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Biochimica et Biophysica Acta 1554 (2002) 170–179

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Characterization of oxidative phosphorylation in the colorless chlorophyte *Polytomella* sp. Its mitochondrial respiratory chain lacks a plant-like alternative oxidase

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Received 30 October 2001; received in revised form 7 May 2002; accepted 30 May 2002

Abstract

The presence of an alternative oxidase (AOX) in *Polytomella* sp., a colorless relative of *Chlamydomonas reinhardtii*, was explored. Oxygen uptake in *Polytomella* sp. mitochondria was inhibited by KCN (94%) or antimycin (96%), and the remaining cyanide-resistant respiration was not blocked by the AOX inhibitors salicylhydroxamic acid (SHAM) or *n*-propylgallate. No stimulation of an AOX activity was found upon addition of either pyruvate, α-ketoglutarate, or AMP, or by treatment with DTT. An antibody raised against *C. reinhardtii* AOX did not recognized any polypeptide band of *Polytomella* sp. mitochondria in Western blots. Also, PCR experiments and Southern blot analysis failed to identify an *Aox* gene in this colorless alga. Finally, KCN exposure of cell cultures failed to stimulate an AOX activity. Nevertheless, KCN exposure of *Polytomella* sp. cells induced diminished mitochondrial respiration (20%) and apparent changes in cytochrome *c* oxidase affinity towards cyanide. KCN-adapted cells exhibited a significant increase of *a*-type cytochromes, suggesting accumulation of inactive forms of cytochrome *c* oxidase. Another effect of KCN exposure was the reduction of the protein/fatty acid ratio of mitochondrial membranes, which may affect the observed respiratory activity. We conclude that *Polytomella* lacks a plant-like AOX, and that its corresponding gene was probably lost during the divergence of this colorless genus from its close photosynthetic relatives.

Keywords: Alternative oxidase; *Chlamydomonas*; Cyanide adaptation; Cytochrome *c* oxidase; *Polytomella*

1. Introduction

All plant mitochondria studied to date, along with those from some protists and fungi, have a respiratory chain with two terminal oxidases: a typical cytochrome *c* oxidase (COX) and an alternative oxidase (AOX). An important difference between these two enzymes is that the AOX is not coupled to oxidative phosphorylation [1]. AOX drains electrons directly from the ubiquinone pool and shows resistance to inhibitors of COX (cyanide and azide) and the bc_1 complex (antimycin and myxothiazol) [1]. Nevertheless, the presence of any of these inhibitors in cells or tissue cultures induces AOX mRNA synthesis, and increases AOX protein levels and activity [2–4]. Other stress conditions, such as chilling [5], increase of reactive oxygen species (ROS) [6] and aging [7], may also induce AOX synthesis. Therefore, it is thought that AOX could protect the cell from oxidative damage during metabolic states in which coupled respiration is inhibited [8–12].

Chemical analysis of photosynthetic pigments, chloroplast microstructural studies, and recently, gene sequence analysis [13] strongly suggest that the land plants (*Streptophyta*) and green algae (*Chlorophyta*) constitute a monophyletic clade called *Chlorobionta*. Green algae share with land plants the presence of AOX and alternative NAD(P)H dehydrogenases in their mitochondrial respiratory chains [14]. The presence of a cyanide resistant respiration or of

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PII: S0005-2728(02)00241-4

an AOX has been described in several chlorophytes, including *Chlamydomonas reinhardtii* [3,15,16], *Selenastrum minutum* [15], *Chlorella pyrenoidosa* [17] and *Nitella clavata* [18].

In this work, we describe the functional characterization of oxidative phosphorylation in mitochondria from the colorless chlorophyte *Polytomella* sp., a unicellular alga closely related to the green genus *Chlamydomonas* [19] that lacks both functional chloroplasts and a cell wall. Taking into account the physiological similarities and the evolutionary relationships between green algae and land plants, one supposes that the colorless alga *Polytomella* sp. could have a respiratory chain similar to that of other chlorobionts. Nevertheless, the presence of several activators known to enhance AOX activity in plants failed to induce an AOX activity in *Polytomella* sp. mitochondria. Exposure of cell cultures to KCN did not induce an AOX activity, but did change some of the kinetic parameters of cytochrome *c* oxidase. These observations support the absence of an AOX activity in the mitochondrial respiratory chain of *Polytomella* sp., and suggest the lack of an *Aox* gene and of the corresponding AOX polypeptide in this alga. We hypothesize that the AOX was lost during the divergence of the genus *Polytomella* from its close photosynthetic relatives.

2. Materials and methods

2.1. Cell cultures

Polytomella sp. strain 198.80 (E.G. Pringsheim) was obtained from the algae culture collection of the University of Göttingen, Germany. The cells were grown in 1 mM sodium phosphate buffer (pH 5.3), 10 mM MES, 0.5% sodium acetate, 5% Beinjerink's solution, and trace elements [20]. The medium was supplemented with 10 μg/ml of vitamin B_1 and 0.5 μg/ml of vitamin B_{12}. Cultures of 2 l were started with an inoculum of 0.2×10^6 cells/ml in 3-l large-bottom flasks, without shaking, at 25 °C.

Cell-wall-less *C. reinhardtii* strain CW15 was grown in TAP media [21] with 1% sorbitol, with agitation at 100 rpm, and under continuous light. The cells were harvested at the late exponential phase of growth.

2.2. Preparation of mitochondria

Mitochondria from *C. reinhardtii* were isolated as previously described [16]. For preparation of *Polytomella* sp. mitochondria, cell cultures were harvested at the stationary phase ($1.8–2.0 \times 10^9$ cells), 24 h after inoculation, by centrifugation at $2500 \times g$ for 8 min at 4 °C. Cells were washed once with 250 mM sucrose, 20 mM MOPS (pH 7.4) and 1 mM EGTA (SME buffer). The pellet was resuspended in 2 ml of SME buffer supplemented with 0.3% fatty acid-free bovine serum albumin. Cells were broken by simple homogenization with a Teflon pestle in a glass homogenizer. The homogenate was suspended in 35 ml of SME buffer and centrifuged at $2500 \times g$ for 10 min; the supernatant was recovered and centrifuged at $9500 \times g$ for 10 min. The pellet, containing the crude mitochondrial fraction, was gently resuspended in 1 ml of SME buffer and incubated in the presence of 0.2 mM ADP and 0.2% fatty acid-free BSA for 10 min at 4 °C. Then, the mitochondrial fraction was diluted to 8 ml final volume with SME buffer and centrifuged at $10{,}500 \times g$ for 10 min. The usual yield was 20–25 mg of mitochondrial protein/l of culture. Protein concentration was determined as previously described [22].

2.3. Oxygen consumption

Oxygen uptake was measured with a Clark-type O_2 electrode. Mitochondria (1 mg protein) were incubated in 2 ml of air-saturated 20 mM MOPS (pH 7.2), 120 mM KCl, and 1 mM EGTA (KME buffer) at 30 °C. Rate values were determined using an oxygen solubility of 400 ng atoms/ml (200 μM) at 2240 m of altitude and 30 °C temperature.

