Oxidation of Substrates in Percoll-purified Mitochondria Isolated from *Kalanchoë daigremontiana*

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*Received December 25, 2003*

**Summary**

Percoll-purified mitochondria isolated from *K. daigremontiana* showed a rather high degree of purity and intactness of the inner membrane. These mitochondria posses malate dehydrogenase and NAD (P)H-malic enzyme activities in which malate dehydrogenase activity was about twenty five folds higher than that with NAD-malic enzyme. The *K. daigremontiana* mitochondria already oxidized succinate, malate, NADH and NADPH without any cofactor. The highest respiration rate was found with succinate, followed by NADH, NADPH and malate. The respiration rate in succinate oxidation by *K. daigremontiana* mitochondria was near twice as far as the rate by malate oxidation. Mitochondria of *K. daigremontiana* oxidized NADH and NADPH with the same way; however the respiration rates in NADH oxidations was slightly higher than that with NADPH. The cooperative oxidation of succinate plus NAD(P)H or NADH plus NADPH normally leads to increase the respiratory rates that were lower than the sum of the individual rates but higher than the individual rates.

**Key words:** Cooperative oxidation, Enzyme, *Kalanchoë daigremontiana*, Mitochondria, Respiratory properties.

**Introduction**

Mitochondria play a central role in energy and carbon metabolism of eukaryotic cell, being the site of both the tricarboxylic acid cycle and oxidative phosphorylation pathways (Siedow and Day, 2000; Vanlerberghe et al., 2002). Over the past 20 years, researchers investigating the mitochondria of plants mainly concentrated in C₃ and C₄ species. There is very little research that is conducted in mitochondria of CAM species. Recently, based on the reports at the 3rd International Congress on Crassulacean Acid Metabolism (CAM 2001), Holtum (2002) has been concluded that most of the research in CAM plants concentrated on three broad categories: circadian rhythms and biological clocks, carbon flux and metabolic control, and phenotypic plasticity. Two features of CAM that are not well understood are the role of mitochondria and regulation of decarboxylation during the light (Holtum, 2002). So that, studying on mitochondria in CAM plants becomes more necessary for providing a completely understanding in whole plant mitochondrial biology.

The respiratory property of *K. daigremontiana* mitochondria with malate is reported by Day (1980), and Wiskich and Day (1982). However, they use washed mitochondria that are not purified on a Percoll gradient. In this study, we used Percoll-purified mitochondria of *K. daigremontiana*...
tiana to investigate the differences in the respiratory properties with succinate, malate, NADH and NADPH in both oxidation of simple substrate and cooperative substrates. Another parts of this work were to investigate the levels of purify and intactness of the inner membrane mitochondria, and activities of malate dehydrogerase (MDH), NAD-malic enzyme (ME) and NADP-ME in leaf and in the mitochondria.

Materials and Methods

1. Plant material

Experimental plants, Kalanchoë daigremontiana were transferred to a growth chamber (KG-50 HLA, Koito Industrial Co., LTD., Japan) with a photoperiod of 12 h light and 12 h dark. Conditions in the growth chamber were 35°C during the light period at a light intensity of 420 to 450 μmol m⁻²s⁻¹, and 25°C during the dark period, and a relative humidity of 70% during both periods.

