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Isolation and Oxidative Properties of Intact Mitochondria from the Leaves of *Sedum praealtum*

A CRASSULACEAN ACID METABOLISM PLANT[1]

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ABSTRACT

A procedure is described for preparing intact mitochondria from leaves of *Sedum praealtum* D.C., a plant showing Crassulacean acid metabolism. These mitochondria oxidized malate, pyruvate, α-ketoglutarate, succinate, NADH, NADPH, and isocitrate with good respiratory control and ADP/O ratios better than those observed in mitochondria from other photosynthetic tissues.

Malate oxidation was very resistant to inhibition by rotenone. Glycine oxidation was very slow with poor respiratory control and was resistant to rotenone inhibition. Antimycin A completely inhibited the oxidation of both NADH and NADPH. The oxidation of isocitrate, malate, succinate, and α-ketoglutarate was partially inhibited by antimycin A and cyanide. Overall rates of substrate oxidation were slow on a protein basis, but purification of the mitochondrial preparations on a linear sucrose gradient removed a large amount of nonmitochondrial protein. The original mitochondrial preparations contained little glycolate oxidase activity, and most of this activity was removed by the sucrose gradient.

Leaf mitochondria from plants exhibiting different pathways of carbon assimilation (C_3, C_4, and CAM[2]) are of interest in relation to photorespiratory metabolism (mitochondrial decarboxylation of glycine) and capacity for malate decarboxylation (through NAD-malic enzyme). A prerequisite for comparative studies is the isolation of functional mitochondria from species representing the three groups.

Although tightly coupled mitochondria have been isolated from etiolated and storage tissues (12, 24), there have been few instances of the isolation of such mitochondria from the leaves of higher plants. Mitochondrial preparations from spinach leaves (C_3) (15) and from tobacco (C_3) (2, 3) exhibited low rates of glycine decarboxylation and no respiratory control. Woo and Osmond (28) also isolated spinach leaf mitochondria but these exhibited poor respiratory control and low ADP/O ratios. More recently, Douce and associates (10, 21) obtained good spinach leaf mitochondrial preparations which oxidized a variety of substrates with good respiratory control and ADP/O ratios comparable to those reported for mitochondria from other plant tissues. These preparations were still contaminated by large amounts of Chl (approximately 40% of the protein in the mitochondrial pellet was of chloroplast origin) and peroxisomes, as indicated in electron micrographs and by high levels of glycolate oxidase activity (10).

Woo and Osmond (29) reported the isolation of bundle sheath mitochondria from NAD-malic enzyme type C_4 plants which decarboxylated glycine, although no oxidative data were given.

The isolation of functional chloroplasts and mitochondria from CAM plants has two major problems in that large amounts of phenolic substances are normally found in succulent leaves and high concentrations of organic acids are associated with CAM plants.

In this paper we describe the isolation of tightly coupled mitochondria from the leaves of the CAM plant *Sedum praealtum* with the help of high concentrations of buffer and PVP-40 in the grinding medium and the use of leaf material detached from plants several hours into the light period when the level of organic acids had dropped. The mitochondrial preparations contained little glycolate oxidase activity (a peroxisomal marker) or Chl.

MATERIALS AND METHODS

Chemicals. All biochemicals were obtained from Sigma Chemical Corporation, St. Louis, Mo.

Plant Material. *S. praealtum* D.C. plants were grown under a 10-h photoperiod (1 h one-half light; 8 h full light; 1 h one-half light; 14 h dark) and a 30/15 C day/night temperature regime. The quantum flux density at soil level under full light was about 600 μE m^{-2} s^{-1}. The plants were watered approximately every other day and fertilized every 2 weeks. Young but fully expanded leaves were chosen for mitochondrial isolation (7th to 14th leaf from apex). Isolations were begun in late morning about 3 h into the full light interval.

