BIOLOGIA PLANTARUM 49 (2): 201-208, 2005

Oxidations of various substrates and effects of the inhibitors on purified mitochondria isolated from *Kalanchoë pinnata*

H.T.K. HONG, A. NOSE* and S. AGARIE

Faculty of Agriculture, Saga University, 1 Honjo-machi, Saga, 840-8502, Japan

Abstract

Kalanchoë pinnata mitochondria readily oxidized succinate, malate, NADH, and NADPH at high rates and coupling. The highest respiration rates usually were observed in the presence of succinate. The high rate of malate oxidation was observed at pH 6.8 with thiamine pyrophosphate where both malic enzyme (ME) and pyruvate dehydrogenase were activated. In CAM phase III of *K. pinnata* mitochondria, both ME and malate dehydrogenase (MDH) simultaneously contributed to metabolism of malate. However, ME played a main function: malate was oxidized *via* ME to produce pyruvate and CO_2 rather than *via* MDH to produce oxalacetate (OAA). Cooperative oxidation of two or three substrates was accompanied with the dramatic increase in the total respiration rates. Our results showed that the alternative (Alt) pathway was more active in malate oxidation at pH 6.8 with CoA and NAD^+ where ME operated and was stimulated, indicating that both ME and Alt pathway were related to malate decarboxylation during the light. In *K. pinnata* mitochondria, NADH and NADPH oxidations were more sensitive with KCN than that with succinate and malate oxidations, suggesting that these oxidations were engaged to cytochrome pathway rather than to Alt pathway and these capacities would be desirable to supply enough energy for cytosol pyruvate orthophosphate dikinase activity.

Additional key words: alternative pathway, CAM, cytochrome pathway, malate dehydrogenase, malic enzyme, oxalacetate.

Introduction

Mitochondrial respiration of plants differs from that of animals by the presence of an alternative (Alt) pathway in the electron-transport chain (ETC). It branches from the cytochrome (Cyt) pathway at ubiquinone (Q) and donates electrons directly to oxygen to form water. The Alt pathway is inhibited by salicylhydroxamic acid (SHAM) and the Cyt pathway is inhibited by KCN. In spite of extensive investigation among higher plants, fungi, yeasts and protozoa, the physiological role of the Alt pathway in ETC is not fully understood. For CAM plants, it has been shown that there was an increase in cyanide-resistant leaf respiration in the phase III of *K. blossfeldiana* (Rustin and Queiroz-Claret 1985), and *K. daigremontiana* (Robinson *et al.* 1992). However, the Alt capacity in CAM mitochondria is probably not great enough to support *in vivo* rates of malate decarboxylation (Wiskich and Day 1982).

Kalanchoë pinnata is a ME type CAM plant. In the phase III, under closure of the stomata, malate is released from the vacuole and oxidatively decarboxylated by NAD(P)-ME to generate pyruvate and CO_2. Pyruvate is phosphorylated to phospho*enol*pyruvate (PEP) by catalysis of pyruvate orthophosphate dikinase (PPDK), and then it is conserved in gluconeogenesis. Recently, the experiment results in our laboratory indicated that PPDK is distributed both in chloroplast and cytosol in *K. pinnata* mesophyll cell (Kondo *et al.* 1998). Under low oxygen, these plants lost phase III in CAM-type diurnal gas-exchange activity (Nose *et al*. 1999). There was an increased of the Alt pathway activity in CAM phase III

Received 9 April 2004, *accepted* 25 August 2004.

Abbreviations: Alt - alternative; CAM - crassulacean acid metabolism; CRR - cyanide resistant respiration; Cyt - cytochrome; ETC - electron transport chain; MDH - malate dehydrogenase; ME - malic enzyme; Mp - purified mitochondria; PEPC - phosphoenolpyruvate carboxylase; PPDK - pyruvate orthophosphate dikinase; Q - ubiquinone; RCR - respiratory control ratio; RuBP - ribulose 1,5-bisphosphate; SHAM - salicylhydroxamic acid; TPP - thiamine pyrophosphate.

Acknowledgements: The author wishes to thank Professor Hans Lambers (University of Western Australia) for his helpful critically comments at the first time on preparing of this manuscript.

* Author for correspondence: fax: (+81) 952 288737, e-mail: nosea@cc.saga-u.ac.jp

of *K. pinnata* leaf (Tsuchiya *et al*. 2001). These results suggest that not only ME but also Alt pathway plays an important role during the malate decarboxylation in the phase III of *K. pinnata* intact leaf and raise some further questions about *K. pinnata* mitochondria. How do the mitochondria contribute to total malate decarboxylation during the phase III? How to reduce the NADPH produced from malate decarbo-xylation via cytosol NADP-ME? How does the cytosolic PPDK activity involve to the mitochondrial ATP synthesis? Is there any relationship between the malate oxidation and the Alt pathway in mitochondria? Are these activities in mitochondria involved with those in intact leaf of *K. pinnata* or not? Based on these view points, we investigated respiratory properties with various substrates and effects of the inhibitors on the Alt pathway in *K. pinnata* mitochondria.