2. Preparation of mitochondria

The midrib and margins of K. daigremontiana leaves were excised and discarded; the remaining tissue was cut into small pieces and ground in a blender (National MX-X 1, Japan) with 150 mL of ice-cold isolation buffer [350 mM mannitol, 250 mM sucrose, 0.1% (w/v) BSA, 1% PVP 40, 5 mM KH₂PO₄, 5 mM EDTA-KOH (pH 7.4), 1 mM DTT and 100 mM HEPES-KOH (pH 7.4)]. After filtration through four layers of sterile Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA) the homogenate was centrifuged at 300 g (Tomy CX-250 refrigerated centrifuge, Japan) for 5 min. The resulting supernatant was centrifuged at 10,000 g for 15 min to pellet the mitochondria. The pellets were resuspended in approximately 10 mL of wash buffer [400 mM sucrose, 5 mM KH₂PO₄, 5 mM EDTA-KOH (pH 7.4), and 50 mM HEPES-KOH (pH 7.4)] and then centrifuged at 500 g for 5 min. The supernatant was again resuspended in 10 mL of wash buffer and centrifuged at 6,000 g for 20 min to pellet mitochondria. The pellets were resuspended in 2.5 mL of wash buffer and then further purified in 16 mL Percoll 27% by centrifugation at 4°C, 52,600 g (P 28S rotor, CP 75β ultracentrifuge, Hitachi Koki Co., LTD, Japan) for 30 min. The mitochondria were found in a band of the lower half of the centrifuge tube, and were extracted from the gradient by pipette. The mitochondria were resuspended in 40 mL of wash medium and pelleted by centrifuging at 12,000 g for 10 min. Final pellets were resuspended in 1 mL of assay buffer contained 400 mM sucrose, 0.1% BSA and 40 mM HEPES-KOH (pH 7.4). The isolated mitochondria were stored on ice until used for the experiments.

3. Preparation of leaf extraction for enzyme assays

The frozen tissue (0.5 g fresh weigh) was homogenized using a mortar and pestle with 0.2 g sea sand and 40 mg of polyvinylpolypyrrolidone in 4 mL of ice-cold extraction buffer. The extraction buffer for MDH, NAD-ME and NADP-ME contained 50 mM Tris-HCl of pH 7.8, 8 mM MgCl₂, 1 mM EDTA-KOH (pH 7.4), 5 mM DTT, 0.2% (w/v) BSA and 0.02% (w/v) Triton X-100. The homogenate was filtered through one layer of Miracloth and the filtrate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was desalted by passing it through a Sephadex G
-25 column that had been equilibrated with the enzyme extraction medium. The desalting extract was used immediately for determination of enzyme activities.

4. Preparation of mitochondria for enzyme assays

The Percoll-purified mitochondria were filtered at room temperature on a column of Sephadex G-25 previously equilibrated with the suspending buffer (400 mM sucrose, 0.1% BSA and 40 mM HEPES-KOH (pH 7.4); thereafter, MDH, NAD-ME and NADP-ME were assayed in mitochondria after lysis with 0.1% (v/v) Triton X-100.

5. Mitochondrial activity

Oxygen consumption was measured using an oxygen electrode type (Rank Brothers England) at 25°C in 2 mL of reaction medium [300 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 10 mM KCl, and 100 mM HEPES-KOH (pH 7.4)]. With succinate as a substrate, the mitochondria were preincubated with 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay. The protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard; RCR and ADP/O ratios value were calculated according to Estabrook (1967). Chlorophyll content was determined according to Arnon (1949).

6. Enzyme activity

Malate dehydrogenase (MDH, L-malate: NAD⁺ oxidoreductase, EC 1.1.1.37) and NAD⁺-dependent ME (EC 1.1.1.39) were assayed according to Pastore et al. (2001). NADP⁺-dependent ME (EC 1.1.1.40) was assayed according to Kondo et al. (1998). Rubisco was assayed according to Du et al. (1996). Malate was measured according to the method of Möllering (1974). PEPC was assayed according to Shaheen et al. (2002).