Preparation of Mitochondria. Approximately 55 g of *S. praealtum* D.C. leaves were cut perpendicular to the leaf axis in slices about 1 cm thick with a sharp razor blade. The leaf slices were vacuum-infiltrated in 250 ml of ice-cold grinding medium containing 0.15 M mannitol, 0.25 M Tricine buffer (pH 8.0), 4 mM cysteine, 5 mM EGTA, 2% PVP-40, and 0.2% BSA. The leaf slices were homogenized for 3 to 4 s with a Polytron PT 35K probe (setting 7). The homogenate was squeezed through four layers of cheesecloth and centrifuged at 1,200*g* for 4 min to remove chloroplasts and cell debris. The supernatant was then centrifuged at 10,000*g* for 10 min. The pellets were resuspended in approximately 40 ml of wash medium containing 0.4 M mannitol, 25 mM Tes (pH 7.6), and 0.1% BSA with a Teflon/glass homogenizer and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in approximately 4 ml of wash medium (washed mitochondria).

Mitochondria (4.5 ml) were purified by centrifugation (25,000 rpm [82,000*g*] in a Beckman L5-50 ultracentrifuge, SW 27.1 rotor, for 2 h) on a linear sucrose gradient (20–60% [w/v]). After

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[2] Abbreviations: CAM: Crassulacean acid metabolism; SHAM: salicylhydroxamic acid; TMPD, tetramethyl-*p*-phenyldiamine; EGTA: ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Table I. *Comparison of Chl Content and Activities of Cyt c Oxidase and Glycolate Oxidase in Washed and Purified Mitochondrial Preparations*

Protein and Chl were estimated, and the activities of Cyt *c* oxidase and glycolate oxidase determined as described under "Materials and Methods."

Preparation	Volume	Protein	Chl	Chl as % of Original Homogenate	Cyt *c* oxidase	Glycolate Oxidase	Cyt *c* Oxidase/ Glycolate Oxidase	Cyt *c* Oxidase/μg Chl
	ml	*mg/ml*	*μg/ml*		*nmol O_2/min·mg protein*			
Washed mitochondria	4.5	16.1	40.0	1.1	116	4.7	25[1]	0.18[2]
Purified mitochondria	1.0	4.9	87.5	0.6	601	1.8	334	1.40

[1] Original leaf homogenate value: 1.29.

[2] Original leaf homogenate value: 0.012.

centrifugation, considerable proteinaceous material was found at the bottom of the ultracentrifuge tube. The mitochondria were located in a single band with an average density of approximately 1.17 g cm^{-3}. They were removed with a large bore syringe, carefully diluted to 0.4 M sucrose by dropwise addition (with stirring) of 10 mM Tes buffer (pH 7.2) + 0.1% BSA, and pelleted at 10,000*g* for 10 min. Final resuspension was in 1 ml wash medium (see above).

Mitochondrial Assays. Mitochondrial respiration was measured polarographically using a Rank O_2 electrode (Rank Bros., Bottisham, Cambridge, U.K.) at 25 C in a standard reaction medium of 0.4 M mannitol, 25 mM Tes (pH 7.2), 5 mM $MgSO_4$, 5 mM KH_2PO_4, and 0.1% BSA, defatted (17). The O_2 concentration in air-saturated medium was taken as 240 μM (11).

Glycolate oxidase was assayed as described by Jackson *et al.* (13). Cyt oxidase was measured polarographically in 1 ml of standard reaction medium containing 0.05 mM Cyt *c*, 10 mM ascorbate, and 5 mM TMPD.

Protein was measured by the method of Lowry *et al.* (19) and ADP/O ratios according to Chance and Williams (5). Chl was determined in 80% acetone by the method of Arnon (1). The amount of mitochondrial protein was corrected for the contribution of broken thylakoids by assuming a protein to Chl ratio of 7 in broken thylakoids (18).

