

Isolation, Purification, and Characterization of Mitochondria from *Chlamydomonas reinhardtii*¹

Mats Eriksson*, Per Gardeström, and Göran Samuelsson

Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden

Mitochondria were isolated from autotrophically grown *Chlamydomonas reinhardtii* cell-wall-less mutant CW 92. The cells were broken by vortexing with glass beads, and the mitochondria were collected by differential centrifugation and purified on a Percoll gradient. The isolated mitochondria oxidized malate, pyruvate, succinate, NADH, and α -ketoglutarate. Respiratory control was obtained with malate (2.0) and pyruvate (2.2) but not with the other substrates. From experiments with KCN and salicylhydroxamic acid, it was estimated that the capacity of the cytochrome pathway was at least 100 nmol O₂ mg⁻¹ protein min⁻¹ and the capacity of the alternative oxidase was at least 50 nmol O₂ mg⁻¹ protein min⁻¹. A low sensitivity to oligomycin indicates some difference in the properties of the mitochondrial ATPase from *Chlamydomonas* as compared to higher plants.

The amount of work published concerning *Chlamydomonas reinhardtii* has increased steadily during the last decade. A large amount of physiological and biochemical work has been done, and after the publication of the methods to transform both the nucleus (Rochaix and van Dillewijn, 1982; Debuchy et al., 1989; Kindle et al., 1989; Kindle, 1990) and the chloroplast (Boynton et al., 1988), the amount of molecular work has also increased.

Many of the processes in a cell are more easily studied in isolated organelles than in intact cells. The cell wall has limited the possibilities to do such isolations from *C. reinhardtii* and other green algae, since it is rigid and tough to break. This problem can, however, be overcome by using cells lacking the cell wall. Such cells can be achieved in two ways. Either a cell wall-deficient mutant can be used (Mendiola-Morgenthaler et al., 1985) or the cell wall can be removed by autolysine (Klein et al., 1983b). This enzyme is excreted by *C. reinhardtii* during mating and it digests the cell wall from the cell so that exchange of cell material can occur. When the cell wall is removed, the cell can be disrupted in different ways, for example by Yeda press (Ryan et al., 1978; Mendiola-Morgenthaler et al., 1985), digitonin treatment (Klein et al., 1983b), or pressing the cells through a syringe needle (Mason et al., 1991). Both cell wall-deficient mutants and protoplasts obtained after au-

tolysine treatment have been used to isolate intact chloroplasts showing photosynthetic carbon reduction (Klein et al., 1983a; Mendiola-Morgenthaler et al., 1985). Despite this, there are only a few publications dealing with isolation of mitochondria (Ryan et al., 1978; Klein et al., 1983b; Kreuzberg et al., 1987; Moyano et al., 1992). To our knowledge no thorough characterization of the respiratory properties of mitochondria from *C. reinhardtii* has been published. Therefore, it is not known whether there are any principal differences between mitochondria of *C. reinhardtii* and mitochondria from higher plants.

In this work we have isolated mitochondria from a cell wall-deficient mutant of *C. reinhardtii* by vortexing the cells with glass beads, collecting the mitochondria by differential centrifugation, and purifying them on a Percoll gradient. It takes about 1 h to get crude mitochondria using this method. We have characterized the respiratory properties of the mitochondria obtained with respect to O₂ uptake rates with different substrates and the effect of some respiratory inhibitors. The results are discussed in relation to what is known about mitochondria from higher plants.

MATERIALS AND METHODS

Algal Strain and Culture Conditions

Chlamydomonas reinhardtii, mutant strain CW 92, was grown in batch cultures at 25°C in continuous light at an intensity of 150 μ mol quanta m⁻² s⁻¹. Bottles containing 2 L of minimal medium were vigorously bubbled with 5% CO₂. The major components of the medium were prepared according to the method of Sueoka (1960), and the trace element solution was prepared according to the method of Hutner et al. (1950).

