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SPECIAL ISSUE RESEARCH PAPER

Malate metabolism in *Hoya carnosa* mitochondria and its role in photosynthesis during CAM phase III

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Abstract

This study investigated the respiratory properties and the role of the mitochondria isolated from one phospho*enol*pyruvate carboxykinase (PCK)-CAM plant, *Hoya carnosa*, in malate metabolism during CAM phase III. The mitochondria showed high malate dehydrogenase (mMDH) and aspartate amino transferase (mAST), and a significant amount of malic enzyme (mME) activities. *H. carnosa* readily oxidized malate via mME and mMDH in the presence of some cofactors such as thiamine pyrophosphate (TPP), coenzyme A (CoA) or NAD^+. A high respiration rate of malate oxidation was observed at pH 7.2 with NAD^+ and glutamate (Glu). Providing AST and Glu simultaneously into the respiratory medium strongly increased the rates of malate oxidation, and this oxidation was gradually inhibited by an inhibitor of α-ketoglutarate (α-KG) carrier, pyridoxal-5′-phosphate (PLP). The mitochondria readily oxidized aspartate (Asp) or α-KG individually with low rates, while they oxidized Asp and α-KG simultaneously with high rates, and this simultaneous oxidation was also inhibited by PLP. By measuring the capacity of the mitochondrial shuttle, it was found that the OAA produced via mMDH seemed not to be transported outside the mitochondria, but mAST interconverted OAA and Glu to Asp and α-KG, respectively, and exported them out via a malate-aspartate (malate-Asp) shuttle. The data in this research suggest that during phase III of PCK-CAM, *H. carnosa* mitochondria oxidized malate via both mME and the mMDH systems depending on metabolic requirements. However, malate metabolism by the mMDH system did not operate via a malate-OAA shuttle similarly to *Ananas comosus* mitochondria, but it operated via a malate-Asp shuttle similarly to *Kalanchoë daigremontiana* mitochondria.

Key words: *H. carnosa* mitochondria, malate-Asp shuttle, malate metabolism, mMDH, mME, starch PCK-CAM.

Introduction

Respiration and photosynthesis are extremely significant biological processes in the life cycle of plant cells. These processes are interdependent because their overall reactions are basically the reverse of each other. The reactants in respiration are the products in photosynthesis, and vice versa. In CAM plants, malate is formed during the night in the cytosol of photosynthesis cells. The malate formed also enters into the mitochondria, where it is equilibrated with the mitochondrial pool of fumarase (Osmond *et al*., 1988; Kalt *et al*., 1990; Holtum *et al*., 2005).

In plant mitochondria, malate could be subjected to some metabolism during night-time as (i) label randomization via fumarase and transported out of the mitochondria, (ii) malate decarboxylation via the NAD-malic enzyme to yield CO_2 and pyruvate, and (iii) malate incorporation into the tricarboxylic acid (TCA) cycle (Osmond, 1978; Kalt *et al*., 1990). In addition, mitochondrial respiratory activity during the night regulates the adenylate and redox balance in the cytoplasm (Holtum *et al*., 2005). The malate accumulated in the vacuoles at night is released into the cytoplasm where it is decarboxylated during the light, and largely incorporated into products of photosynthesis via ribulose 1,5-biphosphate carboxylase and gluconeogenesis (Holtum and Osmond,

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Abbreviations: α-KG, α-ketoglutarate; Asp, aspartate; AST, aspartate amino transferase; CAM, crassulacean acid metabolism; CoA, coenzyme A; COX, cytochrome oxidase; Glu, glutamate; MDH, malate dehydrogenase; ME, malic enzyme; Mp, purified mitochondria; OAA, oxaloacetic acid; PCK, phospho*enol*pyruvate carboxykinase; PEP, phospho*enol*pyruvate; PLP, pyridoxal-5′-phosphate; RCR, respiratory control ratio; TPP, thiamine pyrophosphate.

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1981; Holtum *et al*., 2005). The principal roles proposed for CAM mitochondria during the light include (i) decarboxylation of malate by NAD-ME, (ii) the oxidation of pyruvate to CO_2, (iii) the catabolism of citrate in some species that exhibit diel fluctuation in the citrate pool, and (iv) the provision for energy and substrates required for sucrose synthesis (Raghavendra and Padmasree, 2003; Holtum *et al*., 2005).

Basically, 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 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 cytosolic PCK (cPCK) (Dittrich *et al*., 1973; Winter and Smith, 1996; Cuevas and Podestá, 2000). Each group can also be divided into two subgroups, starch-forming and extrachloroplastic carbohydrate-forming, based on the major carbohydrate reservoir used in their daily cycle (Christopher and Holtum, 1996; Chen *et al*., 2002). Mitochondrial malate decarboxylation 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 (Day, 1980; Rustin *et al*., 1980; Hong *et al*., 2005). In mitochondria of a starch-forming ME-CAM plant, *Kalanchoë daigremontiana*, transamination of Asp via a malate-Asp shuttle is also observed during malate decarboxylation (Day, 1980).