2.4. Cytochrome c oxidase activity

The activity of cytochrome *c* oxidase was measured by oxygen consumption. Mitochondria (1 mg protein) were incubated with 5 mM ascorbate and 1 μM antimycin in 2 ml of KME buffer at 30 °C, and the reaction was started with the addition of 2 mM TMPD as artificial electron mediator [23]. COX activity was also measured spectroscopically in mitochondrial membranes that had been twice frozen at − 72 °C and thawed, by following the oxidation of horse heart cytochrome *c* at 550–540 nm in a double beam spectrophotometer (SLM-AMINCO, DW-2000). For each measurement, 100 μg of mitochondrial protein were added to 1 ml of 250 mM sucrose, 20 mM HEPES (pH 7.2), 1 mM EGTA, 20 mM $MgCl_2$, and 1 μM antimycin at 30 °C.

2.5. Transmembrane electric potential

Changes in absorbance of the internalized lipophylic cation safranin O were used as an indicator of membrane potential [24]. Mitochondria (1 mg protein) were incubated in 3 ml of KME buffer containing 5 mM K_2HPO_4/KH_2PO_4 (pH 7.2) and 8 μM safranin O at 30 °C. The changes in the absorbance difference at 554–520 nm were measured in the double wavelength spectrophotometer. In addition, the difference of electric potential across the inner membrane was estimated by following the distribution of $[^3H]TPP^+$ (tetraphenyl phosphonium) as previously described [25]. Mitochondria (2 mg protein) were incubated in 0.5 ml of KME buffer, containing 5 mM K_2HPO_4/KH_2PO_4 (pH 7.2), and 0.8 μM $[^3H]TPP^+$ (0.05–0.07 μCi/nmol) at 30 °C. Substrates, inhibitors, or uncoupler agents, were added as indicated. After 2 min, the reaction was stopped by centrifugation at $14{,}000 \times g$ for 2 min at 4 °C. The radioactivity

of aliquots of the resulting pellets and supernatants was measured in a scintillation counter. The distribution of [^{3}H]TPP$^+$ was calculated using the Nernst equation, and corrected for the nonspecific binding of the cation to mitochondrial membranes [26].

2.6. ATP synthesis

To determine the rate of oxidative phosphorylation, the incorporation of ^{32}Pi into ATP was measured as previously described [27]. Aliquots of mitochondria (1 mg protein) were incubated in 1 ml of KME buffer containing ^{32}Pi (1–1.3 μCi/μmol), 10 mM glucose, 5 mM K_2HPO_4/KH_2PO_4 (pH 7.2), 1 mg yeast hexokinase (20 units/mg), in the presence of different substrates or respiratory inhibitors, as indicated. After 3 min at 30 °C, the reaction was stopped with ice-cold 5% (w/v) trichloroacetic acid, and the reaction medium was centrifuged to remove denatured protein. An aliquot was withdrawn for extraction of ^{32}Pi from the aqueous phase. Extraction was done by adding a mixture of ammonium molibdate/sulfuric acid, and using acetone plus *n*-butyl acetate as organic solvent. The extraction was repeated at least three times. The extracted aqueous phase was used for determination of ^{32}Pi incorporated into ATP and glucose-6-phosphate by measuring Cerenkov radiation.

2.7. Cytochrome content

Difference spectra were obtained from mitochondrial samples reduced with dithionite versus samples reduced with ascorbate (cytochrome *b* quantification), and from samples reduced with dithonite (cytochromes $a+a_3$) or ascorbate (cytochromes $c+c_1$) versus samples oxidized with persulfate [28]. Measurements were made with 1 mg mitochondrial protein in 1 ml of SME buffer diluted with glycerol 1:1 (v/v) at 25 °C. Cytochrome contents were estimated using the following extinction coefficients: $\varepsilon_{(561-540\ nm)} = 17.5\ mM^{-1}\ cm^{-1}$ for *c*-type cytochromes [29]; $\varepsilon_{(563-578\ nm)} = 26\ mM^{-1}\ cm^{-1}$ for cytochrome *b* [30] and $\varepsilon_{(609-630\ nm)} = 16\ mM^{-1}\ cm^{-1}$ for cytochromes $a+a_3$ [31].

2.8. Mitochondrial fatty acids quantification

Fatty acids extracted from *Polytomella* sp. mitochondria were quantified as described [32], with some modifications. Samples of 10 mg mitochondrial protein were stored at − 70 °C in the presence of 50 μg butyl-hydroxytoluene (BHT)/mg protein until used. After thawing, 1 ml BHT in 0.02% (v/v) methanol, 50 μg phosphatidylcholine-diheptadecanoyl as internal standard, 1 ml 0.9% NaCl, and 2 ml chloroform were added and mixed for 20 s. The suspension was centrifuged at $1600 \times g$ for 5 min. This procedure was repeated once. The organic phase was dehydrated by addition of 1.5 g Na_2SO_4 (anhydrous), then the mixture was filtered through a cotton layer. The solvent was evaporated under a stream of N_2. For trans-esterification of phospholipids and derivatization of free fatty acids to methyl-esters, dried samples were mixed with 0.1 ml toluene, 2 ml anhydrous methanol, and 0.04 ml of sulfuric acid [33]. This mixture was heated at 80 °C for 2 h. The treated samples were mixed with 1 ml 5% NaCl and 2 ml hexane and mixed vigorously. This procedure was repeated once. Then, the samples were evaporated under a stream of N_2 and stored at − 72 °C until chromatographed. Gas chromatography conditions were as follows: a CPSIL 8CB column (film thickness 0.25 mm) using helium as carrier at a flow rate of 1 ml/min at 195 °C, in a Carlo Erba 2300 chromatograph.

2.9. Polyacrylamide gel electrophoresis and immunodetection

Polyacrylamide gel electrophoresis was performed as described [34], and the immunoblotting was carried out as previously reported [35] using conventional antibodies raised against *C. reinhardtii* recombinant fusion AOX1 protein [36], obtained from the cDNA of the *Aox*1 gene [37]. Antibodies were kindly provided by Stacie Nakamoto of the Merchant group at the University of California, Los Angeles.

2.10. Southern blot hybridization

Total *Polytomella* sp. DNA was obtained by phenol–chloroform extraction as described [38]. Cells from 2 l of culture were collected and resuspended in 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 2% Triton X-100, and 1% SDS. Total DNA was extracted from broken cells by two phenol–chloroform extractions (1:1 v/v), followed by an additional chloroform extraction. The resulting aqueous phase was mixed in a 5:1 ratio (v/v) with 3 M sodium acetate to precipitate DNA. The pellet was resuspended in nuclease-free water and treated with RNAse. Aliquots were frozen (− 72 °C) until use. DNA (50 μg) was digested with different restriction enzymes as indicated, and subjected to electrophoresis in a 0.9% agarose gel in 45 mM Tris–borate (pH 8.0), 1 mM EGTA buffer (TBE). The DNA fragments ware transferred to a nylon membrane and subsequently hybridized against a partial cDNA of the Aox gene of *Chlamydomonas* sp. W80 encoding for the putative active site of AOX [a kind gift of H. Miyasaka (The Kansai Electric Power Co., Japan), GeneBank accession number AB009087], labeled with α^{32}P-dCTP by random priming.

