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Respiratory properties and malate metabolism in Percoll-purified mitochondria isolated from pineapple, *Ananas comosus* (L.) Merr. cv. smooth cayenne

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Abstract

An investigation was made of the respiratory properties and the role of the mitochondria isolated from one phospho*enol*pyruvate carboxykinase (PCK)-CAM plant *Ananas comosus* (pineapple) in malate metabolism during CAM phase III. Pineapple mitochondria showed very high malate dehydrogenase (MDH), and low malic enzyme (ME) and glutamate–oxaloacetate transaminase (GOT) activities. The mitochondria readily oxidized succinate and NADH with high rates and coupling, while they only oxidized NADPH in the presence of Ca^{2+}. Pineapple mitochondria oxidized malate with low rates under most assay conditions, despite increasing malate concentrations, optimizing pH, providing cofactors such as coenzyme A, thiamine pyrophosphate, and NAD^+, and supplying individually external glutamate or GOT. However, providing glutamate and GOT simultaneously strongly increased the rates of malate oxidation. The OAA easily permeated the mitochondrial membranes to import into or export out of pineapple mitochondria during malate oxidation, but the mitochondria did not consume external Asp or α-KG. These results suggest that OAA played a significant role in the mitochondrial malate metabolism of pineapple, in which malate was mainly oxidized by active mMDH to produce OAA which could be exported outside the mitochondria via a malate-OAA shuttle. Cytosolic GOT then consumed OAA by transamination in the presence of glutamate, leading to a large increase in respiration rates. The malate–OAA shuttle might operate as a supporting system for decarboxylation in phase III of PCK-CAM pineapple. This shuttle system may be important in pineapple to provide a source of energy and substrate OAA for cytosolic PCK activity during the day when cytosolic OAA and ATP was limited for the overall decarboxylation process.

Key words: Ca^{2+}, malate–OAA shuttle, malate oxidation, NADPH oxidation, PCK-CAM, pineapple mitochondria.

Introduction

Malate decarboxylation is a very important metabolism in plant mitochondria, especially in Crassulacean acid metabolism (CAM) plants in which malate is accumulated in the vacuoles at night and is released into the cytoplasm during the day. Based on malate metabolism, CAM plants can be divided into two groups, ME-CAM and PCK-CAM plants. ME-CAM plants contain significant activities of ME without PCK, and they use ME to decarboxylate malate, generating pyruvate and CO_2. By contrast, PCK-CAM plants contain significant activities of PCK with lower levels of ME, they require the operation of MDH to convert malate to OAA, and then OAA is further converted to PEP and CO_2 by cytosol PCK (Dittrich *et al.*, 1973; Winter and Smith, 1996; Cuevas and Podestá, 2000).

Pineapple, one of the typical PCK-CAM plants, has recently been studied by many researchers. Coté *et al.* (1989) showed that, in intact plants of pineapple, there appeared to be no stimulation of respiratory oxygen uptake in phase III. Cuevas and Podestá (2000) purified a cytosolic MDH (cMDH) from leaves of pineapple, which plays a pivotal role in the interconversion between malate and

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Abbreviations: CAM, Crassulacean acid metabolism; CoA, coenzyme A; GOT, glutamate-oxaloacetate transaminase; MDH, malate dehydrogenase; ME, malic enzyme; Mp, purified mitochondria; OAA, oxaloacetic acid; PCK, phospho*enol*pyruvate carboxykinase; PEP, phospho*enol*pyruvate; RCR, respiratory control ratio; RuBP, ribulose 1,5-*bis*phosphate; TPP, thiamine pyrophosphate.

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OAA, catalysing the reductive reaction during the night, while it oxidizes malate during daytime deacidification. They found that the cMDH in crude pineapple leaf extracts was very active, and OAA reduction by this cMDH enormously exceeded. At any rate, assuming that both malate and cytosolic NAD^+ were sufficient to saturate MDH, the enzyme's V_{max} will limit the maximum malate oxidation activity. Their research not only raises the question whether cMDH is engaged in malate oxidation during the day but also whether mitochondrial MDH (mMDH) could be involved in the daytime conversion of malate to OAA, since the ratio of OAA reduction to malate oxidation in this enzyme is comparably much lower than that exhibited by pineapple cMDH (Hayes *et al.*, 1991; Cuevas and Podestá, 2000).

Leegood and Walker (2003) indicated that, in CAM plants, an increase in cytosolic malate at the beginning of the day is likely to increase flux through PCK by increasing the concentration of OAA. And they found that, in leaves of PCK-CAM pineapple, PCK activity increased during the light period. However, Chen *et al.* (2002) observed that OAA levels in pineapple leaves increased during the dark period, then dropped dramatically to low levels during the light period. The OAA concentration in the cytosol of pineapple during the day varied in the range of 10–25 μM (Chen *et al.*, 2002). These values were much lower than C_4 plants in which the concentration of cytosol OAA was about 150 μM (Leegood and Walker, 2003). From these results, it seems that the low concentration of OAA in the cytosol of pineapple during the day might be limiting for PCK activity during the decarboxylation phase.

Mitochondrial malate oxidation during the day has mostly been investigated in ME-CAM plants in which NAD^+-ME plays an important role in the production of pyruvate and CO_2. This oxidation has not been exploited with mitochondria of PCK-CAM plants, except with a PCK-CAM plant that has a relatively low PCK activity (*Crassuala lycopodioides*), and that possesses NAD-ME and partially oxidized malate in the mitochondria, producing the pyruvate (Peckmann and Rustin, 1992). Until now, and associated with the distinctive pathways of malate (or OAA) decarboxylation in the cytosol of ME-CAM and PCK-CAM plants, the different properties of malate metabolism in mitochondria of PCK-CAM plants and the role of the mitochondrion in this metabolism are not well known.

These questions prompted an investigation into mitochondrial enzyme activities and respiratory property with different substrates. Specifically, the focus was on studying where and how pineapple mitochondria oxidize externally added malate during CAM phase III. The purpose was to find out the metabolism of mitochondrial malate oxidation, the roles of the mitochondria, and the relationship between mitochondria and cytosol in total malate metabolism during the light period of the CAM cycle in pineapple.