Materials and methods

Plant material and mitochondria respiration: *Kalanchoë pinnata* (Lam.) Pers. were vegetatively propagated and grown in plastic pots in a greenhouse with natural light and temperature. Ten days before the experiments, the 3-month-old plants were transferred to a growth chamber (*KG-50 HLA*, *Koito Industrial Co.*, Tokyo, Japan) with 12-h photoperiod. The temperature in the growth chamber was maintained at 25 ^{o}C during the dark period and 35 ^{o}C during the light period with photosynthetically active radiation at the mid-plant height of 420 to 450 μmol m^{-2} s^{-1}. The fifth to seventh leaves, numbered from the apex, were used for the experiments.

Mitochondria of *K. pinnata* were isolated and purified on Percoll gradients as described previously (Hong *et al*. 2004). Oxygen consumption was measured using an oxygen electrode (*Rank Brothers*, Cambridge, UK) at 25 ^{o}C in 2 cm^3 of reaction medium [(300 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM KCl, 100 mM HEPES-KOH (pH 7.4)] and pH was adjusted from 6.8 to 7.8 by adding KOH. The O_2 concentration in air-saturated medium was taken as 258 μM. Respiratory control ratio (RCR) and ADP/O ratio were calculated according to Estabrook (1967). The protein content was measured by the method of Bradford (1976) using bovine serum albumine (BSA) as the standard. Chlorophyll content was determined according to Arnon (1949).

Preparation of leaf extraction and mitochondria for enzyme assays: The leaf sample (0.5 g fresh mass) was homogenized using a mortar and pestle with 0.2 g sea sand and 40 mg PVP in 4 cm^3 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 $MgCl_2$, 1 mM EDTA-KOH (pH 7.0), 5 mM DTT, 0.2 % (m/v) BSA and 0.02 % (m/v) Triton X-100. The homogenate was filtered through one layer of *Miracloth* (*Calbiochem-Novabiochem*, La Jolla, USA). Part of the homogenate was taken for determination of chlorophyll content; the other homogenate was centrifuged at 10 000 *g* for 10 min at 4 ^{o}C. The supernatant was desalted by passing through a *Sephadex G-25* (*PD-10* column, *Pharmacia Biotech AB*, Uppsala, Sweden) that had been equilibrated with the enzyme extraction medium. The desalting extract was used immediately for determination of enzyme activity.

Preparation of mitochondria for enzyme assays: The mitochondria were filtered at room temperature on a column of *Sephadex G-25* previously equilibrated with the suspending buffer contained 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 % (m/v) Triton X-100.

Enzyme assays: 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*. (2000). Rubisco was assayed according to Du *et al*. (1996). PEPC was assayed according to Shaheen *et al*. (2002).

Results

Enzyme activities: PEPC and Rubisco were localized unambiguously in the cytosol and chloroplast, respectively, of *K. pinnata* mesophyll cells (Kondo *et al*. 1998), so that their activities can be used as the indicators of mitochondrial purity. Rubisco activity was null and PEPC activity in mitochondria was approximate by 4.4 % of that in cytosol (Table 1). These results indicated that the mitochondria suspensions did not contain chloroplast components and the cytosol contamination of the mitochondria was rather low.

Activities of NAD-ME, NADP-ME and MDH were detected in leaf extract and in *K. pinnata* mitochondria. NAD-ME activity was higher than NADP-ME activity in leaf extracts of *K. pinnata* (Table 2). Although NAD-ME was predominantly located in the mitochondria, a small amount of NADP-ME was also detected in *K. pinnata*

Table 1. Rubisco and PEPC activities in leaf extract and mitochondria (Mp) of *K. pinnata*. Means ± SD (n = 4 - 5 of separate preparations). Rubisco showed initial activity. Enzyme activities are expressed as: * - [mmol g^{-1}(chlorophyll) min^{-1}], and ** - [mmol g^{-1}(protein) min^{-1}]; nd - not detectable.

Enzyme	Leaf*	Leaf**	Mp**
PEPC	12.97 ± 1.04	0.48 ± 0.04	0.021 ± 0.011
Rubisco	5.99 ± 2.15	0.17 ± 0.03	nd

Table 2. Enzyme activities in leaf extract and in mitochondria (Mp) of *K. pinnata*. Means ± SD (n = 4 - 5). Enzyme activities are expressed as [mmol g^{-1}(protein) min^{-1}].

Enzyme	Leaf	Mp
NADP-ME	0.05 ± 0.01	0.096 ± 0.013
NAD-ME	0.11 ± 0.06	0.950 ± 0.090
MDH	0.60 ± 0.10	18.490 ± 1.970

mitochondria. MDH activity in mitochondria was higher than that in leaf of *K. pinnata*.

Respiration of *K. pinnata* mitochondria with the single substrates: The highest respiration rates were recorded with succinate. They exceeded those for NADH, NADPH and malate (pH 6.8) in the presence of 1 mM TPP, 1.6, 1.9, and 1.8 times, respectively. The ADP/O ratios in succinate oxidation were less than 2, indicating that their oxidations were coupled to two proton-extrusion sites. *K. pinnata* mitochondria were able to oxidase external NADH and NADPH by similar way; however, the rate of NADH oxidation was higher than that of NADPH oxidation (Table 3). Malate oxidation was investigated under three different pH; at pH 7.6, where only MDH was active, at pH 7.2 where both ME and MDH were active and at pH 6.8 where only ME was active (Day *et al.* 1988, Agius *et al.* 1998). NAD^+ was included in all cases to stimulate the optimum ME and MDH activities. *K. pinnata* mitochondria oxidized malate with varied levels depending on pH assay conditions. The rate of malate oxidation was very low at pH 7.6, whereas it increased at pH 7.2 (Table 3). *K. pinnata* mitochondria were able to oxidize malate without any cofactors at pH 6.8; however, its rate was slower than that in the presence of TPP (a pyruvate dehydrogenase activator) or CoA (a ME activator). Addition of TPP or CoA and NAD^+ stimulated malate oxidation via ME. The highest rate of malate oxidation was observed at pH 6.8 in the presence of TPP. In general, before adding ADP, *K. pinnata* mitochondria slowly oxidized malate. Upon ADP addition, high rates of oxygen consumption were measured. In all cases, the ADP/O ratio of malate oxidation was greater than 2 (Table 3), indicating that all three proton-extrusion sites were utilized.