2.11. Aox sequence amplification by PCR

A pair of desoxyoligonucleotides were designed based on two highly conserved regions of plant and *Chlamydomonas* sp. W80 *Aox* genes. Total DNA (1 μg) from *Polytomella* sp., *C. reinhardtii*, or *Mangifera indica* (mango) were used as templates for amplification assays in the presence of 1 mM $MgCl_2$ and 200 pmol of each of the

following primers: 5′-GTC TTG ACA GCT TCC TCC TC-3′ (forward) and 5′-GAG AAC GAG CGG CAT GCA CCT-3′ (reverse). Samples were denatured for 5 min at 94 °C, and subjected to 30 cycles of 1 min denaturation at 94 °C, 1.5 min annealing at 55 °C, and 2 min extension at 72 °C. All standard molecular biology techniques were as described [39]. Sequencing was carried out at the Unidad de Biología Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México.

3. Results

3.1. Oxygen uptake and the effect of respiratory inhibitors

An initial characterization of the respiratory chain of *Polytomella* sp. was carried out with oxymetric measurements in the presence of different substrates and inhibitors. Isolated mitochondria from *Polytomella* sp. were able to oxidize succinate and malate. The addition of glutamate did not increase the rate of malate oxidation (Table 1). In contrast, 10 mM L-lactate, 10 mM D-lactate, 10 mM α-ketoglutarate, or 5 mM pyruvate did not stimulate the rate of respiration. The classical respiratory inhibitors antimycin, myxothiazol and KCN strongly inhibited oxygen uptake (over 90% in all cases) with succinate or malate as substrates (Table 1). The remaining 5–10% of inhibitor-resistant oxygen uptake was not affected by the addition of the typical plant AOX inhibitors, 2 mM SHAM or 2 mM *n*-propylgallate. Nevertheless, 2 mM SHAM in the absence of any other inhibitor was able to partially inhibit respiration (15–20%) with malate or succinate, with estimated I_{max} values (minimal concentration of inhibitor required to achieve maximal inhibition) of 2.5 and 2.8 mM, respectively. The partial inhibition of total respiration by SHAM, but its lack of effect on the KCN-resistant respiration, suggested a nonspecific inhibitory action of SHAM on the respiratory chain of *Polytomella* sp. The oxidation of the quinol analogs duroquinol (29 ± 7 nmol oxygen/min/mg protein, $n=3$) and decylbenzoquinol (44 ± 16 nmol oxygen/min/mg protein, $n=5$), was completely blocked by antimycin (97% inhibition) or myxothiazol (98%), suggesting that all the electron flux is directed through the bc_1 complex. Mitochondria were also able to oxidize NADH (43 ± 18 nmol oxygen/min/mg protein, $n=4$). This oxidation was blocked by KCN (92% inhibition), antimycin (90%), myxothiazol (91%) and rotenone (67%). The oxidation of external NADH and its partial inhibition by rotenone suggested the presence of broken mitochondria in the preparation. A cytochrome *c* oxidase assay following oxygen uptake by added reduced cytochrome *c* in the presence and absence of detergents indicated that 60% of the mitochondrial preparation was intact.

3.2. Membrane potential generation and ATP synthesis

Polytomella sp. mitochondria were able to generate a significant membrane potential, as estimated by changes in the safranin O signal, in the presence of malate or succinate as oxidizable substrates (data supplied for review but not shown). The signal was abolished by the addition of CCCP. Full collapse of the membrane potential also occurred in the presence of KCN or antimycin.

Membrane potential was also quantitatively estimated by measuring the $[^3H]TPP^+$ distribution across the inner mitochondrial membrane. The membrane potential generated by succinate (101 ± 3 mV) or malate (110 ± 6.5 mV) was abolished by KCN (83 ± 6 and 67 ± 3 mV, respectively, $n=3$), antimycin (85 and 76 mV, respectively) and by CCCP (87 ± 3 and 77 ± 1.5 mV, respectively, $n=3$). The membrane potential values obtained were above the threshold value of 80 mV negative inside required to drive ATP synthesis determined for other mitochondrial preparations [40,41]. These results indicated an intact inner membrane, at least in a fraction of the mitochondrial preparation of *Polytomella* sp.

3.3. Attempts to measure alternative oxidase activity in Polytomella sp. mitochondria

To activate a possible oxidized state of the putative AOX, isolated mitochondria were treated with 20 mM DTT for 30 min at 4 °C as previously described for soybean mitochondria [42]. However, the rate of oxygen uptake was inhibited

Table 1
Oxygen uptake and ATP synthesis in *Polytomella* sp. mitochondria

Substrate	Oxygen uptake					ATP synthesis								P/O
	nmol O_2 min^{-1} mg $protein^{-1}$	Percent inhibition				nmol ATP min^{-1} mg $protein^{-1}$	Percent inhibition							
		KCN	Ant	Myx	Rot		KCN	Ant	Myx	Oli	Ven	Cat	CCCP	
Malate	41 ± 11 (7)	93	91	81	64	95 ± 23 (7)	81	80	90	72	80	75	82	1.1
Succinate	92 ± 17 (8)	94	95	96	20	54 ± 19 (7)	90	88	91	75	nd	88	91	0.3
No substrate	3 ± 2 (6)					14 ± 6 (4)							19	

Mitochondria from control cultures were incubated in the presence of the following substrates and inhibitors: 10 mM malate, 10 mM succinate, 1 mM KCN, 200 pmol antimycin/mg protein (Ant), 300 pmol myxothiazol/mg protein (Myx), 1 μM rotenone (Rot), 500 pmol oligomycin/mg protein (Oli), 500 pmol venturicidin/mg protein (Ven), 1 μM carboxyatractyloside (Cat), 500 nM CCCP.
The data shown are the mean ± S.D. with the number of different preparations in parentheses. nd, not determined.