Materials and methods

Plant material

Plants, *Ananas comosus* (L.) Merr. cv. smooth cayenne, N 67–10, were propagated vegetatively and grown in plastic pots in a greenhouse under natural light and temperature. Ten days before the experiments, the 6–8-month-old plants were transferred to a growth chamber (KG-50 HLA, Koito Industrial Co., LTD., Japan) with a of 12/12 h light/dark photoperiod. The temperature in the growth chamber was maintained at 35 °C during the light period and 25 °C during the dark period with photosynthetically active radiation of 420–450 $\mu mol\ m^{-2}\ s^{-1}$ at the top of the plant. Fully expanded mature leaves of pineapple were used for mitochondrial isolation. The leaves were harvested 6–7 h after the beginning of the light period. The harvested leaves were transported to the laboratory, rinsed thoroughly with distilled water, and used for isolating mitochondria.

Isolation of mitochondria

The mitochondria were isolated according to the method of Day (1980) with slight modifications. Approximately 65 g leaves were used for each experiment. The middle part of the leaves was sliced into 0.5 cm thick strips and homogenized with 150 ml of ice-cold isolation buffer [350 mM manitol, 250 mM sucrose, 0.1% (w/v) bovine serum albumin (BSA), 1% (w/v) PVP-40, 5 mM KH_2PO_4, 5 mM EDTA-KOH (pH 7.4), 1 mM dithiothreitol (DTT), and 50 mM HEPES-KOH (pH 7.4)] in a Waring blender (National MX-X1, Japan) for 90 s with rapid stirring. 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. The pellets were resuspended in approximately 10 ml of wash buffer 1 [400 mM sucrose, 5 mM KH_2PO_4, 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 resuspended in 10 ml of wash buffer 2 [600 mM sucrose, 5 mM KH_2PO_4, 5 mM EDTA-KOH (pH 7.4), and 50 mM HEPES-KOH (pH 7.4)] and centrifuged at 6000 *g* for 20 min to collect mitochondria. The pellets were resuspended in 2.5 ml of wash buffer 1 and then further purified in 16 ml of wash buffer 3 [400 mM sucrose, 0.1% (w/v) BSA, 5 mM KH_2PO_4, 5 mM EDTA-KOH (pH 7.4), 50 mM HEPES-KOH (pH 7.4), and 27% Percoll] by centrifugation at 52 600 *g* (P28S rotor, CP75β ultracentrifuge, Hitachi Koki Co., Ltd, Japan) for 30 min at 4 °C. The mitochondria were found in a band in the lower half of the centrifuge tube, and were removed from the gradient by a pipette. The mitochondria were resuspended in 40 ml of wash buffer 1 and collected by centrifuging at 12 000 g for 10 min. Finally, the pellets were resuspended in 1 ml of the buffer contained 400 mM sucrose, 0.1% BSA, and 40 mM HEPES-KOH (pH 7.4).

Oxygen uptake and protein determination

Oxygen consumption was measured using an oxygen electrode (Rank Brothers England) at 25 °C in 2 ml of reaction medium (300 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM KCl, 100 mM HEPES-KOH) and the pH was adjusted from 6.8 to 7.8 with 3 mM KOH. The mitochondria were preincubated with 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay with succinate oxidation. NADH and NADPH oxidations were investigated at pH 6.8 with and without Ca^{2+}. RCR and ADP/O ratios were calculated according to Estabrook (1967). The O_2 concentration in air-saturated medium was taken as 258 μM. The protein content was measured by the method of Bradford (1976) using BSA as the standard.

Enzyme assays

The Percoll-purified mitochondria were filtered at room temperature on a column of Sephadex G-25 equilibrated with the suspending

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buffer [400 mM sucrose, 0.1% BSA, and 40 mM HEPES-KOH (pH 7.4)], and then the mitochondria were collected for the enzyme assays.

Cytochrome *c* oxidase (COX, EC 1.9.3.1), and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), and initial ribulose 1,5-*bis*phosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) were measured in both the Percoll-purified mitochondria and leaf extracts according to Møller and Palmer (1982), Shaheen *et al*. (2002), and Du *et al*. (1996), respectively.

Malate dehydrogenase (MDH, L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) was assayed spectrophotometrically at 340 nm in the OAA-reducing direction in a medium of 100 mM HEPES-KOH (pH 7.4), 10 mM KH_2PO_4, 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1% (w/v) BSA, 0.2 mM KCN, 0.1% (v/v) Triton X-100, and 200 μM NADH. The reaction was started by adding 600 μM OAA. The malate-oxidizing reaction was assayed in 2 ml medium of 100 mM HEPES-KOH (pH 7.4), 10 mM KH_2PO_4, 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1% (w/v) defatted BSA, 0.1% (v/v) Triton X-100, 30 mM malate, 2 mM NAD^+, and 50 mM glutamate. The reaction was started by adding 10 units of glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1).

NAD-ME was assayed in 2 ml medium of 50 mM HEPES-KOH (pH 7.4) containing 2 mM $MnCl_2$, 4 mM DTT, 0.1% (v/v) Triton X-100, 10 mM CoA, 1 μM antimycin A, 500 μM propylgallate, and 2 mM NAD^+. NADP-ME was assayed in 2 ml medium of 50 mM HEPES-KOH (pH 8), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 μM antimycin A, 500 μM propylgallate, 0.1% (v/v) Triton X-100, 2.5 mM EDTA, and 0.5 mM $NADP^+$. The reaction for NAD-ME and NADP-ME was started by adding 10 mM malate (pH 6.8). Measurements were made spectrophotometrically at 30 °C by following the absorbance increase at 340 nm due to NAD^+ or $NADP^+$ reduction.

GOT was assayed according to Bergmeyer and Bernt (1983), and OAA appearance outside mitochondria was assayed basically as described by Pastore *et al*. (2003).

Reagents

Bio-Rad protein kit and Percoll were purchased from the Bio-Rad Laboratory and Amersham Pharmacia Biotechnology (Uppsala, Sweden), respectively. Enzymes were purchased from Roche Diagnostics GmbH, Mannheim, Germany and Sigma Chemical Company. All other reagents were from Wako Pure Chemical Industries and Katayama Chemicals, Japan.