Disintegration with Glass Beads

Two liters of culture were harvested at a Chl concentration of 8 to 12 μ g Chl mL⁻¹ by centrifugation at 400g for 5 min, and the pelleted cells were washed two times in 20 mM Hepes-KOH, pH 7.2. After the cells were washed, they were resuspended in breaking buffer (50 mM Hepes-KOH, pH 7.2, 5 mM EDTA, 0.25 M sorbitol, 4 mM Cys, 0.5% PVP 40, 0.1% BSA) to a concentration of 0.5 g algal pellet mL⁻¹. Five milliliters of algal suspension were mixed with 10 mL

Abbreviations: α -KG, α -ketoglutarate; CAT, carboxyatractyloside; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazine; SHAM, salicylhydroxamic acid; TPP, thiamine pyrophosphate.

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* Corresponding author; e-mail mats.eriksson@plantphys.umu.se; fax 46-90-16-66-76.

of glass beads (Glass beads 425–600 μm , Sigma) in a 50-mL plastic centrifugation tube and vortexed on a bench-top vortex mixer at low speed for 1 min.

Purification

The cell homogenate was rinsed from the glass beads by addition of 30 mL of breaking buffer and centrifuged for 5 min at 2000g. The pellet was discarded and the supernatant was further centrifuged for 10 min at 5000g. The resulting pellet containing crude mitochondria was suspended in assay buffer (10 mM potassium phosphate buffer, pH 7.2, 0.1% BSA, 0.25 M sorbitol, 10 mM KCl, 5 mM MgCl_2) and used for O_2 exchange measurements.

To purify the crude mitochondria they were mixed with 30 mL of 20% Percoll (20% Percoll, 0.25 M sorbitol, 10 mM Mops-KOH, pH 7.2, 1 mM EDTA, 0.1% BSA, 0.5% PVP) and spun for 40 min at 20,000g. The bottom 3 mL were diluted with 40 mL of wash buffer (10 mM potassium phosphate buffer, pH 7.2, 0.1% BSA, 0.25 M sorbitol, 1 mM EDTA) and spun for 10 min at 10,000g. The pellet was resuspended in assay buffer.

Isolation of Pea Mitochondria

The procedure for the isolation of mitochondria from pea leaves (*Pisum sativum* L. cv Kelvedon Wonder) was according to that reported by Hamasur et al. (1990). The major difference was that leaves from *P. sativum* were used instead of *Spinacia oleracea*.

Measurements of O_2 Exchange

Respiratory measurements were done at 25°C using a Hansatech O_2 electrode (Hansatech, Kings Lynn, Norfolk, England) in assay buffer. The measurements were made with 0.1 to 0.4 mg of protein in a total volume of 0.4 mL. The substrates used were malate (10 mM malate, 10 mM glutamate, and 1 mM NAD), succinate (10 mM), pyruvate (1 mM pyruvate, 0.4 mM malate, 0.1 mM CoA, 1 mM NAD, and 0.1 mM TPP), α -KG (20 mM α -KG, 0.5 mM AMP, 0.4 mM TPP, 1 mM NAD, and 0.4 mM CoA), and NADH (1 mM). For the RC measurements 100 μM ADP was added. The inhibition of the ATP/ADP translocator were done with 0.2 μM CAT and the uncoupling was done with 1 μM FCCP. In the inhibition studies of the Cyt oxidase and the alternative oxidase, 1 mM KCN and 2 mM SHAM were used.

Cyt Oxidase Measurements

The intactness of the mitochondria was measured as latency of Cyt *c* oxidase and the yield of the preparation was determined by measuring the amount of Cyt *c* oxidase. The procedure and media used were as described by Wigge and Gardeström (1987).

Chl and Protein Determinations

Chl was extracted in cold methanol and the concentration was calculated according to the method of Porra et al. (1989). The protein content was measured according to the

method of Peterson (1977) using BSA as the standard protein.

