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Cytochrome Oxidase Activity in *Blastocladiella emersonii*[1]

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Abstract. **Studies of cytochrome oxidase in isolated mitochondria of *Blastocladiella emersonii* Cant. and Hyatt show that the enzyme was present in zoospores and throughout the development of ordinary colorless sporangia and of resistant sporangia. The enzyme activity was present in KCl, NaCl, NH_4Cl, and $KHCO_3$ induced resistant sporangia, and was shown to be as active or more active than the enzyme found in ordinary colorless sporangia and zoospores. Interfering substances causing difficulties in the measurement of cytochrome oxidase activity were found in whole cell homogenates of $KHCO_3$ grown resistant sporangia, but not in KCl, NaCl, or NH_4Cl grown thalli. These substances could be removed by dialysis or by sedimentation of the mitochondria.**

Blastocladiella emersonii, a non-filamentous water mold, forms 2 morphologically distinct types of thalli. The resistant sporangiate thalli (RS) have thick, pitted, chitinous walls and are orange-brown in color. The ordinary colorless sporangiate thalli (OC) have thin, non-pitted walls and no pigmentation. Under suitable conditions both sporangial types cleave their protoplasts into uniflagellate zoopores. The RS thalli are metabolically different from the OC thalli. Cantino (3) found that in RS thalli induced with bicarbonate, there was a decrease in oxygen uptake and a loss of certain enzymes including cytochrome oxidase. He concluded that these differences in enzymatic activity were due to the lack of formation of the enzymes rather than to an inhibition of the enzymes.

Although these enzymes were reported missing in mature RS thalli and present in OC thalli, both developed from zoospores which had the capacity to form either of the sporangial types. If suitable concentrations of bicarbonate (3), potassium chloride (9), sodium or ammonium chloride (10, 13), were present in the culture medium, the zoospores developed into RS thalli. If none of these salts were present, and all other conditions in the growth medium were equal, OC thalli formed.

In previous experiments concerning cytochrome oxidase activity in *B. emersonii* RS thalli, activity was measured manometrically or spectrophotometrically with enzyme preparations from homogenates of whole thalli grown with bicarbonate induction (4, 17). Cytochrome oxidase is a particulate enzyme. Ideally, to measure the activity of an enzyme associated with a particular organelle, it would be desirable to isolate the organelle, to measure the activity of the enzyme in a preparation of the isolated fraction, or to attempt to purify the enzyme further.

The purpose of this study was to examine cytochrome oxidase activity in preparations of isolated mitochondria and to compare it with the activity of preparations of whole cell homogenates of *B. emersonii*. Synchronous single generation cultures of OC thalli and RS thalli were examined.

Materials and Methods

Strain 49-1 of *B. emersonii* Cant. and Hyatt from the Berkeley culture collection was used throughout this study. This is a subculture of Cantino's original isolate (6). Stock cultures were maintained on Difco Emerson's YpSs agar. For obtaining large numbers of OC thalli, Difco Cantino PYG broth was used. RS thalli were induced by adding either 0.028 M KCl, 0.03 M NaCl, 0.03 M NH_4Cl, or 0.01 M $KHCO_3$ to the medium. Cultures of OC thalli were grown at 24° with 10 liters of medium in 12-liter carboys on a rotary shaker with a 1-inch throw at 100 rpm. The cultures were given supplemental aeration through a scintered glass sparger. RS cultures were grown at 24° with 750 ml of medium in 2-liter flasks on a reciprocal shaker with a 2-inch throw at 90 oscillations per min. All glassware was treated with dimethyldichlorosilane (Calbiochem, Los Angeles).

Extremely uniform cell suspensions of RS thalli (single sporangia or small clumps of 2 or 3 sporangia), could be consistently produced by autoclaving the medium for a shorter than normal period of time, *i.e.* 750 ml of PYG broth supplemented with bicarbonate or salt in 2-liter flasks for 10 min at 123°. The spore concentrations used for the inoculum in the 2-liter flasks was 5×10^6 spores per

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750 ml of medium and in the 12-liter carboys 5×10^7 spores per 10 liters of medium.

