oxygen on the development of mitochondrial oxidising enzymes.

In this work, hypocotyls of squash grown in 3 % and in 20 % oxygen have been studied to investigate *a*) the relationship between the development of the mitochondrial apparatus and the development of the cell as a whole, by comparing the increase in the mitochondrial enzymes to the increase in the level of total protein under the two conditions; *b*) the changes in some mitochondrial enzymes in comparison with those shown by the mitochondrial proteins, in mitochondrial fractions purified on sucrose gradients.

MATERIALS AND METHODS

Material. Squash seeds (*Cucurbita maxima*), deprived of the teguments, were placed to germinate in open Petri dishes, on a layer of moist filter paper, in the dark at 30° C. The dishes were placed in dessicators through which a constant flow of gas (nitrogen-oxygen mixture, with oxygen 3 % or 20 % accordingly) was maintained. Hypocotyls were collected from the germinating seeds at 48, 72 and 96 hours of germination.

Protein assay [5]. Ten hypocotyls were homogenized in a mortar with 10 % cold TCA (3 : 5 *w/v*); the extract was centrifuged at 2,000×g for 20′ and the supernatant was discarded. The pellet was washed twice with a mixture of ethanol-chloroform-ether (2 : 1 : 2), then resuspended in 5 ml of 1 N NaOH and incubated for 1 hour at 70°–80° C. After centrifugation at 2,000×g for 20′, the supernatant was set aside and the pellet re-extracted twice with 3 ml of hot 1 N NaOH. The pooled supernatants were assayed for protein with the biuret method. Protein in the mitochondrial fraction purified on sucrose gradients were extracted and assayed as described by Lowry [6, 7].

Extraction and purification of the mitochondrial fraction. Hypocotyls, freshly cut from the germinating seeds, were thoroughly homogenized in a mortar with an equal volume of the following extraction medium: 0.4M sucrose; 0,165 M Tricine buffer, pH 7,5; 10 mM KCl; 10 mM $MgCl_2$; 10 mM EDTA; 10 mM dithiothreitol [8]. The homogenate was filtered trough two layers of cheesecloth and centrifuged at 270×g for 10′. The pellet was washed once before discarding. Mitochondria were pelleted from the pooled supernatants by centrifugation at 11,000×g for 30′. The mitochondrial pellet was washed with the same buffer used for the homogenization but not containing EDTA, centrifuged for 30′ at 11,000×g and finally resuspended in the same medium. The mitochondrial fraction was then purified on sucrose gradients according to the procedure described by Breidenbach and Beevers [8]. Linear gradients were obtained from 5.5 ml of 25 % sucrose and 5.5 ml of 60 % sucrose; both solutions were made up in 10 mM EDTA, pH 7.5. Gradients were centrifuged in a SW 40 rotor in a Beckman L/L2 centrifuge for 5 hours at 23,000 rpm, the temperature being kept constant at 2° C. At the end of the run, the mitochondrial fraction was immediately collected from the gradient with a syringe. The fraction was then assayed for enzymatic activities and mitochondrial protein content.

Mitochondrial enzymes assay.

Cytochrome oxidase was assayed as described by Smith [9], by measuring the rate of oxidation of cytochrome *c* (1 mg/3 ml 60 mM potassium phosphate buffer, pH 7.2) reduced with sodium dithionite.

Succinate dehydrogenase was determined according to Hiatt [10] the concentrations of phenazine metasulfate and of KCN being altered to 1.2 mg/ml and 30 mM respectively.

Fumarase was assayed according to Racker [11].

Results

1. *Fresh weight and total protein content.*

Squash seeds, deprived of the membrane, were placed in Petri dishes and allowed to germinate in 20 % and 3 % oxygen for 48, 72 and 96 hours. The development of the germinating seeds was strictly dependent upon the availability of oxygen. At 48 hours of germination the seeds placed in 20% oxygen showed a well developed principal root as well as secondary roots. The average fresh weight of the hypocotyl was 40 mg. In the seeds germinating in 3 % oxygen secondary roots are absent and the hypocotyl appears rudimentary. At 72 hours, secondary roots continue to develop normally in the seeds germinating in 20 % oxygen, whereas they just start becoming visible at this time in the seeds placed in 3 % oxygen. The average fresh weight of the hypocotyl is 320 mg in the first case and only 100 mg in the second. At 96 hours the difference in the development of the root system is still great and the hypocotyl

of seeds germinating in 20 % oxygen averages 720 mg as against 230 mg for the seeds in 3 % oxygen (Table I).