RESULTS AND DISCUSSION

Disintegration

The glass bead method has been found to be very efficient to disrupt algal cell walls. It has been used for thylakoid preparation from a green alga (Berzborn and Bishop, 1973) as well as from diatoms and dinoflagellates (Samuelsson and Prez  lin, 1985), but it is too harsh to use for preparation of intact chloroplasts. We applied this method to isolate intact mitochondria from the cell-wall-less mutant CW 92 of *C. reinhardtii*. This mutant has been shown to be very similar to the wild type with respect to photosynthesis and carbon accumulation (Palmqvist et al., 1990). Mitochondria are much smaller than chloroplasts, and therefore glass beads could be used more successfully to break the cells without damaging the organelle of interest, if the right conditions were chosen. This was only possible for the cell-wall-less mutant. When the method was applied to wild-type cells, the stronger grinding needed to break the cells destroyed the mitochondria. With a disintegration time of 1 min, more than 95% of the cell-wall-less algae were disrupted as estimated by light microscopic examination. The intactness of the released mitochondria was 98 to 99% as revealed by the latency of Cyt *c* oxidase (data not shown).

We also tried to break the algae using a Yeda press. This method has been used for isolation of chloroplasts (Mendiola-Morgenthaler et al., 1985) and mitochondria (Moyano et al., 1992). In our hands this method was not successful; we had to apply such a high pressure to the Yeda press to disrupt the algae that most of the mitochondria were also damaged and no respiratory O_2 uptake was obtained with the resulting mitochondrial fraction.

Yield and Purity

A mitochondrial preparation from 2 L of culture gave 2.9 to 9.6 (6.2 ± 2.0) mg of protein in the crude mitochondrial fraction. It is easy to scale up the preparation 5 to 10 times if necessary. The mitochondrial fraction obtained by differential centrifugation was not pure. It contained membrane fragments from the broken cells, and the Chl concentration was $430 \pm 120 \mu\text{g Chl mL}^{-1}$. The protein to Chl ratio (w/w) varied between 10 and 24 (16 ± 4). This is in the same range as for crude mitochondrial preparations from leaves (Gardestr  m and Edwards, 1985), in which about one-half of the protein content in the crude mitochondrial fraction is estimated to be of nonmitochondrial origin. The yield was 10% as determined from recovery of Cyt *c* oxidase.

The crude mitochondria were purified on a self-generated 20% Percoll gradient. Most of the mitochondria followed the thylakoid fragments and banded at the top of the gradient. The bottom of the gradient was essentially Chl free and contained 10% of the added mitochondria. Thus, the yield of purified mitochondria from the homogenate was only 1%. The reason that most of the mitochondria

band together with the thylakoid fragments might be because either they have the same density or the mitochondria in some way are attached to the chloroplast (Osafune et al., 1972b).

In *C. reinhardtii* both the three-dimensional structure and the number of mitochondria change during the growth cycle (Osafune et al., 1972a). An attempt to isolate mitochondria from synchronously grown cultures 4 h into the 12-h-long light period, when the number of mitochondria would be highest, did not, however, improve the yield or the quality of the isolated mitochondria.

Measurements of Respiration

The purified mitochondria respired malate with a rate of $107 \text{ nmol O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$ and had an RC of 2.4 and a ADP/O ratio of 1.9 (Table I). The crude mitochondria oxidized malate at about the same rate and with a similar RC and ADP/O ratio (Table I). Since the characteristics of the malate oxidation of the crude and purified mitochondria were similar and the yield of the purified mitochondria was low, the characterization of the respiratory properties was done with crude mitochondria. However, most of the experiments were repeated and the results were similar for single experiments with purified mitochondria (data not shown). The respiratory rate with pyruvate was somewhat lower, but the RC and ADP/O ratios were in the same range as with malate. The ratio between the respiratory rate after inhibition of the ATP/ADP translocator (CAT) and uncoupling (FCCP) was comparable with the RC values (malate, 2.2 ± 0.3 ; pyruvate, 3.0 ± 0.6). The isolated mitochondria also showed O_2 uptake with α -KG, NADH, and succinate as substrates. No RC could be demonstrated with these substrates, but the oxidation of α -KG and succinate were stimulated by ADP. Also, addition of SHAM to inhibit the alternative oxidase did not increase the RC for the substrates tested. This shows that high alternative oxidase activity is not the reason for lack of RC. It was necessary to include co-factors to get a maximal O_2 uptake with malate (NAD and glutamate), pyruvate (NAD, malate, CoA, and TPP), and α -KG (NAD, AMP, CoA, and TPP) (see "Materials and Methods"). When working with isolated mitochondria from higher plants it is often necessary to add 0.1 mM ATP to get a maximal respiration with succinate. This inhibited the respiration in the mitochondria

from *C. reinhardtii*. Respiratory O_2 uptake could also be observed with NADPH as substrate, but the rate was so low ($5\text{--}10 \text{ nmol O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$) that it was not possible to do reliable measurements. The respiratory rates are comparable to what is usually reported for mitochondria from higher plants. It should also be noted that in this report we have not corrected the rate of respiration for contamination of nonmitochondrial proteins.