Table 2
Oxygen uptake in control and DTT-treated mitochondria from control or KCN-adapted cells

DTT	nmol O_2 min^{-1} mg $protein^{-1}$			
	mit_C		mit_K	
	+	–	+	–
Succinate	76 ± 11 (3)	85 ± 12 (3)	66 ± 12 (3)	71 ± 14 (3)
+KCN	5 ± 3 (3)	5 ± 2 (3)	6 ± 2 (3)	5 ± 2 (3)
+Pyruvate	6 ± 2 (3)	6 ± 2 (3)	6 ± 3 (3)	6 ± 2 (3)
+AMP	7 ± 3 (3)	5 ± 3 (3)	6 ± 4 (3)	6 ± 3 (3)
+α-KG	7 ± 4 (3)	6 ± 2 (3)	nd	nd

Isolated mitochondria were incubated in SME buffer in the presence of 20 mM DTT during 30 min at 4 °C. After treatment, mitochondria were washed and centrifuged. Additions were 10 mM succinate, 1 mM KCN, 2 mM pyruvate, 2 mM AMP or 5 mM α-ketoglutarate (α-KG). Pyruvate, AMP or α-ketoglutarate were added 1 min after KCN addition.
The data indicate the mean ± S.D. with the number of different preparations in parentheses. nd, not determined.

over 90% by 1 mM KCN (Table 2, mit_C), indicating no activation of a putative plant-like AOX activity. Moreover, subsequent addition of either 5 mM pyruvate, 5 mM α-ketoglutarate, or 5 mM AMP, all of them AOX activators in plants or protists [43,44] did not stimulate oxygen uptake (Table 2).

An AOX activity [3,16] and the presence of two functional *Aox* genes have been identified in *C. reinhardtii* [37]. This *C. reinhardtii* AOX activity was stimulated by the addition of 0.2 mM KCN to the culture medium [3]. We attempted to express a possible AOX activity in *Polytomella* sp. mitochondria by exposing cell cultures to antimycin or KCN [3,5]. Concentrations of 100 pmol/ml antimycin and 0.6 mM KCN completely blocked cell proliferation, although the presence of 0.2 mM KCN maintained the culture population (Fig. 1). The addition of KCN was made 24 h after inoculation, because the addition of the inhibitor at the beginning of the culture abolished cell growth.

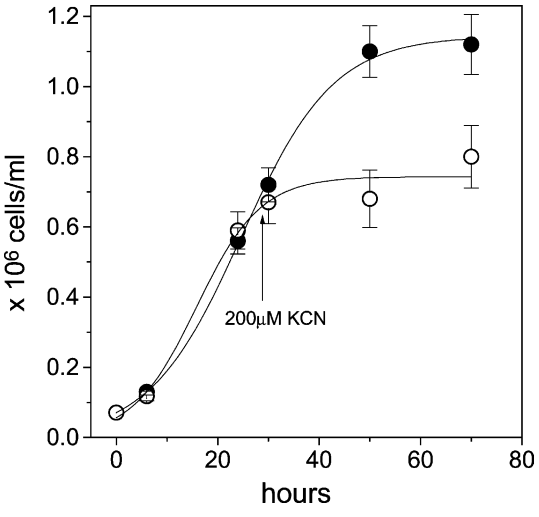

Fig. 1. Cell growth in presence of cyanide. Growth curves of *Polytomella* sp. in control acetate–MES medium (●) and in the presence of 200 μM KCN (○) added 24 h after inoculation (arrow).

Mitochondria isolated from cells adapted to 0.2 mM KCN exhibited a diminished respiratory activity using malate (21% diminished), succinate (18%), NADH (25%) or TMPD/ascorbate (12%) as electron donors. Additionally, DTT-treated mitochondria isolated from KCN-adapted cells also exhibited a high sensitivity to cyanide (Table 2, mit_K); the remaining, low cyanide-resistant respiration was, as in

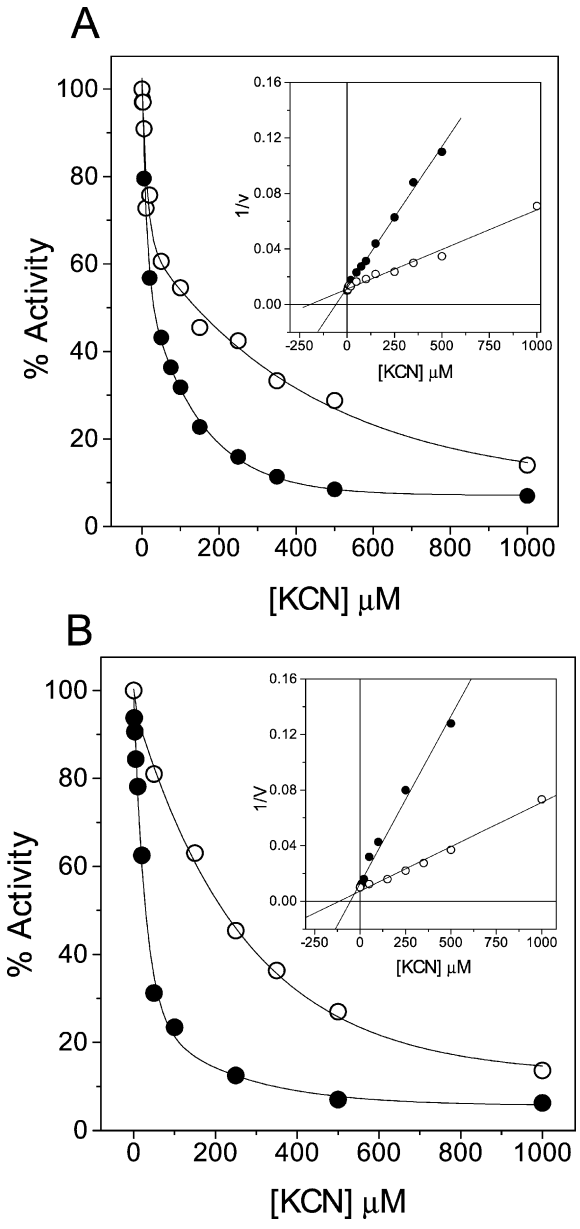

Fig. 2. Inhibition of respiration by cyanide in mitochondria from control and KCN-adapted cells. Isolated mitochondria from control (●) and KCN-adapted cultures (○), were incubated in KME medium at 30 °C, with 10 mM succinate (A), or 10 mM malate (B), and the indicated concentrations of KCN. Representative experiments for each substrate are shown. The Dixon plots for each experiment are shown in the insets. The maximal rates of respiration from mitochondria of KCN-adapted cells in the absence of KCN were 74 ± 22 (5) and 34 ± 12 (5) nmol oxygen min^{-1} mg^{-1} for succinate and malate, respectively.