Results

Purity of mitochondria

PEPC and Rubisco were localized unambiguously in the cytosol and chloroplast, respectively, of pineapple mesophyll cells (Kondo *et al*., 1998), so that their activities can be used as indicators of mitochondrial purity. In pineapple mitochondria, the specific activity of Rubisco was zero and PEPC was approximate 1.7% of that in pineapple cytosol (Table 1). These results indicated that the mitochondrial solutions did not contain chloroplast components and the cytosol contamination of the mitochondria was low. The MDH activity in mitochondria before lysis with Triton X-100 was approximately 5% of that after lysis (data not shown). The COX activity was 18 times higher in mitochondria than in leaf extracts on a protein basis (Table 1). These results indicated that the intactness of the inner and outer mitochondrial membrane was acceptable and the preparation specifically reflected the mitochondrial properties.

Table 1. *Rubisco and PEPC activities in leaf extract and Percoll-purified pineapple mitochondria*

Results shown are means ±SE (*n*=4–5) of separate preparations. ND, not detectable. Rubisco showed initial activity. RuBP was purchased from Roche Diagnostics GmbH Mannheim.

Enzyme	Leaf (nmol min^{-1} mg^{-1} protein)	Mitochondria (nmol min^{-1} mg^{-1} protein)
PEPC (EC 4.1.1.31)	240±13	4±1
Rubisco (EC 4.1.1.39)	120±10	ND
Cyt *c* oxidase (EC 1.9.3.1)	47±4	860±38

Enzyme activities

Activities of NAD-ME, NADP-ME, MDH, and GOT were detected in pineapple mitochondria. MDH activity was very high; by contrast, NAD-ME activity was much lower. Although ME was the predominantly NAD-ME, some NADP-ME was also detected in pineapple mitochondria (Table 2). The activities of NAD-ME and MDH in pineapple mitochondria were different from the results in mitochondria of potato tuber and pea leaf in which MDH activity with NAD^+ was about three times less, and NAD-ME activity was about 5–6-fold higher than those in pineapple mitochondria (Agius *et al*., 1998). In pineapple mitochondria, MDH activity was about 69 μmol min^{-1} mg^{-1} protein and this value was much higher than that of *Kalanchoë blosssfeldiana* (11.5 μmol), (Rustin and Lance, 1986). The NAD-ME activity in pineapple mitochondria was about 0.11 μmol min^{-1} mg^{-1} protein. This rate was not only lower than that in mitochondria of ME-CAM species such as *Aptenia codifolia* (1.29 μmol), and *Prenia sladeniana* (0.46 μmol) but also lower than that in mitochondria of PCK-CAM species such as *Crassula lycopodioides* (0.20 μmol) (Peckmann and Rustin, 1992). Under the same assay conditions, these results were also different from those in concurrent studies with mitochondria of *K. daigremontiana* and *K. pinnata* (Hong *et al*., 2004; HTK Hong, A Nose, S Agarie, unpublished results) which possessed higher NAD-ME and lower MDH activities than those of pineapple (Table 2).

Respiratory properties of pineapple mitochondria

Figure 1 shows typical electrode traces of succinate, NADH, and NADPH oxidations in pineapple mitochondria. The mitochondria readily oxidized succinate and NADH with the respiratory control rates (RCR) and ADP/O ratios typical of these substrates in the mitochondria of CAM plants (Arron *et al*., 1979; Rustin and Queiroz-Claret, 1985). The ADP/O ratios in these oxidations by pineapple mitochondria were less than 2, indicating that these oxidations were

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Table 2. *The comparison of enzyme activities in pineapple mitochondria with mitochondria of* K. daigremontiana *and* K. pinnata

Data are measured under exactly the same experimental conditions. Results shown are means ±SE (n=4–5) of separate preparations. NM, not measured.

Enzyme	Pineapple (μmol mg^{-1} protein min^{-1})	*K. daigremontiana*[a] (μmol mg^{-1} protein min^{-1})	*K. pinnata*[a] (μmol mg^{-1} protein min^{-1})
MDH (EC 1.1.1.37) in malate oxidation	0.92±0.04	NM	NM
MDH (EC 1.1.1.37) in OAA reduction	69±17	16±1.6	18.49±1.97
NAD-ME (EC 1.1.1.39)	0.11±0.02	0.66±0.47	0.95±0.09
NADP-ME (EC 1.1.1.40)	0.050±0.003	0.067±0.023	0.096±0.013
GOT (EC 2.6.1.1)	0.29±0.3	NM	NM

[a] Data based upon that presented by Hong *et al.* (2004; HTK Hong, A Nose, S Agarie, unpublished results).

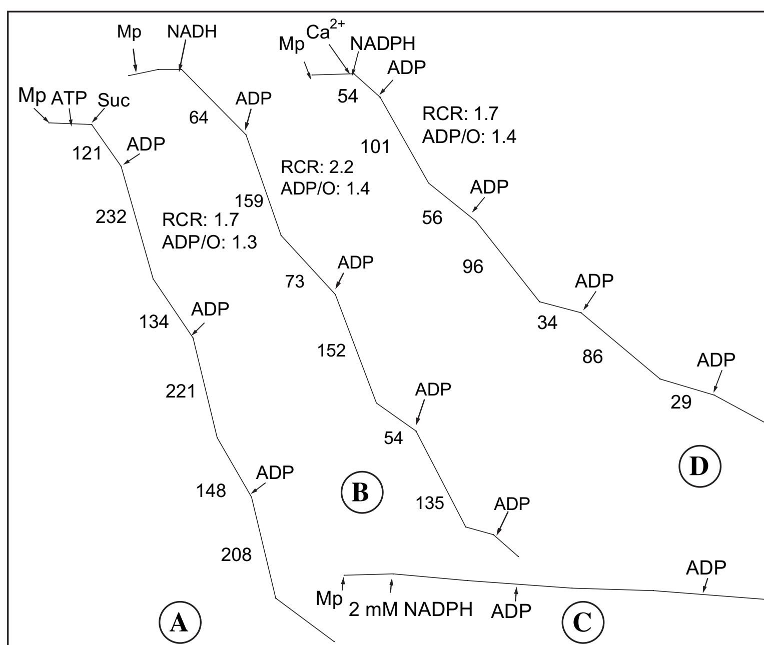

Fig. 1. Respiratory properties of pineapple mitochondria with 10 mM succinate (A), 1 mM NADH (B), 2 mM NADPH (C), and 2 mM NADPH with 1 mM Ca^{2+} (D). Unless otherwise indicated, assay conditions were: 0.16 mM ADP and 10 mM ATP. Numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mp, mitochondria; RCR, respiratory control rate.