RESULTS AND DISCUSSION

Table I compares the degree of contamination by Chl and peroxisomes of conventional washed mitochondrial preparations and purified (by centrifugation through a linear sucrose gradient) preparations obtained from the leaves of *S. praealtum*. The washed mitochondrial preparation contained little Chl (approximately 1.7% of the protein in the preparation was of Chl origin), low levels of both Cyt *c* oxidase activity (on a protein basis) and glycolate oxidase activity (compare with 82 nmol O_2 min^{-1} mg^{-1} protein in spinach preparations; 10). In comparing the leaf homogenate and the washed mitochondrial preparation, there was an approximate 20-fold increase in the ratio of Cyt *c* oxidase activity to glycolate oxidase activity and a 15-fold increase in the activity of Cyt *c* oxidase/μg Chl indicating substantial removal of chloroplasts and peroxisomes from the mitochondrial fraction. Gradient-purified mitochondrial preparations contained more Chl than the washed mitochondrial preparation, although still only constituting 12.5% of total protein, Cyt *c* oxidase activity was higher and glycolate oxidase activity lower. The removal of peroxisomal and other nonmitochondrial protein by the gradient is reflected in the change of the ratio of Cyt *c* oxidase activity to glycolate oxidase activity.

The removal of nonmitochondrial protein from the washed preparation by the sucrose gradient is revealed in Table II where the rates of oxidation of NADH, NADPH, and malate are all higher in the purified preparation than in the washed preparations (all rates on a protein basis). Some degree of respiratory control was lost through purification for all three substrates, presumably because of the time involved in centrifugation and the osmotic stress the organelles suffered. Day and Hanson (7) reported a similar partial loss of control in gradient purified corn mitochondria compared with "cushion" mitochondria (*i.e.* mitochondria centrifuged through a 0.6 M sucrose cushion in the second high speed centrifugation).

Malate, NADH, NADPH, and succinate are all readily oxidized by *S. praealtum* leaf mitochondria (washed) (Fig. 1). Respiratory control values and ADP/O ratios for malate, NADH, and succinate are all comparable with values obtained with mitochondria isolated from both nongreen and green tissue (10, 12).

The oxidation of malate, succinate, and NADH by *S. praealtum* leaf mitochondria suggests that the electron transfer chain, the presence of an NADH dehydrogenase located on the outside of the inner mitochondrial membrane, and the sites of energy conservation are all similar to those previously reported for other plant mitochondria.

ADP/O ratios observed with malate are greater than 2 (Fig. 1), indicating that all three phosphorylation sites are utilized. NADH, NADPH, and succinate oxidation all gave ADP/O ratios of less than 2 (1.2–1.7) indicating that only two coupling sites are utilized. These values are similar to those observed in tightly coupled mitochondria isolated from nongreen and green tissue (10, 12, 16).

It is particularly interesting that *S. praealtum* leaf mitochondria oxidize NADPH (Fig. 1). Koeppe and Miller (16) showed that corn mitochondria oxidize NADPH at approximately half the rate they oxidize NADH. *S. praealtum* leaf mitochondria, however, oxidized NADPH and NADH at similar rates (Fig. 1). The respiratory control values obtained with NADPH as substrate were good (Table II and Fig. 1), whereas corn mitochondria exhibited control values a great deal lower (16).

The oxidation of NADH and NADPH by *S. praealtum* leaf mitochondria was totally resistant to inhibition by rotenone (data not shown), which like piericidin A, inhibits the oxidation of endogenous NADH but has no effect on the oxidation of exogenous NADH (6, 12, 24). The oxidation of NADH and NADPH by these mitochondria was totally inhibited by the addition of antimycin A (Fig. 1). NADH and NADPH oxidation by corn mitochondria revealed the same resistance to rotenone inhibition and sensitivity to antimycin A inhibition (16).

From their studies, Koeppe and Miller (16) concluded that corn mitochondria possess a flavoprotein located on the outer face of the inner mitochondrial membrane, specific for the oxidation of NADPH. It appears that *S. praealtum* leaf mitochondria also

Table II. *O_2 Consumption by Washed and Purified S. praealtum Leaf Mitochondria*

Oxygen uptake was measured as described under "Materials and Methods"; state 3 and state 4 rates are expressed as nmol $O_2 min^{-1} mg^{-1}$ protein with 1 mM NADH, 1 mM NADPH, or 30 mM malate as substrate.