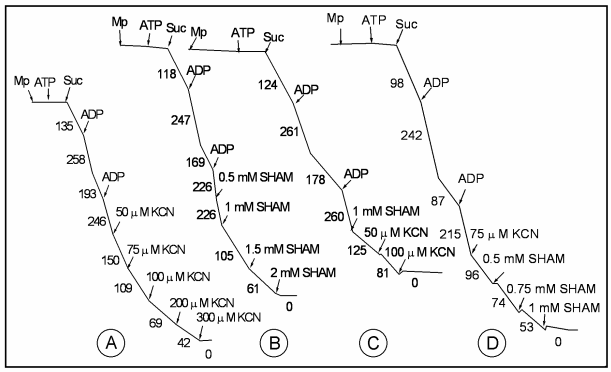

Fig. 1. Effect of KCN (*A*), SHAM (*B*), KCN in the presence of SHAM (*C*), and SHAM in the presence of KCN (*D*) on the succinate oxidation by *K. pinnata* mitochondria. Assay conditions were: 10 mM succinate, 10 mM ATP, 0.16 mM ADP (first adding) and 0.32 mM ADP (last adding). Numbers along the trace refer to μmol(O_2) g^{-1}(protein) min^{-1}.

Table 3. Individual substrate oxidation in *K. pinnata* mitochondria. Assay conditions were 10 mM succinate, 1 mM NADH, 1 mM NADPH, 10 mM malate, 1 mM TPP, 0.1 mM CoA, 0.5 mM NAD^+, and 0.16 mM ADP. 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. Means ± SD (n = 4 - 5) of separate preparations (nd - not detectable).

Substrates	Respiration rate [μmol(O_2) g^{-1}(protein) min^{-1}] state 3	state 4	RCR	ADP/O
Succinate	256 ± 30	119 ± 26	2.2 ± 0.4	1.5 ± 0.4
NADH	155 ± 41	71 ± 14	2.1 ± 0.5	1.4 ± 0.3
NADPH	131 ± 23	60 ± 32	2.5 ± 1.0	1.4 ± 0.4
Malate (pH 7.6, NAD^+)	24 ± 7	nd	nd	nd
Malate (pH 7.2, glutamate, NAD^+)	41 ± 8	32 ± 6	1.3 ± 0.3	2.2 ± 0.1
Malate (ph 6.8)	62 ± 16	31 ± 11	2.0 ± 0.5	2.4 ± 0.3
Malate (pH 6.8, CoA, NAD^+)	105 ± 11	74 ± 9	1.4 ± 0.4	2.4 ± 0.2
Malate (pH 6.8, TPP)	139 ± 25	90 ± 23	1.5 ± 0.2	2.3 ± 0.2

Respiration of *K. pinnata* mitochondria with the multiple substrates: Generally, simultaneous oxidation of these substrates normally increased the respiration rates that were lower than the sum of the individual rates but higher than the individual rates, and it was more marked when succinate was the first substrate. The simultaneous oxidations of malate as a first substrate with other substrates were investigated at pH 6.8 in the presence of TPP (Table 4, Fig. 4) or in the presence of CoA and NAD^+ (Table 5). Addition of NADPH or NADH as the second substrates mainly stimulated the respiration rate that was significantly higher than individual rate, and then addition of NADH as a third substrate did not further increase the simultaneous rate (Table 4, Fig. 4). In our experiments, all of the simultaneous oxidation of substrates gave ADP/O ratios of lower than 2, indicating that two proton-extrusion sites were utilized.

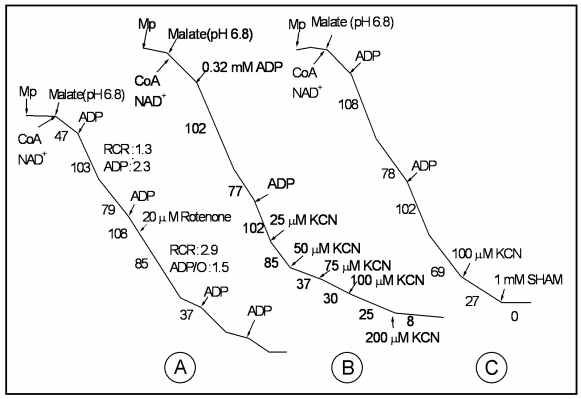

Fig. 2. Effect of rotenone (*A*), KCN (*B*) and SHAM (*C*) on malate oxidation at pH 6.8 in the presence of 0.5 mM CoA and 1 mM NAD^+ by *K. pinnata* mitochondria. Unless otherwise indicated, concentrations were used: 10 mM malate, 0.16 mM ADP (first adding) and 0.32 mM ADP (last adding). Numbers along the trace refer to $\mu mol(O_2)\ g^{-1}(protein)\ min^{-1}$.