Malate oxidation has not been much studied in mitochondria of PCK-CAM plants, except with a PCK-CAM plant that has a relatively low PCK activity (*Crassuala lycopodioides*), that possesses NAD-ME and partially oxidizes malate in the mitochondria, producing pyruvate (Peckmann and Rustin, 1992). Recently, it was discovered that, in a typical extrachloroplastic carbohydrate-forming PCK-CAM plant, *A. comosus*, mMDH played an important role in mitochondrial malate metabolism in which malate was mainly oxidized by active mMDH to produce OAA. This OAA is exported outside the mitochondria via a malate-OAA shuttle. By this shuttle, malate metabolism in *A. comosus* mitochondria not only provided CO_2 for photosynthesis but also supported a source of energy through a subsidiary supplement of OAA for cPCK (Hong *et al*., 2004*b*). Malate oxidations via the shuttles are found in mitochondria of *K. daigremontiana* and *A. comosus* and raise a pertinent question as to whether these shuttle metabolisms are specific or common for mitochondrial malate decarboxylation of CAM plants during the day?

Among CAM plants, *H. carnosa* seems to be an intermediate plant that possesses characteristics of both ME-CAM and PCK-CAM plants. Although it belongs to the PCK-CAM plants, *H. carnosa* has much lower PCK activity than *A. comosus*, and it is a starch-forming PCK-CAM plant which uses starch as the major reciprocating carbohydrate (Holtum and Osmond, 1981; Christopher and Holtum, 1996). *H. carnosa* is a typical CAM plant under non-stressed conditions, but it shifts from CAM to a modified CAM-idling mode of metabolism under water stress (Rayder and Ting, 1983). Furthermore, *H. carnosa* has a clear requirement of oxygen (O_2) and it loses the CAM phase III diurnal gas-exchange under low O_2 condition, similarly to the ME-CAM plant, *K. daigremontiana* (Nose *et al*., 1999). In this research, the metabolism of mitochondrial malate oxidation, the roles of the mitochondria in photosynthesis, and the relationships among cytosol, mitochondrion, and chloroplast in total malate metabolism was investigated during the light period of the starch-forming PCK-CAM cycle in *H. carnosa*.

Materials and methods

Plant material and purification of mitochondria

Plants of *H. carnosa* were propagated vegetatively and grown in plastic pots in a greenhouse under natural light and temperature. Ten days before the experiments, the plants were kept in 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 30 °C during the dark period with a relative humidity of 70%, and photosynthetically active radiation of 420–450 μmol m^{-2} s^{-1} at the top of the plant. 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. Mitochondria were isolated and purified as described previously by Hong *et al*. (2004*b*).

Measurement of mitochondrial enzyme activities

The Percoll-purified mitochondria were filtered at room temperature on a column of Sephadex G-25 equilibrated with the suspending buffer [400 mM sucrose, 0.1% BSA and 40 mM HEPES–KOH (pH 7.4)], and then the mitochondria were collected for the mitochondrial intactness and enzyme assays.

Cytochrome *c* oxidase (COX, EC 1.9.3.1) was measured spectrophotometrically at 25 °C by following the absorbance increase at 550 nm in the Percoll-purified mitochondria before and after lysis with 0.1% (v/v) Triton X-100 according to Møller and Palmer (1982).

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, with and without 0.1% (v/v) Triton X-100, and 200 μM NADH. The reaction was started by adding 600 μM OAA. The percentage latency of these enzymes was calculated as 100×[(rate with Triton)–(rate without Triton)]/(rate with Triton) (Møller and Palmer, 1982; Agius *et al*., 1998).

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, 2 mM NAD^+. NADP-ME was assayed in 2 ml medium of 50 mM HEPES–KOH (pH 8), 10 mM $MgCl_2$, 5 mM DTT, 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

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was started by adding 10 mM malate (pH 6.8). Measurements were made spectrophotometrically by following the absorbance increase at 340 nm due to NAD^+ or $NADP^+$ reduction.

Aspartate amino transferase (AST, EC 2.6.1.1) was assayed according to Bergmeyer and Bernt (1983), and OAA appearance outside mitochondria was assayed basically as described by 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 external NADH dehydrogenase. The reaction was started by adding 0.5 U MDH without malate for the reference cuvette, and 0.5 U MDH with 10 mM malate (pH 7.2) for the assay cuvette. The measurement was followed by the decrease in absorbance at 340 nm using a spectrophotometer (JASCO V-550 UV/VIS, Japan).