Cultures were collected on filter paper or by centrifugation and washed thoroughly with glass distilled water, followed by homogenizing solution: 0.5 M sucrose, 0.01 M phosphate buffer (pH 7.0), 0.001 M EDTA. The cells were chilled on ice.

OC and RS thalli were homogenized with a Tri-R glass-teflon homogenizer at 5000 rpm until at least 90 % breakage had occurred. Zoospores were homogenized according to the method of Lovett (16). The homogenate was centrifuged at 1000*g* for 20 min. The resulting supernatant was called the low-speed supernatant. This supernatant was centrifuged at 15,000*g* for 20 min. The mitochondrial pellet was collected and suspended in a 0.1 M phosphate buffer (pH 7.0). When indicated, the fractions were dialyzed against a 0.1 M phosphate buffer (pH 7.0) for 12 to 16 hr changing the 500 ml buffer volume 3 times. Temperature throughout the isolation procedure was maintained at ice temperature.

The cytochrome oxidase assay was adapted from Smith (19). The rate of cytochrome *c* oxidation was determined by measuring the decrease in absorbancy at 550 nm with a Beckman DB-G spectrophotometer equipped with a Beckman recorder. Both the sample cuvette and reference cuvette contained 0.9 ml phosphate buffer (pH 7.0) and 0.04 ml of the enzyme preparation; 0.06 ml of 0.5 mM reduced cytochrome *c* was stirred rapidly into the sample cuvette and the reaction was measured for 3 to 5 min.

Protein was measured by a modified Folin Phenol method after Griffin and Breuker (11). Rates of the enzymic reactions are expressed as the change in absorbance units per min per mg protein per ml reaction mixture.

Results

The results reported in table I show that mitochondrial preparations from KCl, NaCl, NH_4Cl, and $KHCO_3$ grown RS thalli all exhibited cytochrome oxidase activity and thus indicated the presence of a terminal oxidase. The undialized low-speed supernatants examined from the KCl, NaCl, and NH_4Cl grown thalli all exhibited oxidase activity, whereas, in the low-speed supernatant from the $KHCO_3$ grown RS thalli, oxidase activity was essentially zero. This was in agreement with previous reports (4) where $NaHCO_3$ was used in the growth medium and assays were performed on whole cell homogenates. When the $KHCO_3$ supernatant was dialyzed against the phosphate buffer, cytochrome oxidase activity could be detected with rates comparable to the low speed supernatants of the monovalent cation-induced RS thalli. It was apparent that there were substances that interfered with the assay of cytochrome oxidase present in the $KHCO_3$ grown thalli that were not present in the KCl, NaCl, or NH_4Cl grown thalli. These interfering substances could be removed by sedimentation of the mitochondria or by extensive dialysis. Potassium cyanide (10^{-4} M) completely inhibited enzyme activity as did heating the enzyme preparations to 100°. Washing the mitochondrial pellet with the 0.1 M phosphate buffer or dialysis of the pellet against the buffer did not significantly increase the enzymic activity of the mitochondrial preparations. Phenylthiourea (0.005 M) gave a 5 % inhibition.

Table I. *Cytochrome Oxidase Activity in Mature 72 Hour RS*

Assay mixtures contained 0.04 ml enzyme preparation, 0.06 ml cytochrome *c* and 0.9 ml phosphate buffer (pH 7.0). Absorbance at 550 nm was recorded. Controls with enzyme treated at 100° for 5 min and controls with 10^{-4} M KCN showed no activity.