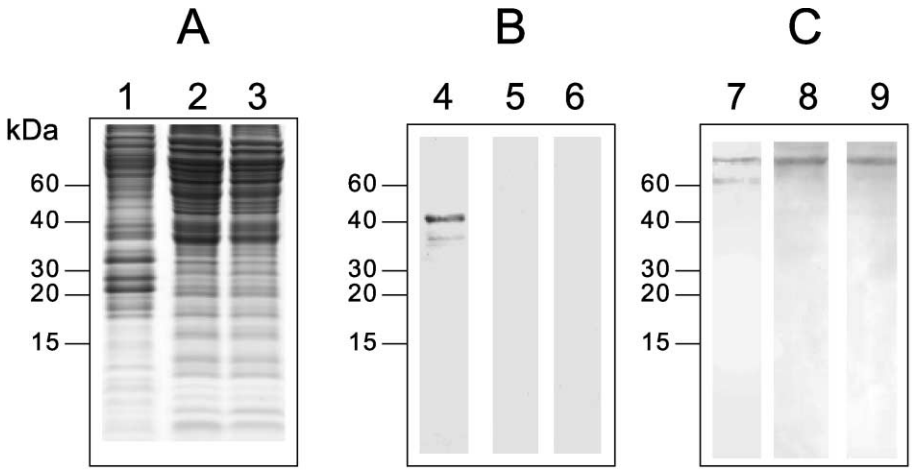

Fig. 3. Coomassie Blue stained polyacrylamide gels and Western blot analysis. Lanes 1, 4 and 7 contained mitochondria from *C. reinhardtii* (40 μg protein). Lanes 2, 5 and 8 had mitochondria from *Polytomella* sp. (40 μg protein). Lanes 3, 6 and 9 contained mitochondria from KCN-adapted *Polytomella* sp. cells (40 μg protein). (A) Coomassie Blue stained 16% polyacrylamide gel. (B) Immunoblots decorated with an anti-AOX antibody raised against the *C. reinhardtii* protein. (C) Immunoblot decorated with an anti-ATPase β subunit antibody raised against the *Polytomella* sp. protein.

control mitochondria, not stimulated by α-ketoacids or AMP, nor was inhibited by 2 mM SHAM or 2 mM *n*-propylgallate.

KCN titration of the rate of respiration with succinate (Fig. 2A) or malate (Fig. 2B) revealed a strong but incomplete inhibition at a concentration of 1 mM in mitochondria

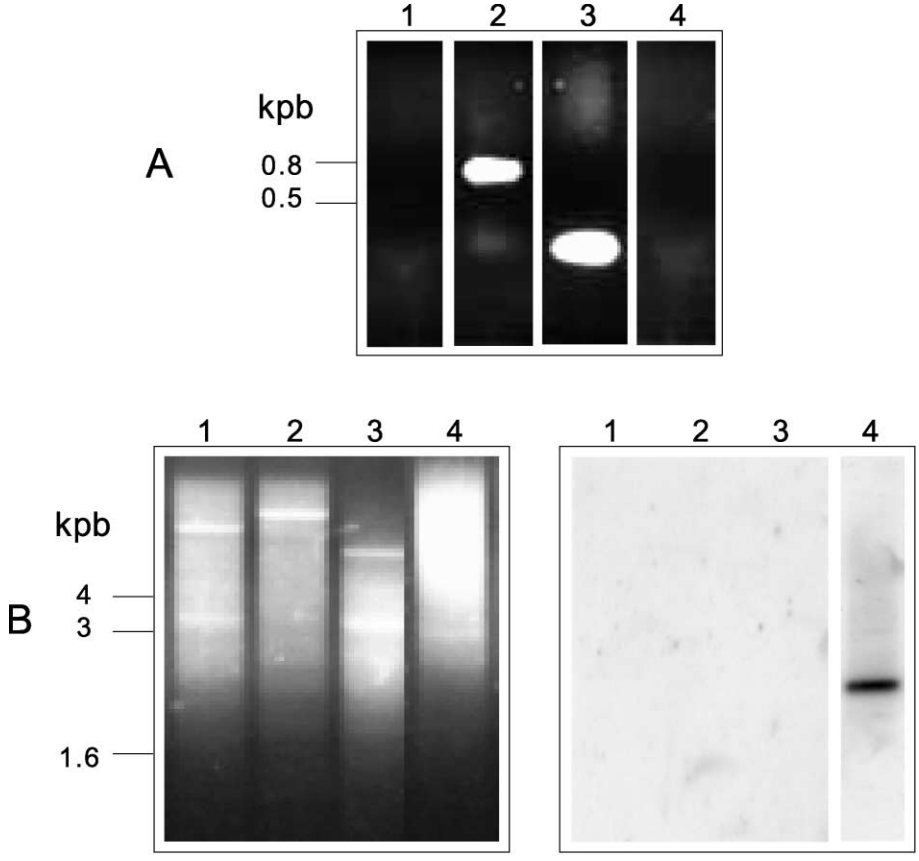

Fig. 4. (A) PCR reactions using as templates total DNA from *Polytomella* sp. (lane 1), *C. reinhardtii* (lane 2) and *M. indica* (lane 3), respectively. PCR conditions were those described in Material and methods. Lane 4 is a control without DNA. (B) Agarose gels and Southern blot analysis of total DNAs from *Polytomella* sp. (lanes 1, 2, and 3) and from *C. reinhardtti* (lane 4) using as probe a fragment of the cDNA of the *Aox* gene of *Chlamydomonas* sp. W08. Total DNAs (50 μg per lane) were treated with the restriction enzymes *Eco*RI (lane 1), *Hin*dIII (lane 2) *Eco*RI–*Hin*dIII (lane 3) and *Pst*I (lane 4). The right panel shows the 0.9% agarose gel stained with ethidium bromide and the left panel the autoradiogram of the corresponding nylon membrane.

Table 3
Mitochondrial cytochrome content

Heme type	pmol mg protein $^{-1}$	
	mit_C	mit_K
A	201 ± 48 (3)	405 ± 74 (4)
B	313 ± 13 (3)	398 ± 59 (3)
C	228 ± 14 (3)	294 ± 37 (3)

Spectra were obtained at room temperature with mitochondria from control (mit_C) and KCN-adapted cultures (mit_K).
Data shown represent mean ± S.E. of three different preparations.

from control and KCN-adapted cells. In both cases, the remaining KCN-resistant respiration was not activated by 5 mM pyruvate or 2 mM AMP, nor was it affected by the plant mitochondrial AOX inhibitors 2 mM SHAM or 2 mM *n*-propylgallate. The Dixon plots (Fig. 2, inserts) showed an apparent diminished affinity of mitochondria towards KCN by exposure to the inhibitor. Moreover, both the overall and KCN-resistant respiration were fully blocked by antimycin or myxothiazol in both types of mitochondria. Altogether, these results suggested the lack of an AOX activity in *Polytomella* sp. mitochondria isolated from cells grown with or without KCN.

3.4. Immunochemical analysis of AOX polypeptides

Using a conventional antibody raised against AOX1 of *C. reinhardtii*, it was possible to detect a polypeptide with apparent molecular mass of 40 kDa in a Western blot of isolated mitochondria from *C. reinhardtii*. In addition, a minor band around 36 kDa was also detected, and was thought to correspond to the AOX2 polypeptide (Fig. 3, panel B, lane 4). In contrast, the same anti-AOX1 antibody was unable to cross-react with mitochondrial proteins from *Polytomella* sp. control cells (Fig. 3, panel B, lane 5) or KCN-adapted cells (Fig. 3, panel B, lane 6). A polyclonal antibody raised against subunit β of the mitochondrial F_1F_0-ATPase of *Polytomella* sp. immunoreacted with a 70 kDa polypeptide of *C. reinhardtii* (Fig. 3, panel C, lane 7) and with mitochondrial proteins from *Polytomella* sp. control cells G or from KCN-adapted cells (Fig. 3, panel C, lane 9).