TABLE I

Effect of oxygen concentrations on the fresh weight and on the protein content of squash hypocotyls at various times of germination.

	48 h			72 h			96 h		
	Fresh weight (g)	Proteins (mg)	Proteins/ Fresh weight	Fresh weight (g)	Proteins (mg)	Proteins/ Fresh weight	Fresh weight (g)	Proteins (mg)	Proteins/ Fresh weight
20% O_2	0.4	4.0	10	3.2	23	7.2	7.2	32	4.5
3% O_2	—	—	—	1.0	3.5	3.5	2.3	10	4.3

All the data are referred to 10 hypocotyls.

Fig. 1 shows the changes in the level of protein and in fresh weight during the first 96 hours of germination, under the conditions of low and high pO_2. In 20 % oxygen the accumulation of protein is considerable between the 48

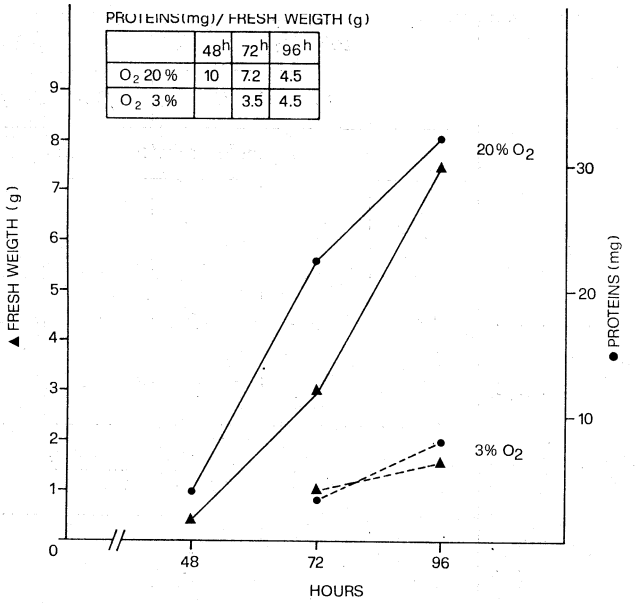

Fig. 1. – Effect of oxygen concentration on the increase in fresh weight (▲) and on the total protein content (●).

and the 72 hours and appears to occur to a much lesser extent in the following 24 hours. The initially high ratio protein/fresh weight falls considerably between 72 and 96 hours, an indication that cell expansion prevails over cell division.

Reliable data for the hypocotyls of seeds germinating in 3 % oxygen can only be obtained at 72 and at 96 hours of germination because of the difficulty encountered in dissecting the hypocotyls at 48 hours. The values obtained at 72 and 96 hours show that at low pO_2 the increase in fresh weight and in protein is greatly reduced. Moreover, at 72 hours of germination the ratio protein/fresh weight is already rather low, a suggestion that under these conditions the cells may already be undergoing enlargement.

2. *Mitochondrial enzyme activities.*

In Table II are reported the values of the activities of cytochrome oxidase, succinate dehydrogenase and fumarase obtained by assaying the mitochondrial fraction (270×g–11,000×g) at different germination times and oxygen concentrations. A considerable increase in the enzyme activities occurs during growth of the hypocotyls in 20% oxygen. The ratio between enzyme activities and total protein content at various times of germination are reported in Table II.

TABLE II

Changes in mitochondrial enzyme activities and in the content of total proteins of squash hypocotyls grown at different concentrations of O_2.

	20% O_2			3% O_2		
	48 h	72 h	96 h	48 h	72 h	96 h
	mμmoles min/10 hypocotyls					
Cytochrome oxidase	700	1525	2070	—	200	450
Succinic dehydrogenase . .	257	800	1035	—	139	347
Fumarase	1525	4300	5650	—	629	1448
Total proteins (mg.) 10 hypocotyls	4	23	32	—	3.5	10
	mμmoles/min / mg total proteins					
Cytochrome oxidase	175	66	65	—	57	45
Succinic dehydrogenase . .	64	35	32	—	39	34
Fumarase	381	187	177	—	180	145

For all three enzymes assayed the ratio activity/total protein decrease between 48 and 72 hours of germination in 20 % oxygen, remaining constant in the following 24 hours. These results indicate that between 48 and 72

hours of germination in 20 % oxygen the synthesis of cytoplasmic protein is greater than the synthesis of mitochondrial enzymes, whereas between 72 and 96 hours of germination the development of mitochondria appears to be directly related to the development of the whole cell. The development of enzyme activities is slowed down considerably in the hypocotyls of seeds germinated in 3 % oxygen: the ratios of succinate dehydrogenase and fumarase activities to total protein content remain constant between 72 and 96 hours of germination. The values of these ratios are the same as those found for the much better developed hypocotyls germinated in 20 % oxygen for 72 hours.