Results

1. Purity and integrity of mitochondria

The ratios of Rubisco and PEPC activities in Percoll-purified mitochondria to those in leaf extract were used as the indicators of mitochondrial purity (Table 1). The PEPC activity in mitochondria was approximate 1.7% of that in cytosol and Rubisco activities were not detected in these mitochondria (Fig. 1 and Table 1). These results indicated that our mitochondria solutions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Leaf (µmol/ng Chl/min)</th>
<th>Leaf (µmol/ng pro/min)</th>
<th>Mitochondria (µmol/ng pro/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPC</td>
<td>15.33±3.26</td>
<td>0.51±0.02</td>
<td>0.018±0.005</td>
</tr>
<tr>
<td>Rubisco</td>
<td>4.39±0.78</td>
<td>0.14±0.02</td>
<td>ND</td>
</tr>
<tr>
<td>NADP-ME</td>
<td>0.70±0.02</td>
<td>0.04±0.01</td>
<td>0.067±0.023</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>4.69±0.68</td>
<td>0.15±0.07</td>
<td>0.66±0.47</td>
</tr>
<tr>
<td>MDH</td>
<td>23.09±7.63</td>
<td>0.77±0.57</td>
<td>16.31±1.65</td>
</tr>
</tbody>
</table>
were not disturbed by chroplast property and the cytosol contamination of the mitochondria were rather low.

The intactness of the mitochondrial inner membrane of Percoll-purified K. daigremontiana mitochondria was estimated by comparing MDH (EC 1.1.137) activities before and after treatment with Triton X-100. The results were showed in Fig. 1. From these data, the integrity levels were calculated about 96.6% indicating that these mitochondria showed a rather high degree of intactness of the inner membrane.

2. Enzyme activities

Table 1 shows the activities of malate dehydrogenase (MDH), NAD-malic enzyme (NAD-ME) and NADP-malic enzyme (NADP-ME) in leaf tissue and in mitochondria isolated from K. daigremontiana. The activities of NAD-ME, NADP-ME and MDH were detected in leaf extract and in Percoll-purified these mitochondria. NAD-ME activity was higher than NADP-ME activity in leaf extracts. This result is similar with the observation of Winter and Smith (1996) who show that in K. daigremontiana, the development of CAM in the course of leaf ontogeny is associated with an increase in NAD-ME activity but a significant decline in NADP-ME activities. In mitochondria of K. daigremontiana, MDH activity was higher than that in leaf. Although NAD-ME was predominantly located in the mitochondria, a small amount of NADP-ME was also detected in these mitochondria (Table 1).

3. Respiratory properties of K. daigremontiana mitochondria

Discontinuous Percoll density gradients had been used for the purification of mitochondria. In our experiment, concentration of 27% Percoll was found to be ideal for purifying of K. daigremontiana mitochondria. Percoll-purified K. daigremontiana mitochondria all readily oxidized succinate, malate, NADH and NADPH as substrates (Fig. 2). Total respiration rates, respiratory control ratio (RCR) and ADP/O ratios differed for the substrates (Table 2).

The results in Fig. 2 show typical electrode traces for the oxidation of substrates by K. daigremontiana mitochondria. The total respiration rates in succinate oxidation (Fig. 2 A) were near similar with that of NADPH oxidation (Fig. 2 B), were slightly lower than that with NADH oxi-
Fig. 2. Individual oxidation of succinate (A), NADPH (B), NADH (C) and malate (D) in Percoll-purified *K. daigremontiana* mitochondria. Assay conditions were as shown in the legend of Table 2.

Table 2. Respiratory properties of Percoll-purified *K. daigremontiana* mitochondria.

Oxygen uptake was measured as described in “Materials and Methods”. Concentrations used were: 10 mM succinate, 1 mM NADH, 1 mM NADPH, 10 mM malate, 400 nmol ADP with succinate as a substrate, and 240 nmol ADP with all another substrates. State 3 refers to the respiration rate of O₂ uptake in the presence of ADP; state 4 refers to the rate upon depletion of ADP. Respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 rates. Each value was the average of four or five independent experiments.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Respiration rate (nmol O₂ min⁻¹mg⁻¹ protein)</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>142±18</td>
<td>66±6</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>NADH</td>
<td>137±28</td>
<td>60±34</td>
<td>2.4±0.7</td>
</tr>
<tr>
<td>NADPH</td>
<td>124±16</td>
<td>60±12</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>Malate</td>
<td>75±12</td>
<td>43±11</td>
<td>1.7±0.3</td>
</tr>
</tbody>
</table>

dation (Fig. 2 C) and near double of that with malate oxidation. The ADP/O ratios with succinate, NADH and NADPH oxidations were less than 2 indicating that its oxidation was coupled to two proton-extrusion sites (Table 2). These ADP/O ratios were similar to the results in washed mitochondria of the other CAM plants such as: *Sedum praealtum* (Arron et al., 1979), *K. daigremontiana* (Day, 1980) and *K. blossfeldiana* (Rustin and Queiroz-Claret, 1985).