Numerous PCR experiments were done to amplify a putative *Aox* gene fragment from *Polytomella* sp. Fig. 4 shows one of such experiments, using the conditions described in Materials and methods. The designed primers were able to generate a PCR product of 662 bp from total DNA of *C. reinhardtii* (Fig. 4, Panel A, lane 2) and a product of 180 bp when using DNA from *M. indica* as template (Fig. 4, Panel A, lane 3). Nucleotide sequence analysis confirmed that the amplification products originated from the corresponding *Aox* genes of these organisms, and were identical to fragments from the sequences previously described (GenBank accession numbers AF314255 and X79329) [37,45]. In contrast, no amplification products were observed in the absence of DNA (Fig. 4, Panel A, lane 4) or when using total DNA from *Polytomella* sp. as template (Fig. 4, Panel A, lane 1). In parallel with these experiments, Southern blot analysis was carried out. Total DNA from *Polytomella* sp. and *C. reinhardtii* were digested with restriction endonucleases, and probed with an *Aox* cDNA fragment of *Chlamydomonas* sp. W80. While this probe readily hybridized with *C. reinhardtii* DNA fragments (Fig. 4, Panel B, lane 4), negative results were obtained with *Polytomella* sp. DNA digested with different restriction enzymes (Fig. 4, Panel B, lane, 4, Panel B, lanes 1, 2, and 3).

The lack of recognition of an AOX polypeptide and of the corresponding *Aox* gene in *Polytomella* sp. mitochondria in this colorless alga DNA also favored the notion of the absence of an alternative oxidase in the respiratory chain of this chlorophyte.

3.5. Cytochrome content and fatty acid composition

How did the presence of KCN in the culture medium modify the activity of the cytochrome *c* oxidase? In an initial attempt to address this question, cytochrome content and fatty acid composition were analyzed in mitochondria obtained from control and KCN-adapted cells.

Spectrophotometric analysis of *Polytomella* sp. mitochondrial membranes indicated an increase in the content of $a+a_3$ cytochromes after exposure to KCN and no significant variation in the content of *b* and *c* type cytochromes (Table 3). In contrast, the activity of COX was drastically decreased in mitochondria from cells adapted to KCN (V_m was four times lower than in control mitochondria). Nevertheless, the affinity ($S_{0.5}$) for cytochrome *c* did

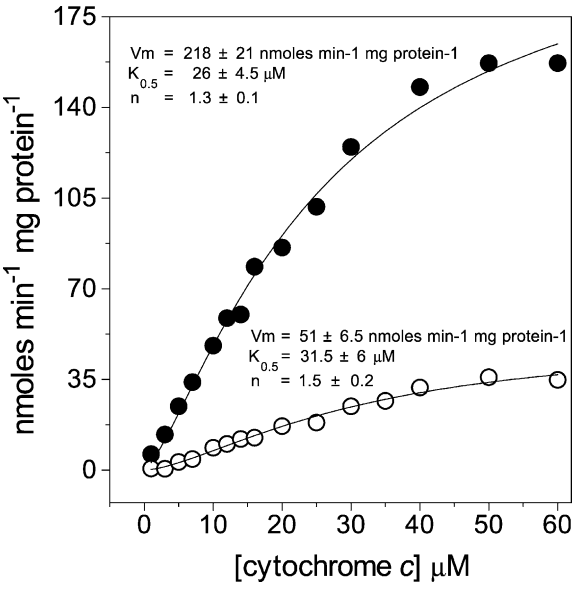

Fig. 5. Cytochrome *c* oxidase activity. Two times frozen–thawed mitochondrial membrane samples from control (●) and KCN-adapted cultures (○) were incubated with the indicated concentrations of reduced horse cytochrome *c*. The nonlinear regression, to determinate maximal velocity (V_m) and substrate affinity ($S_{0.5}$), using the Hill equation was made with Microcal-Origin 3.73 software.

Table 4
Mitochondrial fatty acid content

Fatty acid	μg mg protein^{-1}	
	mit_C	mit_K
C16:0	15 ± 2	25 ± 5.5[a]
C16:1$n-7$	5 ± 0.7	6 ± 1
C16:2$n-7$	39 ± 8	60 ± 20
C18:0	5 ± 4.5	7 ± 6.5
C18:1$n-9$	12 ± 5.5	12 ± 3
C18:2$n-6$	13 ± 2	18 ± 4[a]
C18:3$n-3$	38 ± 5	62 ± 20[a]
Totals[b]	134 ± 17	201 ± 56[a]

Fatty acids of mitochondria from control (mit_C) and KCN-adapted cultures (mit_K) were analyzed by gas spectrography. The values are the mean ± S.D. of five different preparations.

[a] The values are significantly different with respect to controls (*t*-test, at $P<0.05$).

[b] The total content considers other fatty acids (together constituting less than 5%) not shown in this table.

not change, as judged by the titration of COX activity with different cytochrome *c* concentrations (Fig. 5).

The fatty acid content of mitochondrial membranes was determined, since stress conditions such as exposure to respiratory inhibitors, chilling or aging, may induce physical changes in the mitochondrial membrane lipid components. Such changes may lead to leaks in the electron transfer reactions catalysed by respiratory complexes, enhancing ROS production and restricting the overall respiratory activity [10,46].

The major fatty acids in both mitochondrial preparations were α-linolenic acid (18: 3$n-3$) and 7,10-hexadecadienoic acid (16:2$n-7$). These two compounds constituted more than half of total fatty acid content (57% and 60%, respectively) (Table 4). A high content of α-linolenic acid has been previously described in mitochondria from other chlorobionts [47], but the 16:2$n-7$ fatty acid has been reported in low (1–6%) or trace amount in algae cells [48]. The unsaturated/saturated fatty acid ratio in mitochondria from control (5.0) and KCN-adapted cells (5.2) indicated a similar mitochondrial inner membrane fluidity in both preparations. A similar temperature dependence of the COX activity in the range 15–50 °C also suggested a similar membrane fluidity in both mitochondrial preparations (data not shown). The high content of unsaturated fatty acids (84% and 83%, respectively) was in agreement with similar values described for plant mitochondria [48]. The most remarkable effect induced by KCN exposure was the 30% increase in the total content of fatty acids (Table 4). The significant modification in the lipid to protein ratio in the inner mitochondrial membrane may influence the activity of COX.