TABLE III

Specific activities $\left(\frac{\text{m } \mu\text{moles/min}}{\text{mg mitochondrial proteins}}\right)$ *of mitochondrial enzymes in squash hypocotyls grown at different concentrations of* O_2.

	O_2 20%		O_2 3%	
	72 h	96 h	72 h	96 h
Cytochrome oxidase	937	975	430	462
Succinic dehydrogenase	599	500	725	590
Fumarase	508	600	638	540

The activity of cytochrome oxidase appears to behave differently from those of other enzymes: the ratio enzyme activity/total protein in hypocotyls grown in 3 % oxygen is lower that the one detected in hypocotyls grown in 20 % oxygen. This difference would suggest that the cytochrome oxidase activity is affected by low oxygen pressures to a higher extent than other enzymes. To test this hypothesis, the three enzyme activities as well as the total protein content have been assayed in a mitochondrial fraction purified on gradient. The values obtained for the specific activities (enzyme activity/ mitochondrial protein) (Table III) illustrate the enzymatic composition of the mitochondria under different conditions of germination. The specific activities of both succinate dehydrogenase and fumarase remain constant in the mitochondrial fraction obtained from hypocotyls grown in 20 % oxygen after 72 or 96 hours of germination. Closely similar values are observed for the enzymes assayed in the mitochondrial fraction obtained from hypocotyls grown in 3 % oxygen, whereas the specific activity of cytochrome oxydase in this fraction is approximately 50 % of that detected in the mitochondrial fraction extracted from hypocotyls grown in 20 % oxygen. These results support the above mentioned hypothesis that the development of cytochrome oxidase is affected by the availability of oxygen to a higher extent than other enzymes and mitochondrial proteins. The lower specific activity of cytocrome

oxidase observed in mitochondria extracted from hypocotyls grown in 3 % oxygen could however be due to a reduced permeability of the mitochondrial membrane to the substrates or to a different arrangement of the enzyme in the membrane, rather than to a true reduction in the level of the enzyme.

Measurements of cytochrome oxidase activity in mitochondrial fractions treated with digitonin seem to rule out this possibility: in mitochondria from both hypocotyls grown in 20 % oxygen and hypocotyls grown in 3 % oxygen the cytochrome oxidase activity is increased by 100 % after treatment with digitonin.

The results obtained can therefore be interpreted as indicating an effect of oxygen on the development of the enzyme.

Conclusions

The activities of the three mitochondrial enzymes studied (cytochrome oxidase, fumarase and succinate dehydrogenase) were found to increase during the development of the hypocotyl. This increase is drastically reduced when the concentration of oxygen in the air is lowered from 20 % to 3 %. Under both conditions the development of the mitochondrial apparatus appears to be directly related with the synthesis of total protein in the tissue.

In the mitochondrial fraction obtained from hypocotyls grown in 20 % oxygen, after purification on gradient, all three enzyme activities increase proportionally to the mitochondrial proteins. The specific activities of these enzymes remain constant between 72 and 96 hours of germination, suggesting that the development of the three enzymes occurs simultaneously with the development of the population of mitochondria.

In hypocotyls grown in 3 % oxygen the specific activities of the enzymes studied are also found to be constant during the development of the hypocotyl. While the specific activities of succinate dehydrogenase and fumarase are approximately the same as those obtained in the hypocotyls grown in 20 % oxygen, in 3 % oxygen the cytochrome oxidase activity is noticeably lower.

The data presented above seem, as a whole, to indicate that:

a) The reduced availability of oxygen depresses the development of the mitochondrial apparatus, this effect being to an extent directly related to the development of the total protein content of the whole cell.

b) The scarcity of oxygen also induces the formation of mitochondria which appear to contain a much lower concentration of cytochrome oxidase.

The constant interrelation shown by cytoplasm and mitochondria during the course of their development recalls the situation found in Candida [2], which, like higher plants, is a strictly aerobic organism. The pattern shown by the cytochrome oxidase activity is on the contrary closer to the one observed in rats and anaerobic yeasts [1, 12]. The fact that organisms so distantly related show a similar response to changes in oxygen tension may

suggest that such a response is part of a set of metabolic reactions operated by the cell when environmental changes take place. However, the results obtained so far on squash seeds do not allow the formulation of hypotheses on how oxygen might affect the synthesis of cytochrome oxidase.

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