The malate-Asp shuttle was measured according to Scholz *et al*. (1998). Briefly, 100 µl of mitochondrial suspension were mixed with 2 ml of reaction medium (300 mM mannitol, 10 mM KH_2PO_4, 10 mM HEPES–KOH (pH 7.4), 10 mM KCl, 5 mM $MgCl_2$, 2 mM L-aspartate, 1 mM EDTA, 1 mM ADP, 0.14 mM NADH, 6 U MDH, and 4 U AST. Baseline oxidation of NADH was monitored at 340 nm for 4 min (JASCO V-550 UV/VIS, Japan). Malate-Asp shuttle activity was initiated with the addition of 4 mM malate and 4 mM glutamate (final concentration). The difference between the rate of change of absorbance with and without added substrates was normalized to added mitochondrial protein to determine the shuttle capacity.

Oxygen consumption and protein determination

Oxygen consumption by mitochondria was measured using an oxygen electrode (Hansatech, CA1D, Japan) at 25 °C in 2 ml of reaction medium (300 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM KCl, 20 mM HEPES–KOH) and the pH was adjusted from 6.8 to 7.6 with 3 N KOH. In malate oxidations, 0.25 mM CoA (an ME activator) and 0.25 mM TPP (a pyruvate dehydrogenase activator) were included at pH 6.8 to optimize ME activity; 0.25 mM CoA, 0.5 mM NAD^+ (a MDH activator) and 10 mM Glu were included at pH 7.2 to optimize both ME and MDH activities; 0.5 mM NAD^+ and 10 mM Glu were included at pH 7.6 to optimize MDH activity. Respiration control rate (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.

Reagents

The 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 the Sigma Chemical Company. All other reagents were from Wako Pure Chemical Industries and Katayama Chemicals, Japan.

Results

The intactness of H. carnosa *mitochondria*

The intactness of the outer and inner membranes of *H. carnosa* mitochondria was examined by the latency of both COX and NAD^+-MDH, respectively, as described in the 'Materials and methods'. *H. carnosa* mitochondria showed about 95% intactness of the outer membrane and 91% of the inner membrane. These results indicated that the intactness of the inner and outer mitochondrial membrane was acceptable and the preparation specifically reflected the mitochondrial properties.

Properties and activities of enzymes engaged in mitochondrial malate metabolism

The activities of mMDH, mAST, and mME were detected in *H. carnosa* mitochondria (Table 1). The activity of mMDH in *H. carnosa* mitochondria was 26 µmol min^{-1} mg^{-1} protein and this value was much lower than that of *A. comosus* (69 µmol min^{-1} mg^{-1} protein) but significantly higher than those of *K. pinnata* (18.5 µmol min^{-1} mg^{-1} protein) (Hong *et al*., 2004*b*), and *K. blossfeldiana* mitochondria (11.5 µmol min^{-1} mg^{-1} protein) (Rustin and Lance, 1986). Mitochondrial AST activity in *H. carnosa* was about 1.45 µmol min^{-1} mg^{-1} protein and this activity was much higher than that of *A. comosus* mitochondria (0.29 µmol min^{-1} mg^{-1} protein), but it was much lower than that of castor been mitochondria (11.4 µmol min^{-1} mg^{-1} protein at pH 7.0) (Mettler and Beevers, 1980). *H. carnosa* mitochondria showed a considerable amount of NAD-ME and NADP-ME activities (Table 1). The NAD-ME activity in *H. carnosa* mitochondria was about 0.29 µmol min^{-1} mg^{-1} protein. This rate was lower than that of mitochondria isolated from ME-CAM species such as *Prenia sladeniana* (0.46 µmol min^{-1} mg^{-1} protein) and *K. pinnata* (0.95 µmol min^{-1} mg^{-1} protein), but higher than that of mitochondria isolated from PCK-CAM species such as *Crassula lycopodioides* (0.20 µmol min^{-1} mg^{-1} protein) (Peckmann and Rustin, 1992) and *A. comosus* (0.11 µmol min^{-1} mg^{-1} protein) (Hong *et al*., 2004*b*). Although mME was predominantly NAD-ME, some NADP-ME was also detected in *H. carnosa* mitochondria. The NADP-ME activity in *H. carnosa* mitochondria was about 0.058 µmol min^{-1} mg^{-1} protein. This rate was slightly lower than those in mitochondria of ME-CAM species such as *K. daigremontiana* (0.067 µmol min^{-1} mg^{-1} protein) and *K. pinnata* (0.096 µmol min^{-1} mg^{-1} protein), but it was almost the same as that of *A. comosus*

Table 1. *Enzyme activities in* H. carnosa *mitochondria during CAM phase III*

Results shown are means ±SE (n=4–5) of separate preparations.

Malate oxidations	Respiration rate (nmol O_2 min^{-1} mg^{-1} protein)	
	H. carnosa mitochondria	*A. comosus* mitochondria[a]
MDH (EC 1.1.1.37)	26±4	69±17
AST (EC 2.6.1.1)	1.45±0.07	0.29±0.3
NAD-ME (EC 1.1.1.39)	0.29±0.06	0.11±0.02
NADP-ME (EC 1.1.1.40)	0.058±0.014	0.050±0.003

[a] Data based upon that presented by Hong *et al*. (2004*b*).