Table 4. Simultaneous substrate oxidation in *K. pinnata* mitochondria. Means ± SE (n = 4 - 5). Assay conditions were 1 mM NADH, 1 mM NADPH, 0.16 mM ADP, 10 mM malate at pH 6.8 in the presence of 1 mM TPP and 10 mM succinate in the presence of 10 mM ATP.

Substrates	Respiration rate [$\mu mol(O_2)\ g^{-1}(protein)\ min^{-1}$] state 3	state 4	RCR	ADP/O
Succinate + NADH	364 ± 22	202 ± 12	1.8 ± 0.3	1.5 ± 0.2
Succinate + NADPH	329 ± 10	218 ± 16	1.6 ± 0.2	1.2 ± 0.2
NADH + NADPH	231 ± 12	112 ± 23	2.1 ± 0.8	1.5 ± 0.2
Malate + NADH	249 ± 49	181 ± 32	1.4 ± 0.6	1.3 ± 0.3
Malate + NADPH	204 ± 22	128 ± 23	1.7 ± 0.7	1.5 ± 0.2
Malate + NADH + NADPH	216 ± 17	139 ± 31	1.6 ± 0.4	1.3 ± 0.2

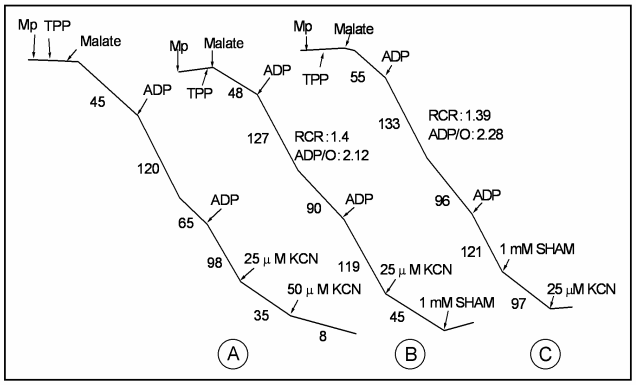

Fig. 3. Effect of KCN (*A*), KCN and SHAM (*B*) and SHAM and KCN (*C*) on the malate oxidation at pH 6.8 in the presence of 1 mM TPP. Assay conditions were: 10 mM malate, 0.16 mM ADP (first adding), and 0.32 mM ADP (last adding). Numbers along the trace refer to $\mu mol(O_2)\ g^{-1}(protein)\ min^{-1}$.

Effect of KCN, SHAM and rotenone on oxidation of various substrates: The oxidation was inhibited by both KCN and SHAM as well as combination of them. Succinate oxidation was uninhibited at the concentrations lower than 50 μM KCN or 1 mM SHAM, it was significantly inhibited at 0.5 - 2 mM SHAM or 50 - 200 μM KCN and fully inhibited at 300 μM KCN or 2 mM SHAM (Fig. 1*A*,*B*). Combination of 1 mM SHAM and 100 μM KCN or 75 μM KCN and 1 mM SHAM completely blocked the electron transport chain (Fig. 1*C*,*D*). The effect of KCN and SHAM on succinate oxidation indicated that *K. pinnata* mitochondria possessed both of Alt and Cyt pathways in the electron transport chain.

The other KCN titration experiments with malate oxidation at pH 6.8 in the presence of CoA and NAD^+ showed that concentrations of KCN from 25 - 50 μM significantly inhibited the malale oxidation. This oxidation was not more significantly inhibited by increasing KCN up to 100 μM; however, it was near fully inhibited by increasing KCN up to 200 μM (Fig. 2*B*). In the presence of 100 μM KCN, the rate of malate oxidation was about 25 % of the uninhibited rate (Fig. 2*B*). Adding 100 μM KCN and 1 mM SHAM fully inhibited this oxidation (Fig. 2*C*). At pH 6.8, malate

oxidation with TPP was more sensitive to KCN than that in malate oxidation with CoA and NAD^+ (Fig. 3). Adding 50 μM KCN inhibited 36 % of the state 3 rate in malate oxidation with CoA and NAD^+, whereas this concentration of KCN near fully inhibited malate oxidation with TPP (Fig. 3*A*). In the presence of TPP, adding 25 μM KCN or 1 mM SHAM inhibited about 62 or 20 % of the state 3 rate, respectively. A combination of 25 μM KCN and 1 mM SHAM or opposite completely inhibited the residual rate (Fig. 3*B*,*C*). NADPH and NADH oxidations were very sensitive to KCN. These oxidations were completely inhibited by 100 μM KCN (Table 5), whereas succinate and malate oxidations were not.