NADH and NADPH were readily oxidized by *K. daigremontiana* mitochondria in the absence of Ca²⁺, an activator of external NAD (P) H dehydrogenase. These mitochondria were capable of oxidizing exogenous NADH and NADPH with near similar way, but the respiration rates with NADH were slightly higher than those with NADPH oxidation (Fig. 2 B and 2 C). NADPH oxidation by Percoll purified *K. daigremontiana* mitochondria showed the respiration rate were rather higher than that by washed mitochondria of *K. blossfeldiana* (104 nmol min⁻¹mg⁻¹ protein) (Rustin and Queiroz-Claret, 1985) and by washed mitochondria of *S. praealtum* (28.8 nmol min⁻¹mg⁻¹ protein, Arron et al., 1979). And the respiration rates of the NADH and NADPH oxidations by Percoll purified mitochondria in our study were near double of that with washed mitochondria of *K. daigremontiana* which is previous reported by Day (1980).
Fig. 2 D shows typical O₂ uptake patterns obtained with *K. daigremontiana* mitochondria oxidizing malate as a substrate. The mitochondria isolated from *K. daigremontiana* oxidized malate (pH 6.8) at similar rates, and all gave ADP/O ratios of greater than 2 (Table 2) indicated that all three proton-extrusion sites were utilized. *K. daigremontiana* mitochondria oxidized malate without any cofactors; however, their oxidation rate was slower than that with other substrates (Table 2 and Fig. 2 D). These results were near similar with those in malate oxidation by washed mitochondria of *S. praealtum* and sunflower (Arron et al., 1980). In general, before adding ADP, these mitochondria slowly oxidized malate. Upon ADP addition, high rates of oxygen consumption were measured (Fig. 2 D).

The respiratory properties of cooperative oxidation by *K. daigremontiana* mitochondria with two substrates were showed in Fig. 3 and Table 3. All of the cooperative oxidation gave ADP/O ratios of lower than 2 indicated that all two proton-extrusion sites were utilized. The simultaneous oxidation of succinate and NADH or succinate and NADPH always had to result in respiratory rates that were lower than the sum of the individual rates but higher than the individual rates (Fig. 3). These simultaneous oxidation rates were about 80% and 87% of the sum of the individual rates with succinate plus NADH and succinate plus NADPH, respectively. In contrast, combination of NADH and NADPH did not increase the respiratory rates and this rate was about 51% of the sum of the individual rates and was near the same rate with NADH or NADPH individual oxidation (Fig. 3 and Table 3). Day and Wiskich (1977) also observed that the addition of succinate to cauliflower mitochondria oxidizing malate resulted in a larger stimulation of oxygen uptake.

**Discussion**

In contrast to mitochondria from animal tissues, plant mitochondria can also oxidize cytosolic NADH and in some cases cytosolic NADPH (Heldt, 1997). Our experiments showed that *K. daigremontiana* mitochondria were able to oxidize both of NADH and NADPH. These results differed from previous report for mitochondria isolated from aged beetroot slices which cannot oxidize exogenous NADPH (Arron et al., 1979, 1980).