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mitochondria (0.050 μmol min^{-1} mg^{-1} protein) under the same assay conditions (Table 1; Hong *et al*., 2004*b*).

Malate oxidation in H. carnosa *mitochondria*

Malate oxidation was investigated under three different pH conditions, at pH 6.8, 7.2, and 7.6 where only mME, both mME and mMDH, and only mMDH were activated, respectively (Day *et al*., 1988; Agius *et al.,* 1998). The results showed that *H. carnosa* mitochondria readily oxidized malate under most of the assay conditions with varying levels depending on pH assay conditions and the cofactors (Table 2). The mitochondria were able to oxidize malate without any cofactors as described in 'Materials and methods' (data not shown); however, its respiration rate was much lower than those with the cofactors. The mitochondria readily oxidized malate in the presence of cofactors with significant respiration rates under most assay conditions, but the highest respiration rate was observed at pH 7.2 where both mME and mMDH were activated and stimulated by supplying external CoA, NAD^+, and Glu (Table 2). *H. carnosa* mitochondria oxidized malate with the respiratory control and ADP/O ratios typical of this substrate in the mitochondria of CAM plants (Day, 1980; Rustin and Queiroz-Claret, 1985). The ADP/O ratio in malate oxidation by *H. carnosa* mitochondria was more than 2, indicating that malate oxidation was coupled with three proton-extrusion sites (Table 2). Malate oxidations under these assay conditions were stimulated by ADP and partially inhibited by rotenone, KCN and SHAM (Fig. 1).

Effect of AST on malate oxidation

Along with rather high mAST activity, it was also found that supplying external AST in the respiratory medium strongly stimulated malate oxidation in the presence of Glu (Table 3). Malate oxidation at pH 6.8 was normally measured in the presence of CoA and TPP without NAD^+, and under this assay condition, adding AST or NAD^+ and Glu separately had no effect on the respiration (data not shown). However, when mMDH and a transamination system outside the mitochondria were activated by supplying external NAD^+ and both AST and Glu to the respiratory medium, malate oxidation was rather stimulated. Thereafter, addition of ADP gave higher respiration rates than those without AST (Table 3). Generally, the addition of AST in the presence of NAD^+ and Glu caused a considerable stimulation in malate oxidation of *H. carnosa* mitochondria. This stimulation was clearly observed in malate oxidations at pH 7.2 and 7.6 where both NAD^+ and Glu were included in the reaction medium, but the higher rate was detected at pH 7.6 (Table 3). In the presence of AST, all of the malate oxidations were gradually inhibited by rotenone, KCN, and SHAM, indicating that the NADH produced by mME and mMDH under these assay conditions was still reoxidized by the electron transport chain (ETC) of *H. carnosa* mitochondria (Table 3).

Table 2. *Malate oxidation in* H. carnosa *mitochondria*

Assay conditions were 20 mM malate, 0.12 mM ADP, 10 mM glutamate (Glu), 0.25 mM CoA, 0.5 mM NAD^+, and 0.25 mM TPP. State 3 refers to the respiration rate of O_2 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. Results shown are means ±SE (n=4–5) of separate preparations.

Malate oxidations	Respiration rate (nmol O_2 min^{-1} mg^{-1} protein)		RCR	ADP/O
	State 3	State 4		
pH 6.8 (CoA, TPP)	59±17	22±10	2.7±0.5	2.4±0.3
pH 7.2 (Glu, NAD^+, CoA)	94±11	34±7	2.8±0.4	2.4±0.2
pH 7.6 (Glu, NAD^+)	71±15	32±6	2.2±0.3	2.3±0.1

The appearance of Asp and α-KG, but not OAA outside H. carnosa *mitochondria*

Although under exactly the same assay condition, malate oxidation in *H. carnosa* mitochondria was significantly stimulated by supplying both external AST and Glu (Table 3) similar to *A. comosus*, OAA formed via mMDH from malate oxidation was directly exported out of *A. comosus* mitochondria via the malate-OAA shuttle (Hong *et al*., 2004*b*) while the appearance of OAA outside *H. carnosa* mitochondria was not detected (Fig. 2A). By contrast, the appearance of both Asp and α-KG outside *H. carnosa* mitochondria were clearly detected at significant rate (139±21 nmol NADH min^{-1} mg^{-1} protein, Fig. 2B).