coupled with two proton-extrusion sites. Pineapple mitochondria oxidized succinate with the rate of 232 nmol O_2 min^{-1} mg^{-1} protein (Fig. 1A) and this rate was much higher than that of ME-CAM mitochondria as *K. blosssfeldiana* (93 nmol) (Rustin and Queiroz-Claret, 1985), *K. fedtschenkoi* (14.8 nmol) (Cook *et al.*, 1995), and *K. daigremontiana* (142 nmol) (Hong *et al.*, 2004). In the same assay conditions, pineapple mitochondria readily oxidized NADH without Ca^{2+} but not NADPH (Fig. 1B, C). NADPH oxidation was also not detected in the absence of Ca^{2+} when NADPH concentrations were increased (data not shown); however, NADPH was rapidly oxidized with gradually increasing rates of supplementary Ca2+ up to 1 mM with an apparent K_m was about 0.39 mM and V_{max} was about 121 nmol O_2 min^{-1} mg^{-1} protein (Fig. 2). In the presence of 1 mM Ca^{2+}, NADH oxidation was stimulated about 32.6% whereas the NADPH oxidation was strongly stimulated (Table 3). NADH oxidation was inhibited 70% by 1 mM EGTA, while 1 mM EGTA completely inhibited NADPH oxidation.

Malate oxidation was investigated under three different pH conditions, at pH 6.8, 7.2, and 7.6 where only ME, both ME and MDH, and only MDH were activated, respectively (Agius *et al.*, 1998; Day *et al.*, 1988). The results showed that pineapple mitochondria oxidized malate with low rates under most of the assay conditions (Figs 3, 4, 5). These results not only differed from previous results in mitochondria of ME-CAM species such as *K. blossfeldiana* (Rustin and Queiroz-Claret, 1985) and *Sedum praealtum* (Arron *et al.*, 1979), but also differed from concurrent results under exactly the same assay conditions in *K. daigremontiana* and *K. pinnata* (Hong *et al.*, 2004; HTK Hong, A Nose, S Agarie, unpublished results). All of these ME-CAM species readily oxidized malate without any cofactors, with respiration rates about 114, 90, 75, and 62 nmol min^{-1} mg^{-1} protein, respectively, but pineapple did not. Pineapple

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mitochondria oxidized malate in the absence of cofactors with very low rates, even at pH 7.2 where both ME and MDH were activated and together contributed their roles in malate oxidation (16 nmol O_2 min^{-1} mg^{-1} protein) (Fig. 4A). For this reason, in the experiments on oxygen uptake with malate as a substrate, NADH was always added as a second substrate after measuring the malate oxidation to confirm the quality of the mitochondria and to make sure of their property to oxidize malate. The respiration rates with malate as a single substrate were extremely low, however, they increased greatly with the addition of NADH (Figs 3, 4, 5). These results confirmed that the quality of mitochondria was acceptable and that the mitochondria oxidized malate with the low rate under the assay conditions.

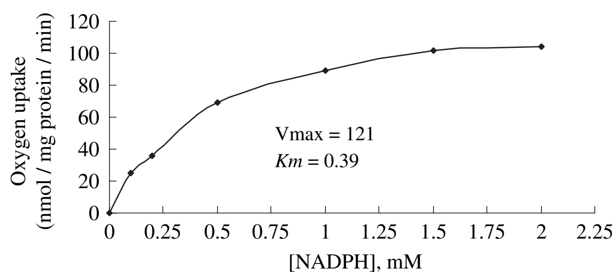

Fig. 2. The effects of NADPH concentrations on state 3 NADPH oxidation with 1 mM Ca^{2+} by pineapple mitochondria. Other conditions were as shown in the Fig. 1D.

Table 3. *Effect of Ca^{2+} on external NAD(P)H oxidation*

NAD(P)H oxidation was determined in an oxygen electrode in assay medium at pH 6.8 (see Materials and methods), using NAD(P)H at a final concentration of 1 mM, 1 mM Ca^{2+} and 1 mM EGTA. ND, not detectable.

Experiments	NADH oxidation (nmol min^{-1} mg^{-1} protein)	NADPH oxidation (nmol min^{-1} mg^{-1} protein)
Control	141 ± 16	ND
1 mM Ca^{2+}	187 ± 13	93 ± 9
EGTA	42 ± 11	ND

It is well known that malate oxidation via ME is stimulated by adding cofactors such as coenzyme A (CoA: an ME activator), thiamine pyrophosphate (TPP: a pyruvate dehydrogenase activator), and NAD^+, whereas malate oxidation via MDH is stimulated by adding NAD^+ or glutamate. Exogenous NAD^+ stimulated malate oxidation via both ME and MDH (Tobin *et al*., 1980; Rasmusson and Møller, 1990). In pineapple mitochondria, at pH 6.8 where ME was strongly activated and malate was oxidized mainly via ME, additions of CoA (Fig. 3A), or TPP (Fig. 3B), or NAD^+ together with CoA and TPP (Fig. 3C) did not significantly stimulate this malate oxidation. These results not only differed from the mitochondria of PCK-CAM species such as *Crassula lycopodioides* in which adding CoA and TPP stimulated malate oxidation (Peckmann and Rustin, 1992), but also differed from mitochondria of ME-CAM species such as *K. daigremontiana* in which adding TPP considerably increased the respiration rate of this oxidation (Wiskich and Day, 1982). The increase in malate concentrations did not stimulate malate oxidation further (Fig. 3B). At pH 7.2, supplying NAD^+ to malate oxidation considerably increased the respiration rate in *K. blossss-feldiana* mitochondria (Rustin and Queiroz-Claret, 1985) whereas in pineapple mitochondria it did not (Fig. 4B). In malate oxidation at pH 7.2, the addition of glutamate to