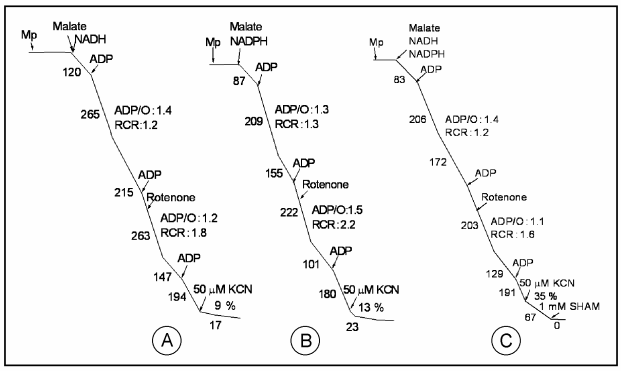

Fig. 4. Effect of KCN and SHAM in the presence of rotenone on the malate oxidation at pH 6.8 in the presence of 1 mM TPP with other substrates. Malate and NADH (*A*), malate and NADPH (*B*), and malate, NADH, and NADPH (*C*). Assay conditions were: 2.0 μM rotenone, 10 mM malate, 1 mM NADH, 1 mM NADPH, 0.16 mM ADP (first and second adding), and 0.32 mM ADP (last adding). Numbers along the trace refer to $\mu mol(O_2)\ g^{-1}(protein)\ min^{-1}$.

Concomitantly with the increase in the respiration rate, an increase in the cyanide resistant respiration of the multiple substrate oxidations was also observed. Addition of 100 μM KCN fully inhibited NADH and NADPH oxidations, whereas this concentration inhibited about 25 % of the state 3 rates in their simultaneous oxidation (Table 5). Cyanide resistant activity increased when succinate or malate at pH 6.8 with CoA and NAD^+ were oxidized concurrently with the second substrate (NADH) and became further increased in addition of third substrate (NADPH) (Table 5). This result was similar to that in soybean mitochondria (Day *et al*. 1988), indicating that Alt pathway was not fully engaged with only one substrate.

Malate oxidation at pH 6.8 with CoA and NAD^+ was inhibited by 20μM rotenone (by 12 % of the state 3 rates), indicating that *K. pinnata* possess a rotenone-sensitive complex I in the mitochondrial inner membrane (Fig. 2*A*). In the presence of rotenone, complex I was inhibited so that two proton-extrusion sites were utilized during malate oxidation, leading to the ADP/O ratio less than 2 instead of higher than 2 as in malate oxidation without rotenone. The effects of rotenone on the simultaneous oxidation of malate at pH 6.8 with TPP and other substrates were also investigated (Fig. 4).

Table 5. Effect of KCN on individual and simultaneous substrate oxidation by *K. pinnata* mitochondria. Assay conditions were 10 mM succinate in the presence of 10 mM ATP, 10 mM malate at pH 6.8 in the presence of 0.1 mM CoA, and 0.5 mM NAD^+, 1 mM NADH, 1 mM NADPH, 0.32 mM ADP, and 100 μM KCN. Typical results of four independent experiments.

Substrates	Rate of O_2 consumption $[\mu mol(O_2)\ g^{-1}(protein)\ min^{-1}]$		
	control	+KCN	[%]
NADH	155	0	0
NADPH	131	0	0
Succinate	246	69	28
Malate	102	27	26
Succinate + NADH	383	161	42
Succinate + NADPH	328	140	43
NADH + NADPH	219	55	25
Malate + NADH	190	88	46
Malate + NADPH	158	60	38
Malate + NADH + NADPH	206	104	50

Interestingly, the ADP/O ratios in these simultaneous oxidations were always less than two, whereas these rates were higher than two in the individual malate oxidation at pH 6.8 with TPP (Tables 3, 4). The explanation for this phenomenon is not clear, it seemed that in these multiple oxidations without rotenone, the complex I was simultaneously and competitively activated with other substrate dehydrogenases in which latter substrate dehydrogenases were superior, therefore complex I was less active; leading to the proton-extrusion complex I side was uncoupled. As a result, the effect of rotenone on the complex I side was not detected and the ADP/O ratios were observed less than two, and then adding rotenone did not make more important change in these ratios. Concomitantly, the increasing of CRR was also observed in these cases. The rates of these simultaneous oxidations in the presence of 50 μM KCN were higher than those of the individual malate oxidations at pH 6.8 with TPP (Figs. 3*A*,4).

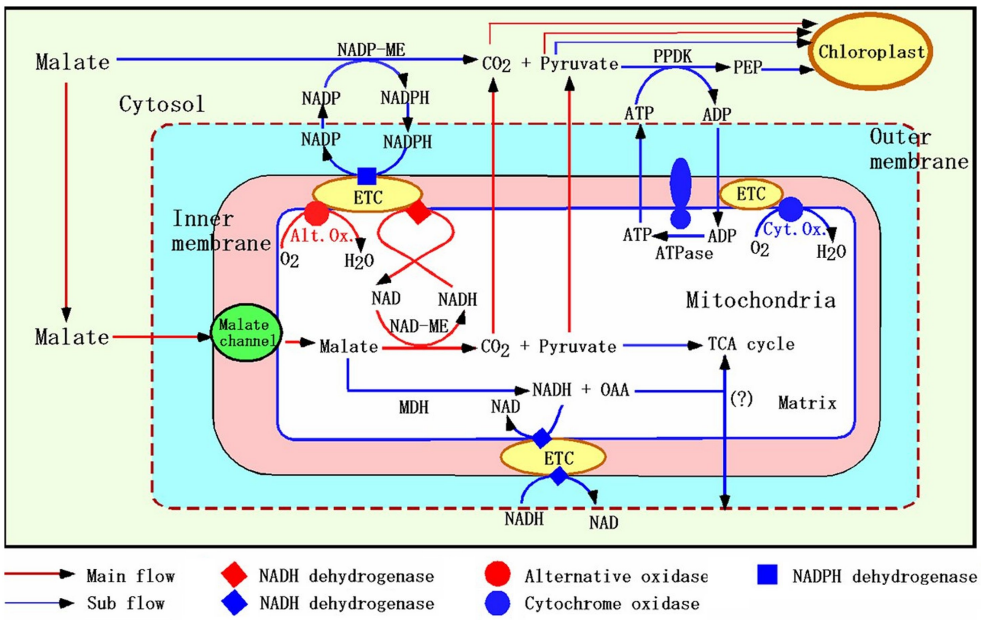

Fig. 5. Organization of malate-oxidizing systems in cytosol and mitochondria of *K. pinnata.* Alt.Ox.: alternative oxidase, Cyt.Ox.: cytochrome oxidase.