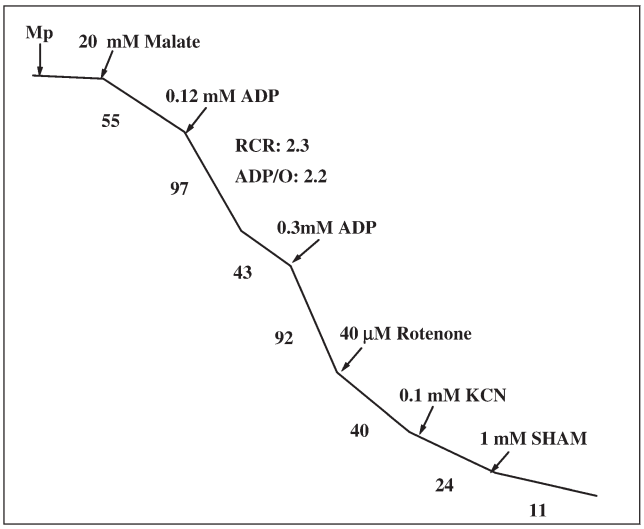

Fig. 1. A typical trace showed the effect of rotenone, KCN, and SHAM on malate oxidation by *H. carnosa* mitochondria. Malate was oxidized at pH 7.2 with 10 mM Glu, 0.25 mM CoA, and 0.5 mM NAD^+. Numbers along the trace refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mp, mitochondria.

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Table 3. *Effects of AST, rotenone, KCN, and SHAM on malate oxidation by* H. carnosa *mitochondria*

Unless otherwise indicated in Table 1, 10 mM Glu and 0.5 mM NAD^+ were included in reaction medium for three different pH levels, and assay conditions were 20 mM malate, 0.12 mM ADP (first addition), 0.3 mM ADP (second addition), 10 U AST, 40 μM rotenone, 0.1 mM KCN, and 1 mM SHAM. Results shown are means ±SE (n=3–4) of separate preparations.

Substrates and inhibitors	Malate oxidations (nmol mg^{-1} min^{-1} protein)		
	pH 6.8	pH 7.2	pH 7.6
Malate	33±8	52±11	43±9
ADP	59±17	94±11	71±15
AST	91±11	121±13	126±18
ADP	116±14	134±17	43±16
Rotenone	78±9	82±11	90±8
KCN	50±7	42±9	50±10
SHAM	13±5	11±7	14±8

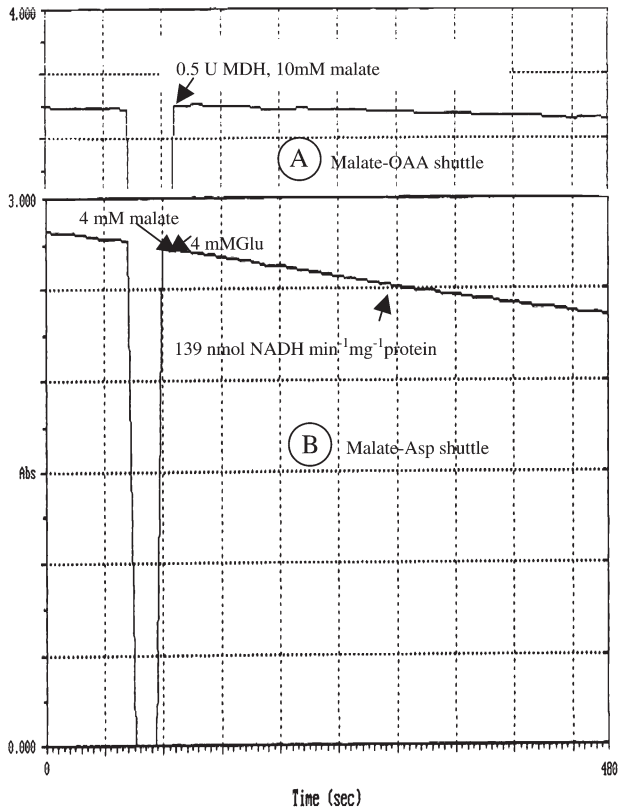

Fig. 2. The appearance of OAA (A), and Asp and α-KG (B) outside *H. carnosa* mitochondria. The experiment of OAA, and Asp and α-KG were assayed according to Pastore *et al.* (2003) and Scholz *et al.* (1998), respectively as described in the 'Materials and methods'. The measurement was followed by the decrease in absorbance at A_{340} nm using a spectrophotometer (JASCO V-550 UV/VIS, Japan).

Effect of PLP on the oxidations of α-KG, Asp, and malate

Differing from *A. comosus* (Hong *et al.*, 2004*b*) but similar to *K. daigremontiana* (Day, 1980), *H. carnosa* mitochondria readily oxidized α-KG or Asp as single substrates, but these oxidations required cofactors such as CoA and TPP, and their O_2 uptake rate was much lower than that of malate oxidation. The simultaneous oxidations of both Asp and α-KG showed a higher O_2 uptake rate than those of the single substrate oxidations (Table 4). In this study, the simultaneous oxidations of both Asp and α-KG by *H. carnosa* mitochondria were gradually inhibited by PLP, a specific inhibitor of the phosphate translocator (Hampp *et al.,* 1985; Laloi, 1999). These oxidations were slightly inhibited by adding 0.1 mM PLP, but they were completely inhibited by 0.3 mM PLP (Table 4). Inhibition by PLP on respiration rates was also detected in malate oxidation at pH 7.2 with AST and Glu, in which it was gradually inhibited following an increase in PLP concentrations, and it was completely inhibited by 0.5 mM PLP (Table 4).