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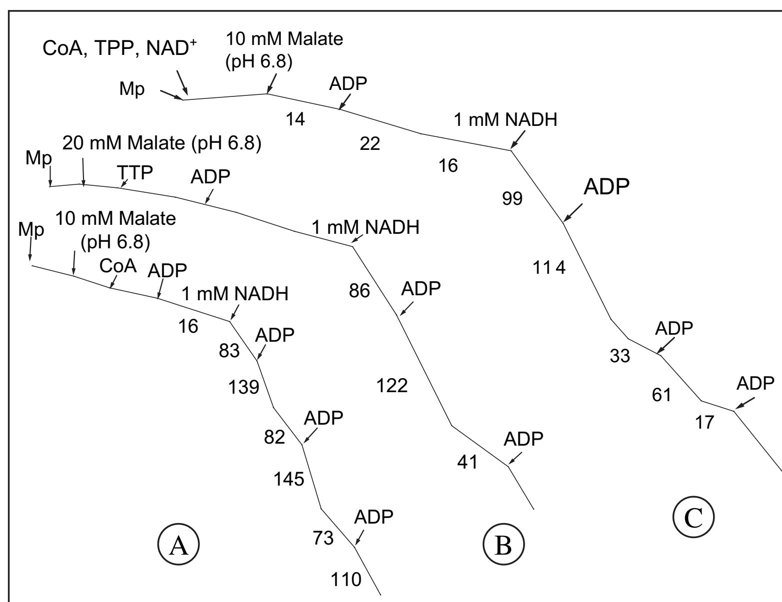

Fig. 3. Malate oxidation at pH 6.8 with CoA (A), CoA and TPP (B), and CoA, TPP, and NAD^+ (C). Unless otherwise indicated, assay conditions were: 0.16 mM ADP, 0.1 mM CoA, 1.5 mM TPP, and 0.5 mM NAD^+. Numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

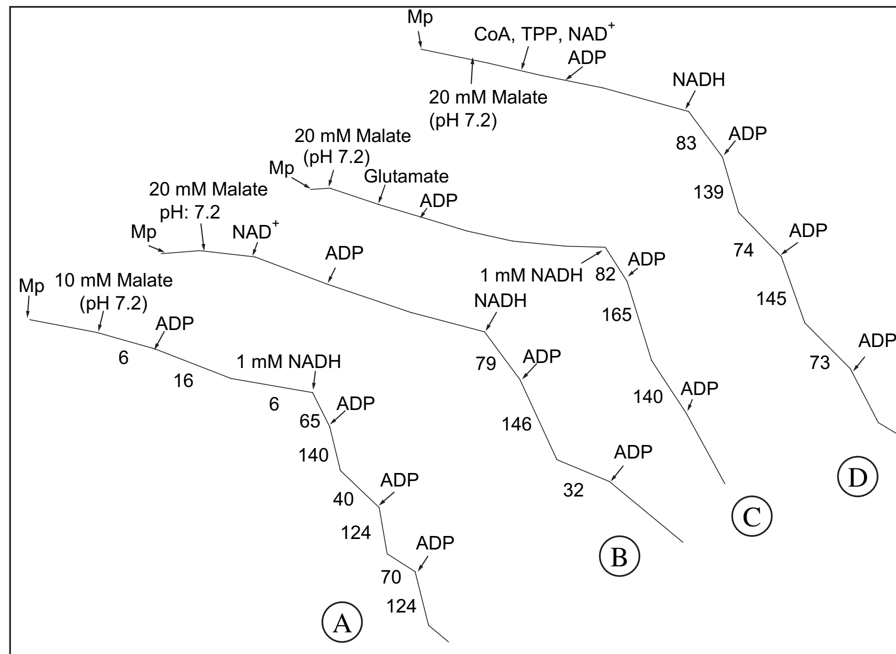

Fig. 4. Malate oxidation at pH 7.2 with malate (A), malate and NAD$^+$ (B), malate and glutamate (C), and malate, CoA, TPP, and NAD$^+$ (D). Unless otherwise indicated, assay conditions were: 0.16 mM ADP, 1.5 mM TPP, 0.1 mM CoA, 0.5 mM NAD$^+$, and 10 mM glutamate. Numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

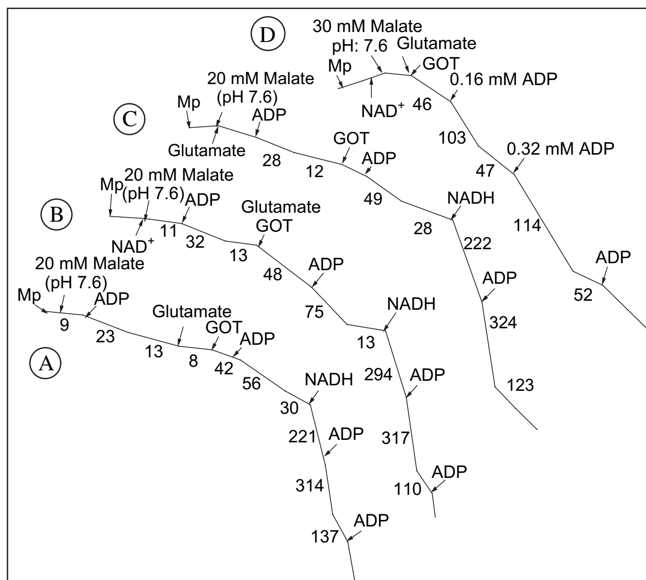

Fig. 5. Malate oxidation at pH 7.6 with malate (A), malate and NAD$^+$ (B), malate and glutamate (C), and malate with glutamate and GOT (D). Unless otherwise indicated, assay conditions were: 0.16 mM ADP, 0.5 mM NAD$^+$, 10 U GOT, and 10 mM glutamate. Numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

remove OAA by transamination (Fig. 4C), or the addition of CoA, TPP, and NAD$^+$ (Fig. 4D) to optimum ME and MDH activities still did not increase the respiration rates. At pH 7.6 where MDH was mainly activated and malate oxidation was mostly operated via MDH, malate was oxidized with rather higher rates than those of the same oxidation at pH 6.8 or 7.2, however, these rates were still much lower than those in other substrate oxidations (Fig. 5A). Adding NAD$^+$ to malate oxidation at pH 7.6 slightly increased the respiration rates (Fig. 5B). Addition of glutamate significantly stimulated malate oxidation with mitochondria of *K. daigremontiana* (Wiskich and Day, 1982), but not with pineapple (Fig. 5C). However, when a transamination system outside mitochondria was provided by adding both glutamate and GOT to the respiratory medium, malate oxidation was stimulated, and thereafter, the addition of NADH on these oxidations gave much higher rates than those without glutamate and GOT (Fig. 5A–C). This stimulation was more clearly detected in Fig. 5D where both glutamate and GOT were supplied just after adding malate.