The respiratory properties of mitochondria isolated from *K. daigremontiana* with NADH

![Fig. 3. Cooperative oxidation of two substrates in Percoll-purified *K. daigremontiana* mitochondria. Assay conditions were as shown in Table 3.](image-url)
Table 3. Respiratory properties of Percoll-purified K. daigremontiana mitochondria with two substrates.
Concentrations used were: 10 mM succinate, 1 mM NADH, 1 mM NADPH, 10 mM malate, 240 nmol ADP for NADH plus NADPH, and 320 nmol ADP for succinate plus with NADH or NADPH. Each value was the average of four or five independent experiments.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Respiration rate (nmol O_2 min^{-1} mg^{-1} protein)</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td></td>
</tr>
<tr>
<td>Succinate + NADH</td>
<td>223±23</td>
<td>127±19</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Succinate + NADPH</td>
<td>231±19</td>
<td>141±23</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>NADH + NADPH</td>
<td>134±20</td>
<td>52±21</td>
<td>2.9±1.4</td>
</tr>
</tbody>
</table>

(70 nmol min^{-1} mg^{-1} protein) and NADPH (64 nmol min^{-1} mg^{-1} protein) was reported by Day (1980), using washed mitochondria. Compared with these results, our results showed near double rate (137±28 nmol min^{-1} mg^{-1} protein for NADH and 124±16 nmol min^{-1} mg^{-1} protein for NADPH), possibly because we used Percoll-purified mitochondria. Percoll gradient markedly decreased the degree of contamination and showed high rate (Fredlund et al., 1991).

The property of absorbing malate has been detected in many plant mitochondria in which malate can be decarboxylated in the mitochondrial matrix through the action of NAD-ME to produce pyruvate (Artus and Edwards, 1985), which is oxidized by the TCA cycle. Our data showed that K. daigremontiana mitochondria possessed a larger ME activity, and readily oxidized malate. This phenomenon fits with previous results on malate oxidation by mitochondria isolated from K. daigremontiana (Day, 1980) and K. blossfeldiana (Rustin and Lance, 1986).

Previous experiments with other mitochondria plants have shown that the Cytochrome (Cyt) chain is not saturated by oxidation of any one substrate. For example, in Iris bulb mitochondria the respiration rates with the combination of succinate and NADH are always significantly lower than the sum of the single rate (Hemrika-Wagner et al., 1986). In our research, for K. daigremontiana mitochondria, when the second substrate (NADH or NADPH) was simultaneously present with the first substrate (succinate), the overall rate of respiration was always significantly higher than the individual rates. However, the respiration rate in simultaneous oxidation of NADH and NADPH was not higher than the individual rates (Fig. 3 and Table 3). This result was deferred with potato mitochondria in which the oxidation of two substrates NADPH and NADH leads to increase the respiration rate (Arrabaca et al., 1992). These results suggested that the Cyt chain is saturated by individual oxidation of NADH and NADPH but not saturated by individual succinate oxidation in K. daigremontiana mitochondria.

References
パーコール法で純化した Kalanchoe daigremontiana
（コダカラベンケイソウ）のミトコンドリアにおける基質酸化特性

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平成15年12月25日 受理

摘 要
インテクトな内膜を持つ高純度のミトコンドリアをコダカラベンケイソウ葉からパーコール法で調整した。純化したミトコンドリアにおいて、高いリング酸脱水素酵素（MDH）、NAD 及び NADP 依存リング酸酵素（ME）活性が認められ、MDH活性は NAD-ME活性より25倍以上高かった。純化ミトコンドリアはコハク酸、リング酸、NADH、NADPHを単独の基質として補助因子なしで高い効率で酸化することが観察された。最も高い呼吸速度（基質酸化速度）はコハク酸を基質にしたときに認められ、次いで NADH、NADPH、リング酸の順で低く、コハク酸を基質とした呼吸速度はリング酸の場合の約2倍高かった。また、NADHを基質にした呼吸速度は NADPH 基質の呼吸速度よりわずかに高かった。コハク酸と NAD(P)H、あるいは NADH と NADPH を基質として同時に与えた時の呼吸速度は、個々の基質のもとでの呼吸速度より高くなったが、それらの和より低かった。