Effect of Asp and α-KG on malate oxidation

Figure 3 shows the effects of α-KG and Asp on malate oxidation by *H. carnosa* mitochondria with and without a transamination system. In the absence of Glu and AST, the addition of α-KG on malate oxidation only slightly affected the rate of O_2 uptake but this rate markedly decreased by further adding Asp. This rate was recovered by supplying with both external Glu and AST, but it was gradually decreased by PLP (Fig. 3A). By contrast, supplying α-KG for malate oxidation in the presence of both Glu and AST led to a decrease in the O_2 uptake rate which was clearly decreased by further adding Asp. This inhibition was recovered by adding malate and ADP, and it was still partially inhibited by KCN and SHAM (Fig. 3B).

From these data, it is possible to suggest that *H. carnosa* mitochondria oxidized malate via mME and mMDH to produce pyruvate with CO_2 and OAA, respectively. However, when a transamination system inside and outside mitochondria was provided by adding both AST and Glu to the respiratory medium, malate oxidation by mMDH in *H. carnosa* mitochondria was operated via a malate-Asp shuttle (Fig. 4), but not via a malate-OAA shuttle as in *A. comosus* mitochondria (Hong *et al.*, 2004*b*).

Discussion

The most interesting observation in this study was that, although *H. carnosa* belonged to the PCK-CAM group, the mitochondria oxidized malate in a different way from the mitochondria of the PCK-CAM, *A. comosus* (Hong *et al.*, 2004*b*), but similarly to mitochondria of ME-CAM, *K. daigremontiana* (Day, 1980, Hong *et al.*, 2004*a*). *H. carnos*a mitochondria readily oxidized malate with significant rates and coupling in the presence of the cofactors at

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Table 4. *Effects of pyridoxal-5′-phosphate (PLP) on the oxidations of α-ketoglutarate (α-KG), aspartate (Asp) and malate by* H. carnosa *mitochondria*

Assay conditions were 20 mM malate, 5 mM Asp, 5 mM α-KG, and 10 U AST. The Asp and α-KG was oxidized at pH 6.8 with 0.25 mM CoA and 0.25 mM TPP. Malate was oxidized at pH 7.2 with 0.25 mM CoA, 0.5 mM NAD^+, and 10 mM Glu. State 2 refers to the respiration rate of O_2 uptake in the presence of substrate. (–) No addition. Results shown are chose from the typical traces of triplicate preparations for each substrate.

Substrates and inhibitors	Asp oxidation	α-KG oxidation (nmol mg^{-1} min^{-1} protein)	Malate oxidation
State 2	13	19	58
ADP	21	27	97
α-KG	32	–	–
Asp	–	55	–
AST	–	–	102
ADP	41	37	151
0.1 mM PLP	29	3	132
0.2 mM PLP	1	14	104
0.3 mM PLP	0	0	63
0.4 mM PLP	0	0	21
0.5 mM PLP	0	0	0

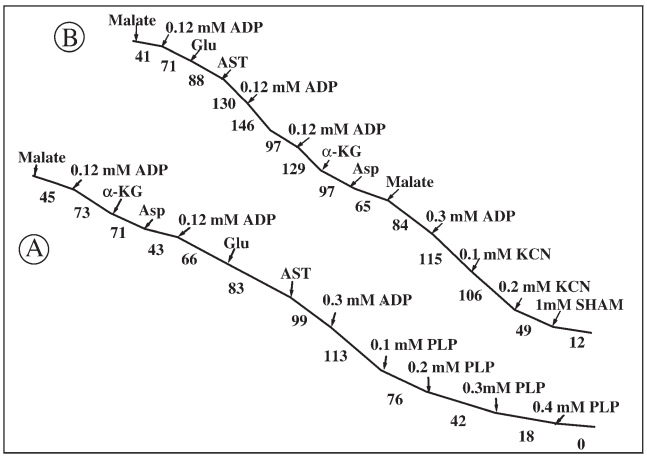

Fig. 3. Effect of α-KG and Asp in the presence of AST on malate oxidation by *H. carnosa* mitochondria. Unless otherwise indicated, the assay conditions were 20 mM malate, 10 mM Glu, 5 mM α-KG, 5 mM Asp, and 10 U AST. Malate was oxidized at pH 7.2 with 0.25 mM CoA and 0.5 mM NAD^+. Numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein.