Discussion

Pyruvate orthophosphate dikinase (PPDK) accumulates in both the chloroplasts and cytosol in *K. pinnata* mesophyll cells; however, the role of cytosolic PPDK in the carbon metabolism is still unclear (Kondo *et al.* 1998). In *K. pinnata,* pyruvate produced from cytoplasmic malate decarboxylation, is transported into the chloroplasts to be phosphorylated to PEP by PPDK and the energy source of this process comes from photosynthesis (Kondo *et al.* 1998). If the role of cytosolic PPDK was the same with chloroplast PPDK, a question arises to where the energy source of this process will come? At mitochondrial levels, our results showed that *K. pinnata* mitochondria were able to easily oxidize external NADH and NADPH at significant rates (Table 3), suggesting that in CAM phase III, NADPH produced during malate oxidation *via* cytosolic NADP-ME would be oxidized by external NADPH dehydrogenase which was located outside the mitochondrial inner membrane. In our study, NADH and NADPH oxidations were more sensitive to KCN than that with succinate and malate oxidations (Table 5). This fits well to observation of Wiskich and Day (1982) with mitochondria of *K. daigremontiana* and cauliflower in which external NADH oxidation is largely cyanide-sensitive. These oxidations could produce proton gradient in concomitance with enough production of the ATP. *In vivo*, therefore, cytosol NAD(P)H oxidations would supply energy, ensuring that ATP/ADP ratio was maintained at high value in the cytosol, supplying enough energy courses which was possible to be reused for pyruvate phosphorylation in the cytosol to PEP by cytosolic PPDK.

Kalanchoë pinnata mitochondria possessed a large activity of NAD-ME and MDH (Table 2), supplying enough enzyme activities for mitochondrial malate metabolism. However under *in vivo* conditions, we did not know how these enzymes contribute to malate metabolism in *K. pinnata* mitochondria and which enzyme plays an important role in the process? Thus, we tried to investigate malate oxidation under various conditions, where ME or MDH or both of them operated and were stimulated. The data showed that *K. pinnata* mitochondria readily oxidized malate in all of investigated assay conditions (Table 3), suggesting that both ME and MDH enzymes were involved in the mitochondrial malate oxidation. Previous studies suggested that cytoplasmic pH may regulate malate decarboxylation by CAM mitochondria (Day 1980). At the beginning of the light phase of CAM plant, cytoplasmic pH was slightly alkaline (about 7.5). It dropped during the light phase (about 6.6 - 7.0), rather increased at midday (about 7.2) and recovered again in the late-day-to-early-dark phase (Hafke *et al.* 2001). A lower pH may lead to higher intramitochondrial substrate level in *K. daigremoniana* (Day 1980). From our study, in all investigated malate oxidations, the low respiration rates was observed at pH 7.6, where only MDH was activated, while the much higher rates was observed at pH 6.8, where ME was activated. These rates were strongly increased in the addition of TPP to malate

oxidation at pH 6.8, where both ME and pyruvate dehydrogenase were activated (Table 3). It was possible that similarly as other ME type CAM plants, in the phase III of *K. pinnata*, malate was released from vacuole to cytosol, decreasing cytosplasmic pH and this might fit to the high capacity of malate decarboxydation at low pH in these mitochondria. In the presence of TPP, pyruvate dehydrogenase was operating. Under the activation of this enzyme, the pyruvate produced from malate oxidation *via* ME was further metabolized to TCA cycle. This process produced NADH and oxidation of this NADH would increase the respiration rate. Based on all these data it was possible to postulate that ME played a main function in mitochondrial malate metabolism of *K. pinnata* in which malate was mainly oxidized *via* ME to produce pyruvate and CO_2 rather than via MDH to produce OAA.

In malate and succinate oxidations, KCN did not completely block total respiration rates and the CN-resistant oxygen consumption was affected by SHAM (Figs. 1, 2), indicating that *K. pinnata* mitochondria posses both Cyt pathway and Alt pathway in the ETC. However, the effective range of KCN with *K. pinnata* mitochondria was lower than 0.5 mM whereas this range were 1 mM with *K. blossfeldiana* mitochondria (Rustin and Queiroz-Claret 1985) suggesting that substrate oxidations by *K. pinnata* mitochondria was more sensitive to KCN than that with *K. blossfeldiana* mitochondria. It has been shown that the oxidation of malate *via* ME essentially proceeds through Alt pathway, whereas the oxidation of malate via MDH appears to be strongly linked to the Cyt pathway (Rustin *et al.* 1980). Our results indicated that in CAM phase III of *K. pinnata* mitochondria, malate oxidation at pH 6.8 in the presence of CoA and NAD^+ showed much higher Alt pathway capacity than that without CoA and NAD^+ (Table 5, Fig. 2*B*,*C*). This result suggest that there was a relationship between ME activity and Alt pathway during mitochondrial malate oxidation and in the phase III of *K. pinnata* mitochondria, Alt pathway appears to be more active where ME operated and was stimulated. This result fits to the previous observation of Tsuchiya *et al*. (2001) in which an increase in the Alt pathway capacity was observed during the phase III of *K. pinnata* leaf respiration. It agrees also with the suggestion of Nose and Takashi (2001) who suggested that the cyanide resistant respiration plays an important role during the light phase of ME-CAM.