4. Discussion

The method reported in this work for the isolation of mitochondria from the colorless chlorophyte *Polytomella* sp. yielded a preparation that was able to generate an electrochemical gradient that supported uncoupler-sensitive ATP synthesis. In a previous report, respiratory controls were obtained with the mitochondria from *Polytomella caeca* [49], although energy-dependent processes or membrane potential were not described. The partial inhibitory effect of rotenone on the oxidation of NADH suggested that a fraction of the *Polytomella* sp. mitochondrial preparation was broken; nevertheless, the remaining intact fraction was able to generate a transmembrane electric potential and to support ATP synthesis. The ATP synthesis was more than 70% inhibited by 200 pmol of oligomycin/mg protein, a concentration that is similar to the one found for maximal inhibition in plant mitochondria during state 3 respiration [50]. In contrast, in *C. reinhardtii*, the ADP-stimulated respiration was only partially inhibited (35%) by a much higher concentration of oligomycin (12.5 nmol/mg protein) [16].

The rate of respiration in mitochondria isolated from *C. reinharditi* was partially inhibited (50%, 20% and 70% inhibition for malate, succinate and exogenous NADH, respectively) by 1 mM KCN and the subsequent addition of 2 mM SHAM totally blocked respiration, indicating the presence of a plant-like respiratory chain in this photosynthetic chlorophyte [16]. In contrast, the full inhibition of oxygen uptake by KCN, antimycin or myxothiazol with either oxidizable substrates or quinol analogs suggested the lack of an active alternate electron pathway in *Polytomella* sp. mitochondria. The AOX activity in plant and protist mitochondria is variable since it depends on cell culture conditions [2,3,11], type of tissue, the developmental stage of the organism [45,51] and the presence of activators such as pyruvate, AMP, IMP or GMP [1,44]. However, in all *Polytomella* sp. preparations, neither the KCN-resistant respiration nor the ubiquinol/oxygen oxidoreductase activity was stimulated by α-ketoacids or AMP.

The AOX in higher plants is present as a homodimer, which exists in a nonlinked (reduced) or covalently linked (oxidized) forms, the former being five times more active than the latter [42]. The ratio between these two forms is regulated by the redox state of the interdimeric disulfide bond. However, treatment with DTT to reduce the putative disulfide bridge and generate an active reduced homodimer [42] failed to enhance a cyanide-resistant respiration in *Polytomella* sp. mitochondria. It is relevant that the two *Aox* genes reported in *C. reinhardtti* lack the conserved cysteines present in plant sequences, which have been related to the regulatory disulfide bond formation and α-keto acid activation [37]. In consequence, this redox regulation of plant-like AOX may not be present in chlamydomonad algae at all.

The stress condition that resulted from the addition of KCN did not induce an AOX activity in *Polytomella* sp., suggesting a nonfunctional state of the protein or the absence of a corresponding *Aox* gene, as discussed below. However, the exposure of cultures to KCN affected the

activity of COX. Our data suggest that the increase in the fatty acid content or in the lipid/protein ratio in mitochondria from KCN-adapted cells could affect the activity of COX from *Polytomella* sp. Changes in activities of membrane proteins may be induced by physical changes in the mitochondrial membranes during stress conditions [46]. In this case, the COX activity and the affinity of the binuclear center towards KCN was modified. The apparent lower affinity for cyanide in mitochondria from KCN-adapted cells may be related to a lower flux control exerted by COX on electron transport induced by the presence of KCN. The presence of a higher amount of *a*-type cytochromes did not correlate with the diminished activity of COX in mitochondria from KCN-adapted cells (Fig. 5). Thus, the cytochrome ($a+a_3$) accumulation is probably associated to nonfunctional proteins.

Altogether, the above data indicates that *Polytomella* sp. does not have a plant-like AOX, and most probably lacks a corresponding *Aox* gene. *Polytomella* therefore differs from other chlorophyte algae, such as *Chlamydomonas* [16] and *Chlorella* [17]. The absence of an AOX activity has also been observed in another colorless chlorophyte, *Polytoma uvella* [52]. Is it possible to relate the loss of photosynthesis with the loss of *Aox* genes? The change from photosynthetic to heterotophic life style increases the mutation rate and induces some structural modifications in the plastid-located genes related to photosynthesis [53]. These modifications may also occur in nucleus-located genes related to chloroplast or other organelle activities. Accumulated mutations could eventually inactivate these genes, making the loss of photosynthetic capacity irreversible [54]. Light exposure of potato leaves increases dramatically the expression of the nucleus-localized *Aox* and *Nad1* (encoding the internal NAD(P)H dehydrogenase of mitochondria) genes [55]. This light-induction suggests that these genes are related to photosynthetic function and light metabolism, and that they may be regulated by chloroplast activity. It is therefore possible to visualize a role for AOX during light-metabolism. In dark–light transitions, the chloroplast NAD(P)H/NAD(P) ratio increases due to a highly active photosynthetic chain, eventually producing a photoinhibitory effect, diminishing the efficiency of the process, and leading to ROS production and concomitant damage [56]. In these conditions, the formation of NAD(P)H and ATP exceeds the cell demand. The existence of alternate, nonphosphorylating mitochondrial components (AOX and several NAD(P)H dehydrogenases) could diminish photoinhibition by removing the reduced intermediaries exported from the chloroplast, dissipating the light energy and avoiding ROS formation, even when ADP availability is limited. Although plant cells posses well-characterized systems that avoid overreduction, like activation of Calvin's cycle and increased nitrogen assimilation [56], the participation of AOX could be important.

With the loss of the whole autotrophic pathway, the mutation rate and inactivation of the light metabolism-related genes could accelerate. In the case of *Polytomella* sp., the possible evolutionary loss of the *Aox* gene may be explained by random drift, assuming a nonessential activity of the encoded AOX enzyme after the loss of photosynthesis. Alternatively, but less likely, the *Aox* gene may still be present, and may be expressed in a different stage of development or in other physiological conditions in the life cycle of *Polytomella* sp. that were not explored in this work, like the processes of encystment and excysment.

Acknowledgements

The authors wish to thank Drs. E. Davidson and M.P. King (Thomas Jefferson University, Philadelphia), M. Gómez-Lim (CINVESTAV, Irapuato, Mexico), and A. Gómez-Puyou (IFC, UNAM, Mexico) for their support in performing some experiments; Drs. L. Ongay y G. Codiz (Unidad de Biología Molecular, IFC, UNAM, Mexico), M. Vázquez-Acevedo (IFC, UNAM, Mexico) and M. West (Unidad de Microscopía, IFC, UNAM, Mexico) for their expert technical help; Drs. M. Gómez-Lim (CINVESTAV, Unidad Irapuato, Mexico), H. Miyasaka (The Kansai Electric Power Co., Japan), S. Merchant (University of California at Los Angeles), and S. Nakamoto (University of California at Los Angeles), for kindly providing us with antibodies or DNA probes that were used in the initial and final stages of this work; and to Drs. A. Atteia (IFC, UNAM), M. Gómez-Lim (CINVESTAV, Unidad Irapuato, Mexico) and D.W. Krogmann (Purdue University), for their critical comments on the manuscript. This work was supported by grants 27754N from CONACyT, Mexico; and IN202598 from DGAPA, UNAM, Mexico. ARP received a fellowship from DGAPA-DGEP (UNAM), and a graduate student grant from PAEP (UNAM).