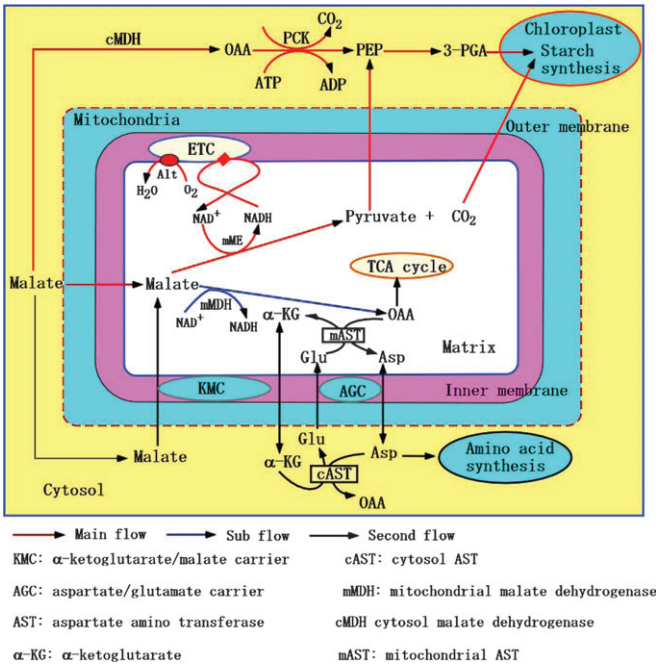

Fig. 4. Organization of the malate-oxidizing system in *H. carnosa* mitochondria. ALT, alternative; ETC, electron transport chain; OAA, oxaloacetic acid; PCK, phospho*enol*pyruvate carboxykinase; PEP, phospho*enol*pyruvate; and PGA, phosphoglycerate.

different pH levels (Table 2). By contrast, *A. comosus* mitochondria only oxidized malate poorly under the same assay conditions (Hong *et al.*, 2004*b*). At pH 6.8, malate was mainly oxidized via mME to produce pyruvate and CO_2 in *H. carnos*a mitochondria similarly to *K. daigremontiana* mitochondria (Day, 1980, Hong *et al.*, 2004*a*). Therefore, when mME was maximally activated by CoA and TPP, malate oxidation via mME was significantly stimulated. The highest rate in malate oxidation was detected at pH 7.2 in the presence of CoA, NAD^+, and Glu (Table 2). It might be because under this assay condition, both mME and mMDH were maximally activated and OAA formed from malate oxidation via mMDH was transaminated inside the mitochondria by Glu (Aguis *et al.*, 1998). The stimulated O_2 uptake rate by Glu indicated that Glu could be imported into the mitochondrial inner membranes and transaminated the OAA produced from malate by mMDH. Malate oxidation at pH 7.2 in the presence of CoA, NAD^+, and Glu was partly inhibited by rotenone, KCN, and SHAM (Fig. 1), indicating that NADH produced by both mME and mMDH during this oxidation were reoxidized to NAD^+ by the mitochondrial ETC, and complex I, alternative (Alt) and cytochrome (Cyt) pathways were activated in this process.

A general property of mitochondrial malate oxidation between *H. carnosa* and *A. comosus* was the fact that supplying both external AST and Glu to respiratory medium significantly increased malate oxidation (Table 3). However, under exactly the same assay condition, OAA formed from malate oxidation by mMDH could export out of *A. comosus* mitochondria via a malate-OAA shuttle (Hong *et al.*, 2004*b*), where OAA was limited to cross the inner membrane of *H. carnosa* mitochondria (Fig. 2A). By contrast, the active mAST of *H. carnosa* mitochondria rapidly interconverted OAA with Glu to Asp and α-KG, respectively, and exported them out via a malate-Asp shuttle (Fig. 2B).

The AST was well known as a catalyst for the reversion of Glu and OAA to α-KG and Asp, respectively. Though *A. comosus* mitochondria possessed a small amount of mAST, they did not oxidize Asp and α-KG as the simple

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substrates, even in the presence of CoA, TPP, and NAD^+ (Hong *et al*., 2004*b*) while *H. carnosa* mitochondria could oxidize Asp and α-KG (Table 4) similarly to *K. daigremontiana* mitochondria (Day, 1980). Genchi *et al*. (1991) reported that the α-KG carrier purified from corn mitochondria is completely inhibited by PLP, and a similar result was observed in *H. carnosa* mitochondria. PLP inhibited the simultaneous oxidation of α-KG and Asp and it also inhibited malate oxidation in the presence of AST (Table 4). These data indicated that *H. carnosa* mitochondria possessed a carrier of α-KG, and PLP caused an inhibition on this carrier. The inhibition by both Asp and α-KG on malate oxidation and the recovery of this inhibition by adding both Glu and AST (Fig. 3A) or malate (Fig. 3B) together with their inhibition by PLP confirmed that Asp and α-KG could be imported inside and exported outside the *H. carnosa* mitochondria.

Based on these results, together with previous results obtained with intact leaves (Black *et al*., 1996; Christopher and Holtum, 1996), 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 PCK-CAM rhythm for *H. carnosa* (Fig. 4). In this phase, malate was mainly oxidized in the cytosol to produce OAA via cMDH. The cPCK converted OAA to PEP and CO_2 for further starch synthesis in the chloroplast. Malate could also be imported from the cytosol to the mitochondria. In the mitochondrial matrix, malate could be oxidized by mME to produce pyruvate and CO_2 or catalysed by mMDH to form the OAA. Under assay conditions, where the mitochondria were supporting a transamination system by adding external AST and Glu, the OAA formed by mMDH and Glu could rapidly be changed to Asp and α-KG, respectively by active mAST. Then, Asp and α-KG could be exported outside the mitochondria via the malate-Asp shuttle (Fig. 4). These results suggested that during the light time, mME and mMDH systems were operated in parallel in *H. carnosa* mitochondria to control and regulate the overall malate metabolism, depending on CO_2 requirement and amino acid synthesis.