Induction ion	Rate: Low-speed supernatant	Rate: Mitochondrial pellet
	Δ *A per min per mg protein per ml*	
K^+	0.080	0.274
Na^+	0.089	0.223
NH_4^+	0.073	0.300
HCO_3^-	0.000	0.310

RS thalli induced with KCl or with $KHCO_3$ were examined at various ages from the zoospores to maturity (72 hr). Mitochondrial preparations from all cases examined were of comparable activity. The undialyzed low-speed supernatants from the KCl grown thalli were also of comparable activity throughout ontogeny. In the $KHCO_3$ grown thalli, interfering substances appeared in the mitochondrial supernatants as early as 24 hr, but these substances could be removed by dialysis. Heating to 100° and 10^{-4} M KCN completely inhibited enzyme activity in all mitochondrial preparations.

Cytochrome oxidase activity was demonstrated in zoospores and in various stages of developing OC thalli (table II). The cytochrome oxidase activity

Table II. *Cytochrome Oxidase Activity in Zoospores and in OC Thalli*

Assay mixtures contained 0.04 ml enzyme preparation, 0.06 ml cytochrome *c* and 0.9 ml phosphate buffer (pH 7.0). Absorbance at 550 nm was recorded. Controls with enzyme treated at 100° for 5 min and controls with 10^{-4} M KCN showed no activity.

Age of thalli	Rate: Low-speed supernatant	Rate: Mitochondrial pellet
	Δ *A per min per mg protein per ml*	
Zoospores	0.033	0.150
13 hr OC	0.083	0.246
17 hr OC (mature)	0.038	0.140

found in zoospores was of the same magnitude as the activity found in all other stages examined in the developmental history of *B. emersonii*.

Discussion

These results show that cytochrome oxidase activity occurred not only in zoospores and in OC thalli of *B. emersonii*, but also in RS thalli induced by all of the methods used in this study. In addition, the enzyme activity present in the RS thalli was shown to be as great or greater per mg mitochondrial protein than the activity present in OC thalli and in zoospores. Therefore, contrary to previous reports (3, 4, 5), cytochrome oxidase activity was not lost in mature bicarbonate-grown RS thalli. It was present and was shown to be quite active in *in vitro* preparations of isolated mitochondria.

It was reported that polyphenol oxidase may act as the terminal oxidase in bicarbonate-induced RS thalli (3, 4, 5). When 0.003 M phenylthiourea, which completely inhibits polyphenol oxidase but not cytochrome oxidase (12), was incorporated into the growth medium, "absolutely colorless, but otherwise structurally-normal viable resistant sporangial plants will form" (2). Since it was possible to show considerable cytochrome oxidase activity that was not inhibited appreciably by 0.005 M phenylthiourea, and since inhibition of polyphenol oxidase did not appear to affect the development of bicarbonate-grown thalli, the best candidate for terminal respiratory oxidase in RS thalli is cytochrome oxidase.

Polyphenol oxidase present during the development of bicarbonate-induced RS thalli (4) may have interfered with the measurement of cytochrome oxidase activity (1, 8, 20). Cantino and Horenstein (4) stated that most of the polyphenol oxidase of *B. emersonii* was firmly bound to the cell wall and part of the polyphenol oxidase was immediately solubilized following homogenization, and that it could not be sedimented out in centrifugal fields as high as 20,000*g*. Thus none of the polyphenol oxidase would be associated with the mitochondrial fraction. However, the polyphenol oxidase system could have generated the interfering substances encountered in the low-speed supernatant of the bicarbonate-grown thalli (14, 15, 20). The inhibitory activity of these substances appears to be an artifact of homogenization and is probably not significant *in vivo* where compartmentalization of the respiratory enzymes occurs.

The cytochrome oxidase activity measured in zoospores was comparable to the activity measured in OC thalli and in RS thalli. Since the cytochrome oxidase activity of the mitochondria *in vitro* was comparable in all stages, whereas QO_2 varied considerably (7, 18), the differences in QO_2 may be due to the contribution of mitochondria to the dry wt rather than to differences in *in vivo* activity of the mitochondria. The presence of cell walls in OC thalli, and thicker cell walls in RS thalli (3) distort the QO_2 picture, whereas the oxidative activity of the mitochondria was comparable in all types.

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