In *K. pinnata* mitochondria, the multiple substrate oxidations lead to an increase in the respiration rate. This rate was higher than the individual rates but lower than the sum of individual rates. This result was similar to what has been described in cauliflower mitochondria (Day *et al*. 1976), *Iris* bulb mitochondria (Hemrika-Wagner *et al*. 1986), *Arum italicum* spadices (Tenreiro *et al*., 1992) and potato mitochondria (Arrabaca *et al*. 1992), in which presence of two or three substrates normally produced O_2 uptake rates far in excess of those obtained with the two or three substrates separately. The characteristic by the additive of the individual rates in the simultaneous oxidation of two or three substrates indicated that the oxygen uptake of *K. pinnata* mitochondria was not saturated with one substrate.

It has been observed also that the cooperative oxidation of two or three substrates enhanced Alt pathway capacity, suggesting that one of the Alt pathway functions in the mitochondria is to provide for noncompeting oxidation of two (or more) substrates by employing two (or several) dehydrogenases of the respiratory chain (Shugaev and Vyskrebentseva 1999). In our results, *K. pinnata* mitochondria also showed the same trend that was the simultaneous oxidation of the substrates generally enhancing the Alt pathway (Table 5, Fig. 4). A combination of succinate with NADH or NADPH not only dramatically enhanced respiration rate but also increased the Alt pathway capacity (Tables 4, 5). This trend was even clearer when three substrates, malate, NADPH and NADH were oxidized (Fig. 4*C*). In these cases, the respiration rate was not dramatically enhanced; however the capacity of Alt pathway was increased. These results indicated that Alt pathway was not fully engaged with just one substrate, suggesting that *K. pinnata* mitochondria employed two (or three) dehydrogenases in the respiratory chain and these dehydrogenases simultaneously functioned in the cooperative oxidation of two (or three) substrates.

Taking into account the results obtained with whole leaves (Kondo *et al*. 1998, Nose and Takashi 2001, Tsuchiya *et al*. 2001) together with those obtained with isolated mitochondria in our study, it is possible to suggest that the relationship of malate decarboxylation in cytosol and mitochondrial matrix of *K. pinnata* followed a system shown in Fig. 5. The malate, stored in the vacuole of *K. pinnata* during the night, was released to the cytoplasm, where it became a substrate for both cytosolic and mitochondrial ME during the day. The decarboxylation of malate in *K. pinnata* during the day was catalyzed by cytoplasmic NADP-ME, and mitochondrial MDH and NAD-ME. In cytosol, NADP-ME independently operated with the TCA cycle to produce CO_2 and pyruvate. Pyruvate was transported into the chloroplasts to be further phosphorylated to PEP by chloroplasts PPDK or was directly phosphorylated to PEP by cytosol PPDK (Fig. 5). Malate from cytosol could also enter into the mitochondrial matrix and to be mainly decarboxylated by NAD-ME to produce CO_2 and pyruvate. It is possible to suggest that there were two pyruvate metabolizing systems operating in tandem in *K. pinnata* mitochondria, depending on the pyruvate dehydrogenase activity. In the absence of TPP, the produced pyruvate was transported outside mitochondria and further phosphorylated to PEP by cytosolic or chloroplast PPDKs. In the presence of TPP, the pyruvate was further metabolized in the TCA cycle. In CAM phase

III of *K. pinnata*, cytoplasmic malate decarboxylation mainly via cytosolic NADP-ME would release NADPH and this NADPH was reduced by external NADPH dehydrogenases which were located on the outer membrane of mitochondria. This process might supply sufficient energy for cytosolic PPDK activity. Concomitantly, mitochondrial malate decarboxylation mainly via mitochondrial NAD-ME would release NADH. This process and further NADH metabolism would connect to Alt pathway, leading to an increase in the phase III Alt producing capacity.

References

Agius, S.C., Rammuson, A.G., Åkerlund, H.E., Møller, I.M.: Dynamic changes in the redox level of NAD in potato tuber mitochondria oxidizing malate. - In: Møller, I.M., Gardeström, P., Glimelius, K., Glaser, E. (ed.): Plant Mitochondria: From Gene to Function. Pp. 343-346. Backhuys, Leiden 1998.

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Arrabaca, J.D., Ribeiro-Lima, I.M.R., Tenreiro, A.M.: Interaction between the oxidation of external NADH and NADPH in plant mitochondria. - In: Lambers, H., Van der Plas, L.H.W. (ed.): Molecular, Biochemical and Physiological Aspects of Plant Respiration. Pp. 125-131. SPB Academic Publ., The Hague 1992.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Day, D.A.: Malate decarboxylation by *Kalanchoë daigremontiana* mitochondria and its role in Crassulacean acid metabolism. - Plant Physiol. **65**: 675-679, 1980.