Malate oxidation via mME to produce pyruvate and CO_2 was a general metabolism for the mitochondria of many ME-CAM species in which the pyruvate formed could be exported outside the mitochondria or oxidized by the TCA cycle inside the mitochondria (Spalding *et al*., 1979; Day, 1980; Rustin *et al*., 1980). Under the assay conditions used in this study, external acetyl-CoA was not supplied to *H. carnosa* mitochondria, so it was probable that the pyruvate formed via the mME system could not be converted in the next reactions of the TCA cycle, and they might be accumulated in the mitochondrial matrix or exported to the respiratory medium. Holtum *et al*. (2005) reported that, in CAM species, that use starch as a temporary repository for carbon, PEP or pyruvate produced during malate decarboxylation in the light must be imported into the chloroplast and converted to starch. *H. carnosa* is a starch PCK-CAM specie and it also possesses a small amount of pyruvate phosphate dikinase (PPDK) of about 12 nmol min^{-1} mg^{-1} protein in the chloroplast (Holtum and Osmond, 1981; Black *et al*., 1996). This PPDK might be necessary for converting pyruvate to PEP inside the chloroplast of *H. carnosa*. Therefore, it is possible to suggest that during the decarboxylation phase of *H. carnosa*, when the CO_2 and PEP produced by cPCK were insufficient for starch synthesis in the chloroplast, the pyruvate formed by the mME system could be exported outside the mitochondria and converted to PEP by chloroplast PPDK for starch synthesis.

In this study, malate oxidation via mME was less sensitive with KCN than via mMDH or both mME and mMDH (Table 3). Thus, it seemed that NADH produced from malate oxidation via mME was reoxidized by the ETC in which their electrons were more connected to Alt respiration rather than Cyt respiration in *H. carnosa* mitochondria. The pyruvate generated intramitochondrially during malate oxidation could activate Alt oxidize (AOX) (Day *et al*., 1994); hence the pyruvate inside the mitochondria could stimulate the Alt respiration. Malate metabolism via the mME system in *H. carnosa* mitochondria helps to understand why under low O_2 concentration, both *H. carnosa* and *K. daigremontiana* lost phase III of CAM-type diurnal gas-exchange, but *A. comosus* still exhibited a pattern of diurnal gas-exchange (Nose *et al*., 1999; Nose and Takashi, 2001).

Malate metabolism via mMDH to produce OAA was clearly reported in *A. comosus* mitochondria in which the OAA formed by mMDH could be exported outside the mitochondria via the malate-OAA shuttle (Hong *et al*., 2004*b*). By contrast, *H. carnosa* mitochondria did not possess the malate-OAA shuttle, but they could rapidly transaminate the OAA formed by mMDH to Asp in the presence of Glu and AST, and export the produced Asp and α-KG outside the mitochondria via the malate-Asp shuttle (Fig. 4). This metabolism seemed not to play an important role in supplying OAA for cPCK activity, similar to *A. comosus* mitochondria, but it might supply OAA for Asp transamination or for the TCA cycle in *H. carnosa* mitochondria. The Asp transamination was reported in *K. daigremontiana* mitochondria and it might be important for the mitochondria when respiratory chain activity was restricted by energy charge (Day, 1980). In *H. carnosa*, malate metabolism via the mME system was more connected to Alt respiration and this respiration seemed not to produce much energy inside the mitochondria. However, malate metabolism by the mMDH system via the malate-Asp shuttle could support the energy, because this shuttle could import the reducing equivalents of cytosolic NADH to the mitochondria. The NADH

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imported via this shuttle could reduce complex I, so oxidation of imported NADH was associated with greater energy than that of cytosolic NADH oxidation by the externally facing dehydrogenase (Siedow and Day, 2000). The energy formed by this shuttle might be necessary for amino acid metabolism in *H. carnosa*.

In conclusion, *H. carnosa* used the cPCK, mME, and mMDH systems to decarboxylate malate during CAM phase III. Malate metabolism in the mitochondria could involve in both CAM photosynthesis and amino acid synthesis. Malate metabolism via mME system in *H. carnosa* mitochondria was operated in a similar way to the mitochondria of other ME-CAM species, and this metabolism might play an important role in CAM photosynthesis during the decarboxylation phase. Malate metabolism by mMDH system via the malate-Asp shuttle seemed to play a physiological role in supplying the energy and the substrates of Glu and α-KG for amino acid metabolism in the cytosol of *H. carnosa*. This point would be an interesting topic of amino acid metabolism for future studies of starch PCK-CAM.

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