State 3 respiration refers to the rate of O_2 uptake in the presence of ADP, and state 4 refers to the rate upon depletion of ADP, *i.e.* when all ADP has been phosphorylated (5). RCR = respiratory control ratio, Mw = washed mitochondria, Mp = gradient purified mitochondria.

Substrate	Mitochondria	State 3	State 4	RCR
NADH	Mw	18.9	4.3	4.4
	Mp	177.0	47.7	3.7
NADPH	Mw	20.3	3.3	6.2
	Mp	180.3	43.0	4.2
Malate	Mw	20.3	4.2	5.8
	Mp	113.7	30.3	3.8

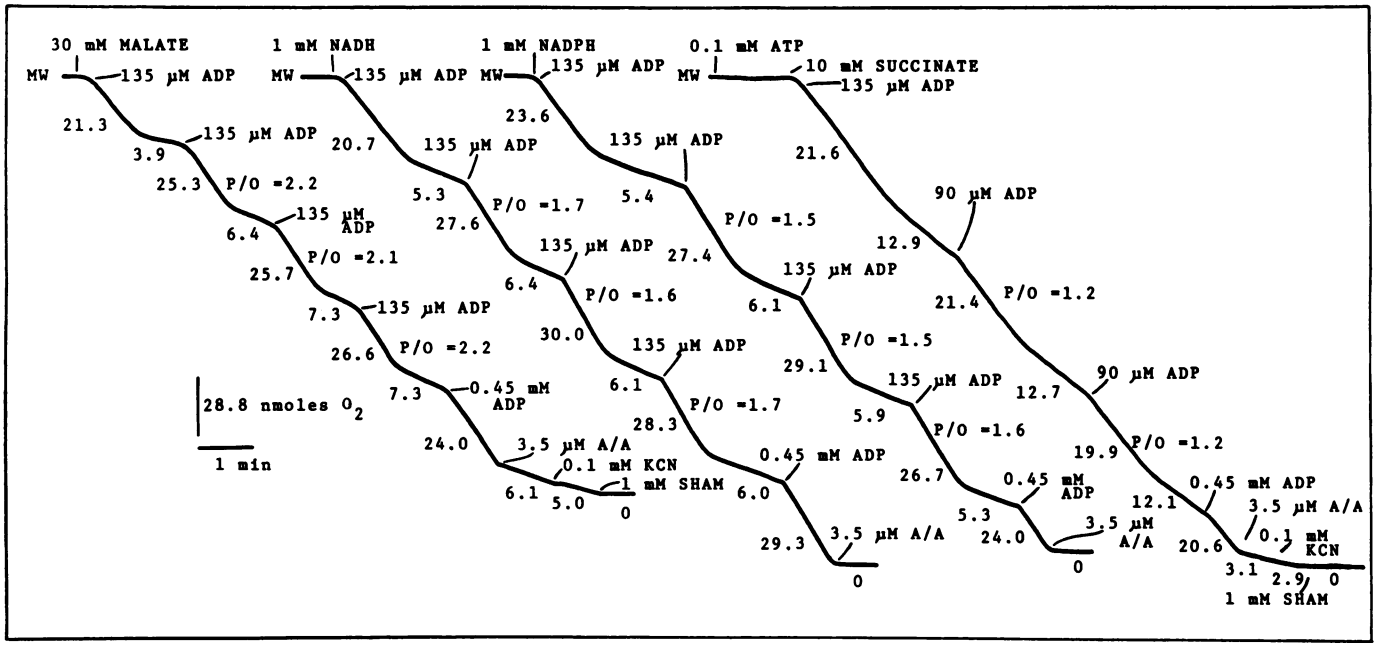

FIG. 1. Effect of antimycin A, KCN, and SHAM on malate, NADH, NADPH, and succinate oxidation by *S. praealtum* leaf mitochondria. Concentrations given are final concentrations in reaction medium. Numbers on traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mw: washed mitochondria; A/A: antimycin A.

possess a NADPH dehydrogenase (it should be noted that the oxidation of NADPH reported here was not due to microsomal contamination, since purification of conventional washed mitochondrial preparations did not result in any loss of NADPH oxidase activity relative to NADH oxidase activity).