Day, D.A., Moore, A.L., Dry, I.B, Wiskich, J.T, Azcon-Bieto, J.: Regulation of non-phosphorylating electron transport pathways in soybean cotyledon mitochondria and its implications for fat metabolism. - Plant Physiol. **86**: 1199-1204, 1988.

Day, D.A., Rayner, J.R., Wiskich, J.T.: Characteristics of external NADH oxidation by beetroot mitochondria. - Plant Physiol. **58**: 3-42, 1976.

Du, Y.C., Nose, A., Kawamitsu, Y., Murayama, S., Wasano, K., Uchida, Y.: An improved spectrophotometric determination of the activity of ribulose 1,5-bishosphate carboxylase.- Jap. J. Crop Sci. **65**: 714-721, 1996.

Estabrook, R.W.: Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. - Methods Enzymol. **10**: 41-45, 1967.

Hafke, J., B., Neff, R., Hütt, M., T., Lüttge, U., Thiel, G.: Day-to-night variations of cytoplasmic pH in a crassulacean acid metabolism plant. - Protoplasma **216**: 164-170, 2001.

Hemrika-Wagner, A.M., Gude, H., Marissen, N., Vander Plas, L.H.W., Verleur, J.D.: Compartmentation of alternative oxidase in plant mitochondria. - Plant Cell Physiol. **27**: 499-503, 1986.

Hong, H.T.K., Nose, A., Agarie, S.: Oxidation of substrate in Percoll-purified mitochondria isolated from *Kalanchoë daigremontiana*. - Bull. Fac. Agr. Saga Univ. **89**: 121-129, 2004.

Kondo, A., Nose, A., Ueno, O.: Leaf inner structure and immunogold localization of some key enzymes involved in carbon metabolism in CAM plants. - J. exp. Bot. **49**: 1953-1961, 1998.

Kondo, A., Nose, A., Yuasa, H., Ueno, O.: Species variation in the intracellular localization of pyruvate, Pi dikinase in leaves of Crassulacean-acid-metabolism plants: an immunogold electron-microscope study. - Planta **210**: 611-621, 2000.

Nose, A., Soda, Y., Sunami, A., Wasano K.: Effects of low oxygen concentration on CAM-type CO_2 exchanges in *Kalanchoë daigremontiana*. - Jap. J. Crop Sci. **68** (extra issue 1): 72-73, 1999.

Nose, A., Takashi, H.: Low oxygen effects on diurnal gas exchanges and malate contents in three CAM species: Is cyanide-insensitive respiration indispensable to malic enzyme (ME)-type CAM? - In: Holtum, J.A.M. (ed.): Proceedings of the 3rd International Congress on Crassulacean Acid Metabolism. P. 18. James Cook University School of Tropical Biology, Townsville 2001.

Pastore, D., Trono, D., Laus, M.N., Fonzo, N.D., Passarella, S.: Alternative oxidase in durum wheat mitochondria. Activation by pyruvate, hydroxypyruvate and glyoxylate and physiological role. - Plant Cell Physiol. **42**: 1373-1382, 2001.

Robinson, S.A., Ribas-Carbo, D.Y.M., Giles, L.C., Osmond, B., Siedow, J.N., Berry, J.A.: Measurements of the engagement of cyanide-respiration in the Crassulacean acid metabolism *Kalanchoë daigremontiana* with the use of on-line oxygen isotope discrimination. - Plant Physiol. **100**: 1087-1091, 1992.

Rustin, P., Moreau, F., Lance, C.: Malate oxidation in plant mitochondria via malic enzyme and the cyanide-insensitive electron transport pathway - Plant Physiol. **66**: 457-462, 1980.

Rustin, P., Queiroz-Claret, C.: Changes in oxidative properties of *Kalanchoë blossfeldiana* leaf mitochondria during development of Crassulacean acid metabolism. - Planta **164**: 415-422, 1985.

Shaheen, A., Nose, A., Wasano, K.: *In vivo* properties of phosphoenolpyruvate carboxylase in Crassualcean acid metabolism plants. Is pineapple CAM not regulated by PEPC phosphorylation? - Environ. Control Biol. **40**: 343-354, 2002.

Shugaev, A.G., Vyskrebentseva, E.I.: Cooperative substrate oxidation by mitochondria from Castor been hypocotyls. - Russ. J. Plant Physiol. **48**: 165-170, 1999.

Tenreiro, A.M., Vaz-Pinto, V., Arrabaca, J.D.: Simultaneous oxidation of substrates and the effects of specific inhibitors in mitochondria from *Arum italicum* spadices. - In: Lambers, H., Van der Plas, L.H.W. (ed.): Molecular, Biochemical and Physiological Aspects of Plant Respiration. Pp. 117-123. SPB Academic Publ., The Hague 1992.

Tsuchiya, T., Sasaki, H., Uehara, N, Nose, A.: Effects of low oxygen conditions on cyanide resistant respiration in two CAM plants (*Kalanchoë pinnata and Ananas comosus*). - Jap. J. Crop Sci. **70** (extra issue 2): 139-140, 2001.

Wiskich, J.T., Day, D.A.: Malate oxidation, rotenone-resistance and alternative path activity in plant mitohondria. - Plant Physiol. **70**: 959-964, 1982.