The fact that RC is obtained with malate and pyruvate as substrates and that reasonable ADP/O ratios are observed shows that the mitochondria have a reasonably efficient coupling between electron transport and oxidative phosphorylation. Thus, the mitochondria are sufficiently intact to make a characterization of their respiratory properties feasible.

The respiration with different substrates showed different sensitivity to inhibitors of the Cyt pathway (KCN) and the alternative pathway (SHAM) (Fig. 1). Cyanide inhibited the respiration by 20 to 50% with malate, pyruvate, and succinate as substrates (Fig. 1A). Subsequent addition of SHAM resulted in an almost complete inhibition of the O_2 uptake. From the residual rate after KCN addition the capacity of the alternative oxidase can be estimated to be at least $50 \text{ nmol O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$. The oxidation of NADH was inhibited by 70%, whereas the oxidation of α -KG was unaffected by KCN. The high sensitivity of external NADH oxidation to KCN is similar to what is normally observed in plant mitochondria (Møller and Lin, 1986). This low activity of the alternative oxidase when using NADH as substrate was not stimulated by pyruvate as reported by Millar et al. (1993), who observed a pyruvate-mediated stimulation (100–400%) of the alternative oxidase activity in mitochondria from soybean shoots. The lack of effect by KCN on α -KG oxidation can be explained by the low rate of oxidation of this substrate not saturating the alternative oxidase. SHAM alone had almost no effect on the respiration except when NADH was used as substrate (Fig. 1B). Thus, the capacity of the Cyt pathway is at least $100 \text{ nmol O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$ (equal to the rate of malate oxidation). The inhibition of NADH oxidation by SHAM alone is opposite from what is found in higher plants (Møller and Lin, 1986) and might indicate a direct effect of SHAM on external NADH oxidation. In inhibitor and O_2 discrimination studies of intact *C. reinhardtii* cells, Weger et al. (1990) reported no effect of KCN or SHAM

Table I. Respiration of *Chlamydomonas mitochondria*

Respiratory rates in $\text{nmol O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$. All values are means \pm SD (number of preparations).

Mitochondria/Substrate	Respiratory Rate	RC		ADP/O
		State 3/state 4	FCCP/CAT	
Purified mitochondria				
Malate	108 ± 35 (3)	2.4 ± 0.1 (2)	2.7 ± 0.2 (2)	1.9 ± 0.1 (2)
Crude mitochondria				
Malate	102 ± 23 (12)	2.0 ± 0.4 (11)	2.2 ± 0.3 (11)	1.7 ± 0.3 (5)
Pyruvate	76 ± 13 (5)	2.2 ± 0.4 (4)	3.0 ± 0.6 (4)	1.8 ± 0.2 (4)
NADH	67 ± 9 (5)			
Succinate	54 ± 9 (5)			
α -KG	27 ± 8 (5)			

alone. With isolated mitochondria, KCN alone inhibited the oxidation of all substrates tested except α -KG (Fig. 1). The difference in results between intact cells and isolated mitochondria indicates that mitochondria in vivo operate well below their maximal capacity. Also, growth condition has been reported to affect the capacity of the alternative oxidase in *Chlamydomonas* (Goyal and Tolbert, 1989).