References

[1] G.C. Vanlerberghe, L. McIntosh, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 702–706.
[2] G.C. Vanlerberghe, L. McIntosh, Plant Physiol. 105 (1994) 867–874.
[3] A. Goyal, N.E. Tolbert, Plant Physiol. 89 (1989) 958–962.
[4] H. Yukioka, S. Inagaki, R. Tanaka, K. Katoh, N. Miki, A. Mizutani, M. Masuko, Biochim. Biophys. Acta 1442 (1998) 161–169.
[5] G.C. Vanlerberghe, L. McIntosh, Plant Physiol. 100 (1992) 1846–1851.
[6] A.M. Wagner, FEBS Lett. 368 (1995) 339–342.
[7] C. Hiser, L. McIntosh, Plant Physiol. 93 (1990) 312–318.
[8] E.F. Elstner, W. Oswald, Proc. R. Soc. Edinb. 102B (1994) 131–154.
[9] V.N. Popov, R.A. Simonian, V.P. Skulachev, A.A. Starkov, FEBS Lett. 415 (1997) 87–90.
[10] A.C. Purvis, Physiol. Plant. 100 (1997) 165–170.
[11] D.P. Maxwell, Y. Wang, L. McIntosh, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8271–8276.
[12] Y. Amor, C. Mordechai, A. Levine, FEBS Lett. 477 (2000) 175–180.
[13] D. Moreira, H. Le Guayader, H. Philippe, Nature 405 (2000) 69–72.
[14] I.M. Moller, A.G. Rasmusson, Trends Plant Sci. 3 (1998) 21–27.
[15] H. Weger, R. Guy, D.H. Tupin, Plant Physiol. 93 (1990) 356–360.

[16] M. Eriksson, P. Gardeström, G. Samuelsson, Plant Physiol. 107 (1995) 479–483.
[17] D.F. Sargent, C.P.S. Taylor, Plant Physiol. 49 (1972) 775–778.
[18] E. Ross, Am. J. Bot. 25 (1938) 458–463.
[19] M. Melkonian, B. Surek, Bull. Soc. Zool. Fr. 120 (1995) 191–208.
[20] A. Atteia, R. van Lis, J. Ramírez, D. González-Halphen, Eur. J. Biochem. 267 (2000) 2850–2858.
[21] D.S. Gorman, R.P. Levine, Proc. Natl. Acad. Sci. U. S. A. 54 (1965) 1665–1669.
[22] M.A.K. Markwell, S.M. Hass, L.L. Biber, N.E. Tolbert, Anal. Biochem. 87 (1978) 206–210.
[23] R. Moreno-Sánchez, B.A. Hogue, C. Bravo, A.H. Newman, A.S. Basile, P.K. Chiang, Int. J. Biochem. 41 (1991) 1479–1484.
[24] A. Zanotti, G.F. Azzone, Arch. Biochem. Biophys. 201 (1980) 255–265.
[25] R. Moreno-Sánchez, S. Rodríguez-Enríquez, A. Cuéllar, N. Corona, Arch. Biochem. Biophys. 319 (1995) 432–444.
[26] H. Rottenberg, J. Membr. Biol. 81 (1984) 127–138.
[27] R. Moreno-Sánchez, J. Biol. Chem. 260 (1985) 4028–4034.
[28] C. Bravo, M. Vargas-Suárez, S. Rodríguez-Enríquez, H. Loza-Tavera, R. Moreno Sánchez, J. Bioenerg. Biomembranes 33 (2001) 289–301.
[29] M. Degli Esposti, G. Lenaz, Arch. Biochem. Biophys. 289 (1991) 303–312.
[30] J.W. Priest, J.W. Hadjuk, J. Biol. Chem. 257 (1992) 20188–20195.
[31] K.A. Gray, M. Grooms, H. Myllykallio, C. Moomaw, C. Slaugther, F. Daldal, Biochemistry 33 (1994) 3120–3127.
[32] L. Folch, M. Less, C.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 265–275.
[33] W.W. Christie, in: W.W. Christie (Ed.), Gas Chromatography and Lipids, The Oily Press, Scotland, 1989, pp. 64–84.
[34] H. Shägger, T.A. Link, W.D. Engel, G. von Jagow, Methods Enzymol. 126 (1986) 224–237.
[35] D. González-Halphen, M.A. Lindorfer, R.A. Capaldi, Biochemistry 27 (1988) 7021–7031.
[36] S.S. Nakamoto, PhD thesis in Biochemistry and Molecular Biology, University of California, Los Angeles, 2001, pp. 47–74.
[37] M. Dinant, D. Baurain, N. Coosemans, B. Joris, R.F. Matagne, Curr. Genet. 39 (2001) 101–108.
[38] X. Pérez-Martínez, M. Vázquez-Acevedo, E. Tolkunova, S. Funes, M.G. Claros, E. Davidson, M.P. King, D. González-Halphen, J. Biol. Chem. 275 (2000) 30144–30152.
[39] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor, New York, 1989.
[40] G.F. Azzone, T. Pozzan, S. Massari, M. Bragadin, Biochim. Biophys. Acta 501 (1978) 296–306.
[41] H. Woelders, T. Van der Velden, K. Van Dam, Biochim. Biophys. Acta 934 (1988) 123–134.
[42] A.L. Umbach, J.N. Siedow, Plant Physiol. 103 (1993) 845–854.
[43] A. Millar, J.T. Wiskich, J. Whelan, D.A. Day, FEBS Lett. 329 (1993) 259–262.
[44] W. Jarmuszkiewicz, A.M. Wagner, M.J. Wagner, L. Hryniewiecka, FEBS Lett. 411 (1997) 110–114.
[45] A. Cruz-Hernández, M.A. Gómez-Lim, Planta 197 (1995) 569–576.
[46] A.C. Purvis, R.L. Shewfelt, Physiol. Plant. 88 (1993) 712–718.
[47] K. Edman, I. Ericson, Biochem. J. 243 (1987) 575–578.
[48] G.A. Thompson Jr., Biochim. Biophys. Acta 1302 (1996) 17–45.
[49] D. Lloyd, B. Chance, Biochem. J. 107 (1968) 829–837.
[50] S. Krömer, M. Stitt, H.W. Heldt, FEBS Lett. 226 (1988) 352–356.
[51] Y. Ito, D. Saisho, M. Nakazono, N. Tsutsumi, A. Hirai, Gene 203 (1997) 121–129.
[52] D.A. Webster, D.P. Hackett, Plant Physiol. 41 (1965) 1091–1100.
[53] A.M. Nedelcu, J. Mol. Evol. 53 (2001) 670–679.
[54] R. Rumpf, D. Vernon, D. Schreiber, C.W. Birky, J. Phycol. 32 (1996) 119–126.
[55] A.S. Svensson, A.G. Rasmusson, Plant J. 28 (2001) 73–82.
[56] M.H.N. Hoefnagel, O.K. Atkin, J.T. Wiskich, Biochem. Biophys. Acta 1366 (1998) 235–255.