Although the oxidation of both NADH and NADPH by *S. praealtum* leaf mitochondria was completely inhibited by antimycin, both malate and succinate oxidation were only partially inhibited by antimycin and cyanide (Fig. 1). Addition of SHAM, an inhibitor of the alternative respiratory pathway in plant mitochondria (25), strongly inhibited this residual respiration.

S. praealtum leaf mitochondria oxidized other Krebs cycle intermediates such as α-ketoglutarate, pyruvate, and isocitrate (Fig. 2). Rates of oxidation of these intermediates were lower than the rates of oxidation of both malate and NADH (see Fig. 1). Spinach leaf mitochondria also oxidized α-ketoglutarate and pyruvate at lower rates than they oxidized malate (10).

The effects of rotenone and NAD on malate oxidation by *S. praealtum* leaf mitochondria are shown in Figure 3. Retenone only partially inhibited malate oxidation and addition of NAD to the rotenone-resistant rate did not stimulate O_2 uptake (*cf.* 4, 9). Malate oxidation by *S. praealtum* leaf mitochondria in the presence of rotenone exhibited ADP/O ratios of 1.7 (*i.e.* less than 2; Fig. 3), indicating the presence of a nonphosphorylating rotenone bypass similar to that reported by Brunton and Palmer (4) in wheat mitochondria.

Previous results with spinach mitochondria (10) revealed that the absence of such a substantial rotenone-resistant bypass and may be indicative of inherent functional differences between CAM and C_3 leaf mitochondria.

The fact that *S. praealtum* leaf mitochondria possess a substantial rotenone-resistant bypass for the oxidation of endogenous NADH (Fig. 3) might explain the slightly low ADP/O ratio obtained with malate (Fig. 1, compare with spinach mitochondria; 10), since the bypass may function even in the absence of rotenone.

Neuberger and Douce (22) reported that potato mitochondria possess a NAD translocator on the inner membrane. Translocation of NAD into the mitochondrial matrix results in a stimulation of the NAD-malic enzyme located there (20). The oxidation of malate by *S. praealtum* leaf mitochondria does not appear to respond in the same way to exogenous NAD addition.

Glycine oxidation by *S. praealtum* leaf mitochondria was extremely slow (6 nmol O_2 min^{-1} mg^{-1} protein compared to 25 nmol O_2 min^{-1} mg^{-1} protein with malate as substrate with the same washed mitochondria preparation). Purified mitochondrial preparations exhibited no detectable glycine oxidation, presumably because the glycine decarboxylase enzyme is very labile, becoming rapidly inactive in dilute solution (21) and the purification procedure was lengthy. It may be that we obtained such low rates of glycine oxidation either because our preparations inherently lacked the large scale capacity to oxidize this substrate or they lost the ability with time through enzyme deactivation. The glycine oxidation which could be measured in washed mitochondrial preparations of *S. praealtum* exhibited respiratory control values of 1.4 to 1.6, an ADP/O ratio of 2.3 (indicative of three phosphorylating sites), and was resistant to inhibition by rotenone and partially resistant to inhibition by antimycin and cyanide. Douce *et al.* (10) also reported glycine oxidation by spinach leaf mitochondria coupled to three sites of phosphorylation. The rate of glycine oxidation in the spinach preparations was about the same as the rate of malate oxidation, whereas glycine oxidation by *S. praealtum* mitochondria was a great deal slower than malate oxidation.

Von Willert and Schwöbel (27) have reported a partial characterization of the respiratory properties of mitochondria isolated from the leaves of *Mesembryanthemum crystallinum* plants in the CAM mode. These mitochondria did not oxidize exogenous NADH, were poorly coupled with malate/glutamate and α-ketoglutarate as substrate, and exhibited lower than theoretical ADP/O ratios. The authors concluded that their mitochondrial preparations were contaminated by ATPases inasmuch as the addition of EDTA to the reaction medium improved the ADP/O ratios, and were partially uncoupled because the addition of an uncoupler only stimulated succinate oxidation by 45% whereas maize root mitochondria were stimulated by more than 200%.