In this study, the appearance of OAA outside the mitochondria was clearly detected by the assay as described in Fig. 6. In this assay, external NADH oxidation was prevented by EDTA and EGTA and the appearance of OAA outside the mitochondria was monitored by using the OAA detecting system consisting of 0.2 mM NADH plus 0.5 U MDH (Pastore *et al.*, 2003). Under *in vitro* assay conditions, NADH was observed to be rapidly oxidized by pineapple mitochondria (Fig. 1B) and this oxidation was inhibited about 70% by 1 mM EGTA (Table 3). Under the experimental conditions described in Fig. 6, addition of a larger amount of 10 mM EDTA and 10 mM EGTA strongly inhibited the external NADH dehydrogenase, and the addition of both MDH and malate caused the clearly decreasing absorbance of the spectrophotometer (Fig. 6B),

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whereas a similar decrease was not observed in individual mitochondria after adding MDH without malate (Fig. 6A). NADH oxidation in the presence of EDTA and EGTA before and after adding MDH and malate were about 73±19 and 297±48 nmol NADH min^{-1} mg^{-1} protein, respectively, indicating that OAA was being exported outside the pineapple mitochondria.

It had been indicated that OAA, the product of MDH activity, was also a strong inhibitor of several Krebs cycle dehydrogenases (Rustin *et al*., 1980). This tendency was also detected in pineapple mitochondria (Fig. 7). The addition of external OAA inhibited succinate oxidation. The inhibition levels varied depending on OAA concentrations and they were rapidly overcome by adding exogenous NADH. The inhibition and recovery of succinate oxidation while providing external OAA and NADH to the respiration medium were nearly similar to that in cauliflower mitochondria (Rustin *et al*., 1980).

Furthermore, pineapple mitochondria also showed GOT activity (Table 2) with a similar amount to that in soybean cotyledon mitochondria (Day *et al*., 1988). GOT was well known as a catalyst for the reversible reaction of glutamate and OAA to αKG and Asp, however, the mitochondria did not readily oxidize Asp (Fig. 8A) and α-KG (Fig. 8B) as the simple substrates, even in the presence of CoA, TPP, and NAD^+ (Fig. 8C). Simultaneous addition of α-KG and malate did not increase the rates of oxygen consumption (Fig. 8D). From these observations, it seemed the mitochondrial OAA must be transaminated or decarboxylated in the cytosol via a malate–OAA shuttle in order to maximize malate-dependent respiration (Fig. 9).

Discussion

It was found that pineapple mitochondria readily oxidized NADH with the high rates and coupling (Fig. 1A) similarly to mitochondria of ME-CAM species (Arron *et al*., 1979; Rustin and Queiroz-Claret, 1985; Hong *et al*., 2004; HTK

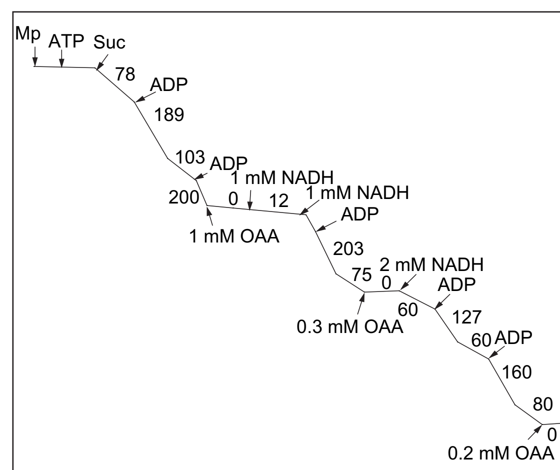

Fig. 7. The import of externally added OAA into the pineapple mitochondrial matrix to inhibit the succinate oxidation. Unless otherwise indicated, concentrations used were: 10 mM succinate, 10 mM ATP, and 0.16 mM ADP. Numbers along the trace refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

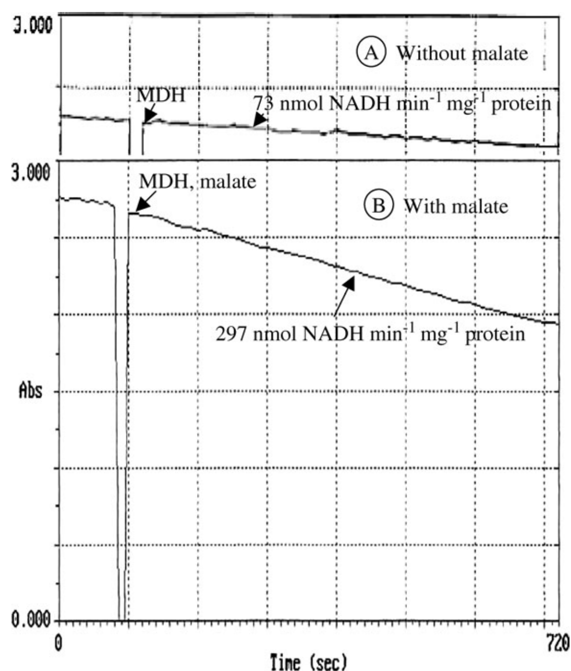

Fig. 6. The appearance of OAA outside pineapple mitochondria. The experiment was assayed according to Pastore *et al*. (2003). Mitochondria were incubated at 25 °C in 2 ml of reaction medium with the addition of 0.2 mM NADH plus 10 mM EGTA and 10 mM EDTA to inhibit the NADH DHEx. The reaction was started by adding 0.5 U MDH without malate for the reference cuvette (A), and 0.5 U MDH with 10 mM malate (pH 7.2) for the assay cuvette (B). The measurement was followed by the decrease in absorbance at A_{340} nm using a spectrophotometer (JASCO V-550 UV/VIS, Japan).