Oligomycin is an inhibitor of the mitochondrial F_0F_1 -ATPase (Lardy et al., 1958). In leaf mitochondria from higher plants oligomycin has been shown to be a specific inhibitor of the mitochondrial ATPase (almost full effect with 0.05 μ g/mL) without any significant effect on the chloroplast ATPase, even at a 100 times higher concentration (Krömer et al., 1988). We compared the effect of oligomycin on mitochondria from *C. reinhardtii* with mitochondria from *P. sativum* (Fig. 2). The ADP-stimulated respiration was inhibited by 85% with 0.1 μ g oligomycin/mL when the *P. sativum* mitochondria were measured, whereas the *C. reinhardtii* mitochondria were nearly unaffected at this concentration. A concentration as high as 4 μ g/mL only inhibited the *C. reinhardtii* mitochondria by 35%. The lack of inhibition by oligomycin was not due to uncoupling, since the RC was unaffected in the presence oligomycin (results not shown). This low inhibitory effect

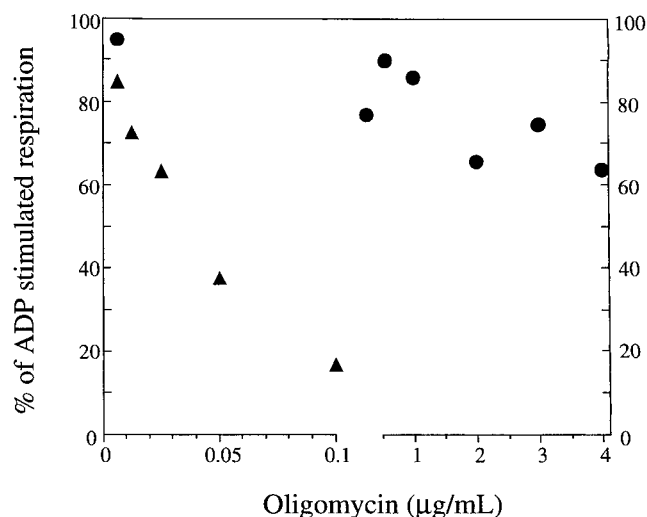


Figure 2. Inhibitory effect of oligomycin expressed as percentage of ADP-stimulated respiration remaining after inhibition. \blacktriangle , *P. sativum* mitochondria; \bullet , crude *C. reinhardtii* mitochondria. Both were measured with malate as substrate.

by oligomycin indicates a difference between the mitochondrial ATPase of *C. reinhardtii* and those from higher plants. This is possibly due to different properties of the oligomycin sensitivity-conferring protein, but further studies are needed to verify this.

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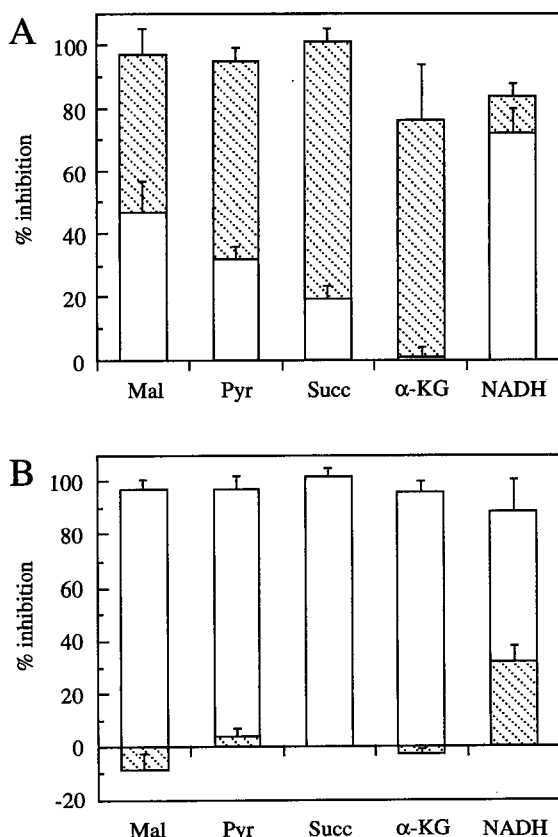


Figure 1. The effect of 1 mM KCN (open columns) and 2 mM SHAM (hatched columns) as percentage inhibition of the O₂ uptake of crude *C. reinhardtii* mitochondria. For A, KCN was added before SHAM, and for B, SHAM was added before KCN. Each column represents the mean \pm SD of five measurements. Mal, Malate; Pyr, pyruvate; Succ, succinate.

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