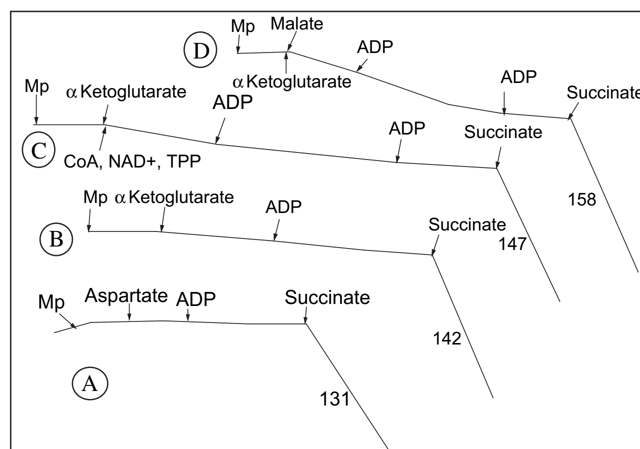

Fig. 8. Aspartate oxidation (A), α-ketoglutarate oxidation (B), α-ketoglutarate oxidation in the presence of CoA, TPP, and NAD^+ (C), and malate oxidation with α-ketoglutarate (D). Assay conditions were: 10 mM aspartate, 10 mM α-ketoglutarate, 10 mM succinate, 0.16 mM ADP, 1.5 mM TPP, 0.1 mM CoA, and 0.5 mM NAD^+. Numbers along the trace refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

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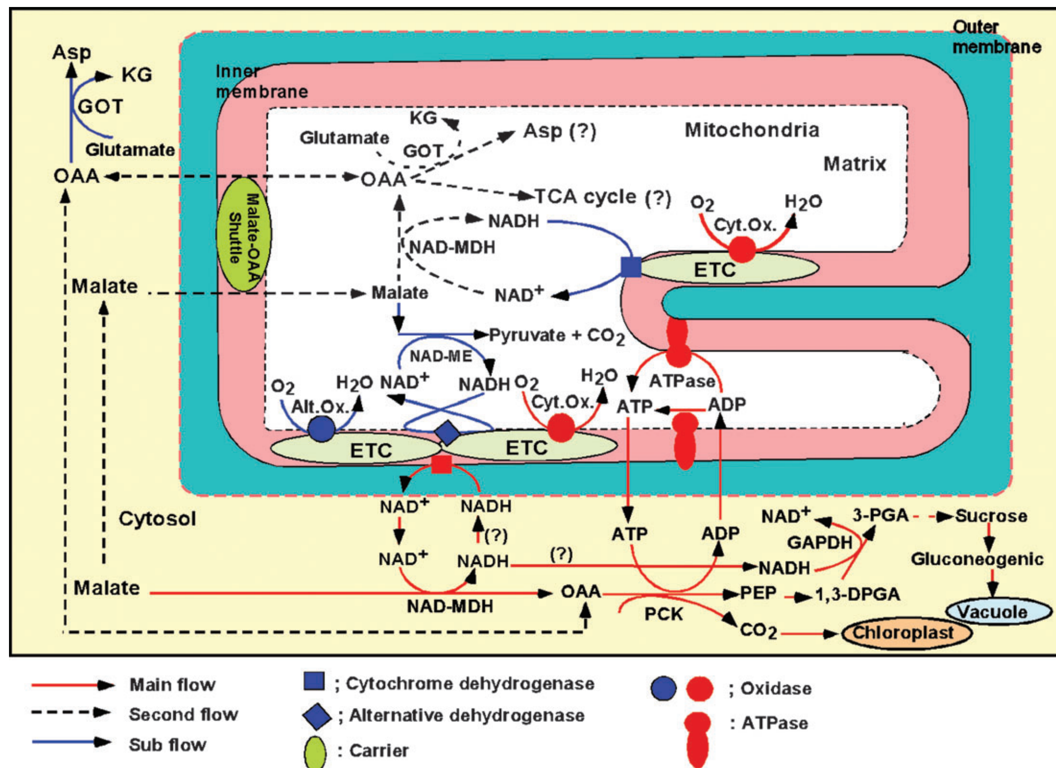

Fig. 9. Organization of the malate-oxidizing system in pineapple mitochondria. Alt.Ox, alternative oxidase; Cyt.Ox, cytochrome oxidase; GOT, glutamate-oxaloacetate transaminase; OAA, oxaloacetic acid; KG, α-ketoglutarate; Asp, aspartate; 3-PGA, 3-phosphoglycerate; 1,3-DPGA, 1,3-diphosphoglycerate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Hong, A Nose, S Agarie, unpublished results), except that mitochondria of these ME-CAM species readily oxidized NADPH without Ca^{2+}. Pineapple mitochondria only oxidized NADPH with significant rates and coupling in the presence of high Ca^{2+} concentrations (Fig. 1D). These results suggest that, as in other plant mitochondria (Møller, 2002), NADH and NADPH oxidations in pineapple mitochondria were due to two separate external NADH and NADPH dehydrogenases, respectively, and, in addition, external NADPH dehydrogenase activity in pineapple definitely required Ca^{2+} whereas that of NADH dehydrogenases did not (Figs 1C, 2; Table 3).

The main finding of this study was that pineapple mitochondria oxidized malate in a different way from mitochondria of ME-CAM plants. Mitochondria of ME-CAM plants such as *Sedum praealtum* (Arron *et al.*, 1979), *K. blossfeldiana* (Rustin and Queiroz-Claret, 1985), *K. fedtschenkoi* (Cook *et al.*, 1995), and *K. daigremontiana* (Hong *et al.*, 2004) usually oxidized succinate and malate with rather similar rates. However, pineapple mitochondria rapidly oxidized succinate while they poorly oxidized malate. The mitochondria showed low rates of malate oxidation under most of the assay conditions, unless supplied with both external glutamate and GOT (Fig. 5D). The optimization of the enzyme activities by changing pH, providing cofactors, and supplementing glutamate to remove OAA also did not stimulate malate oxidation. These results suggest that the respiration of pineapple mitochondria during CAM phase III was low and dependent on malate. Furthermore, individual addition of external glutamate, Asp or α-KG with or without the cofactors, and addition of both α-KG plus malate did not increase the rates of oxygen consumption (Fig. 8), indicating that mitochondrial malate oxidation was operated neither via MDH or ME as usual nor via the malate/aspartate shuttle.

Cuevas and Podestá (2000) found, in crude extracts of pineapple leaves, that the reaction of OAA reduction by cMDH was much faster than malate oxidation and that purified cMDH seemed to carry out both reactions of OAA reduction and malate oxidation. Concomitantly, it was found that pineapple mitochondria showed very high mMDH and low mME activities. In pineapple mitochondria, the rate of OAA reduction was calculated at about 75-fold faster than malate oxidation (Table 2). It was also found that the OAA could export out of (Fig. 6) and import into (Fig. 7) the mitochondrial inner membrane. Thus, the occurrence of high cMDH and mMDH, together with the mitochondrial permeability to both malate and OAA, could allow the operation of a malate–OAA shuttle in the inner membrane of pineapple mitochondria.

Normally, the OAA uptake system in plant mitochondria has a high affinity for OAA. The OAA carrier in plant

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mitochondria usually has low K_m, for example, the K_m value for the uptake carrier of OAA into potato tubers mitochondria was 0.18 mM (Hanning *et al.*, 1999). Thus, the low K_m value of the OAA carrier allows it to compete successfully with cytosolic or matrix malate dehydrogenase (Douce and Neuburger, 1997). In pineapple mitochondria, the addition of 0.2 mM external OAA completely inhibited succinate oxidation (Fig. 7), indicating that the mitochondria easily take up OAA, and with a small amount of OAA was sufficient to cause a significant effect on the rates of oxygen consumption. The mitochondria also exported OAA (Fig. 6), and did not consume α-KG and Asp (Fig. 8). These results completely differed from *K. daigremontiana* mitochondria which can oxidize α-KG and Asp with significant rates (Day, 1980). Furthermore, in *K. daigremontiana* mitochondria, the respiratory chain can be passed by supplying Asp and α-KG with malate, and the transamination of Asp provided an internal source of OAA, but not external OAA. The Asp and α-KG system may be important in *K. daigremontiana* when respiratory chain activity is restricted by energy charge (Day, 1980). By contrast, it seemed that Asp and α-KG did not contribute to malate metabolism in pineapple mitochondria. Therefore, the increasing rates of oxygen consumption when the mitochondria were supplied with both glutamate and GOT externally was attributable to a stimulation of OAA efflux from the mitochondria by the external GOT. The GOT consumed OAA by transamination in the presence of glutamate to form Asp and α-KG, thereby stimulating malate uptake into the mitochondria. As a result, OAA removal, malate oxidation stimulation, and the formed NADH oxidizing increased the respiration rates.

The OAA uptake system was clearly detected in pineapple mitochondria and the activity of mitochondrial GOT was also present at significant rates, however, adding external glutamate to the pineapple mitochondria suspension did not cause an increase in oxygen consumption. Therefore, it is not clear whether the matrix of pineapple mitochondria could contribute further to the *in vivo* system for removing OAA in order to synthesize Asp from glutamate, similarly to that described for other plant mitochondria by Siedow and Day (2000).

Based on these results, together with previous results obtained with intact leaves (Cuevas and Podestá, 2000; Chen *et al.*, 2002; Leegood and Walker, 2003), it is possible to suggest that a scheme summarizing the total malate metabolism in both the cytosol and mitochondrion could occur during the decarboxylation phase of CAM rhythm for pineapple (Fig. 9). In this phase, malate was mainly oxidized in the cytosol to produce OAA via cMDH. The PCK converted OAA to PEP and CO_2. Malate could also import from the cytosol to the mitochondria by the shuttle. In the mitochondrial matrix, malate could be catalysed by very high mMDH to form the OAA. Also, the ME was present at low levels in pineapple mitochondria, hence a little malate could be oxidized by low mitochondrial ME to produce pyruvate and CO_2. The OAA formed by mMDH activity could be exported outside the mitochondria via the malate–OAA shuttle (Fig. 9).

The operation of the shuttle and the capacity of the OAA reversible exchange in the inner membrane of pineapple mitochondria made them likely as a link between the mitochondrion and the cytosol in total malate metabolism during the decarboxylation phase. While the details of the carbon flow through the cMDH in pineapple CAM phase III awaits further study, a possible suggestion for the shuttle metabolism in pineapple mitochondria during the decarboxylation phase was that under the conditions where cMDH activity was insufficient to supply OAA at the required rates for the PCK activity, the mitochondrial malate oxidation could produce OAA and export the OAA to the cytosol via the shuttle. The exported OAA could become the available substrate for PCK activity to decarboxylate and PEP synthesis. By contrast, when OAA in the cytosol exceeded the required amount for PCK activity, the cytosolic OAA could be taken up into the mitochondria.

Lea *et al.* (2001) suggest that PCK may play a key role in both amino acids in C_4 plants and carbohydrate metabolism in CAM plants. This study's results also suggested that, under the experimental conditions where both glutamate and GOT were present, the exported OAA from the pineapple mitochondria could convert to form Asp and α-KG. Thus, it seems that the export OAA system in pineapple mitochondria could play a physiological role in amino acid metabolism, but this function is for future study.

Hoefnagel *et al.* (1998) showed that plant mitochondria have a greater capacity for ATP synthesis than photophosphorylation in the chloroplasts, due to an ATP/ADP translocator. Chloroplasts exhibit a far lower capacity for ATP export than mitochondria, thus mitochondria maintain most of the cytosolic ATP pool. From the current study, it is possible to suggest that the malate–OAA shuttle in pineapple mitochondria may also have contributed their role in the cytosolic ATP pool. By this shuttle, mitochondrial malate metabolism possibly provided the reducing equivalents for mitochondrial ATP synthesis to support the cytosolic PCK reaction in the decarboxylation phase.

In addition, cytosolic PEP in pineapple could be catalysed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to form 3-phosphoglycerate (3-PGA) in a reaction that consumed one NADH similarly to other PCK-CAM plants (Winter and Smith, 1996). The 3-PGA was further contributed in sucrose synthesis via gluconeogenesis. The NADH formed by cytosolic malate oxidation could also be used for the reductive step (GAPDH) in gluconeogenesis or oxidize directly via external NADH dehydrogenases in pineapple (Fig. 9). This point would be an interesting topic of energy metabolism for future studies of CAM.

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As a temporal conclusion in this study, the malate–OAA shuttle in pineapple mitochondria might operate as a supporting system for the mitochondrion and the cytosol in controlling and regulating malate metabolism in order to supply OAA for PCK activity during the decarboxylation phase of the PCK-CAM plant. In other words, the MDH on either side of the mitochondrial membrane are linked by this shuttle in the daytime conversion of malate to OAA during the decarboxylation phase. In addition, it seems that pineapple mitochondria not only support ATP for cytosolic PCK activity, but also contribute in supplying the substrate OAA for PCK activity of the decarboxylation phase during the day and for Asp synthesis in the cytosol.

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