Conclusions

These results indicate that mitochondria isolated from the leaves of *S. praealtum* oxidize Krebs cycle intermediates with good

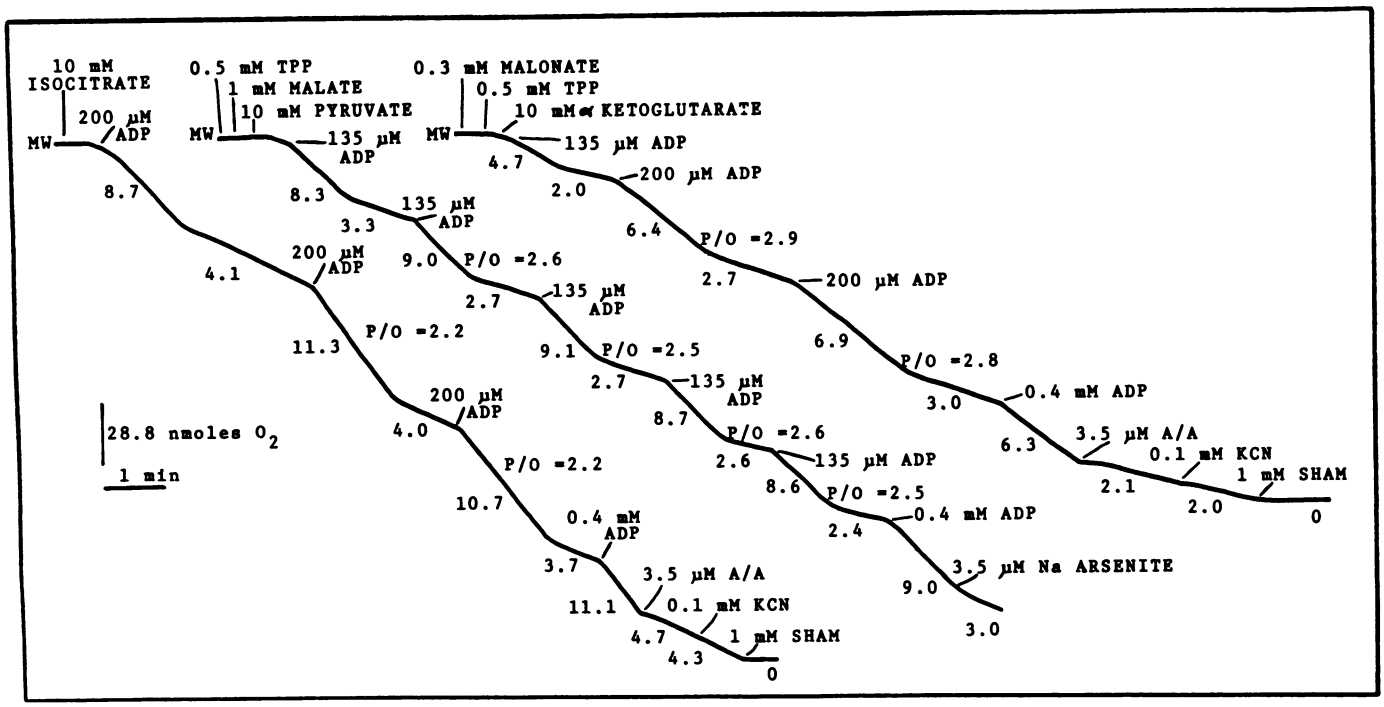

FIG. 2. Oxidation of isocitrate, pyruvate, and α-ketoglutarate by *S. praealtum* leaf mitochondria. Concentrations given are final concentrations in the reaction medium. Numbers on traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mw: washed mitochondria; A/A: antimycin A.

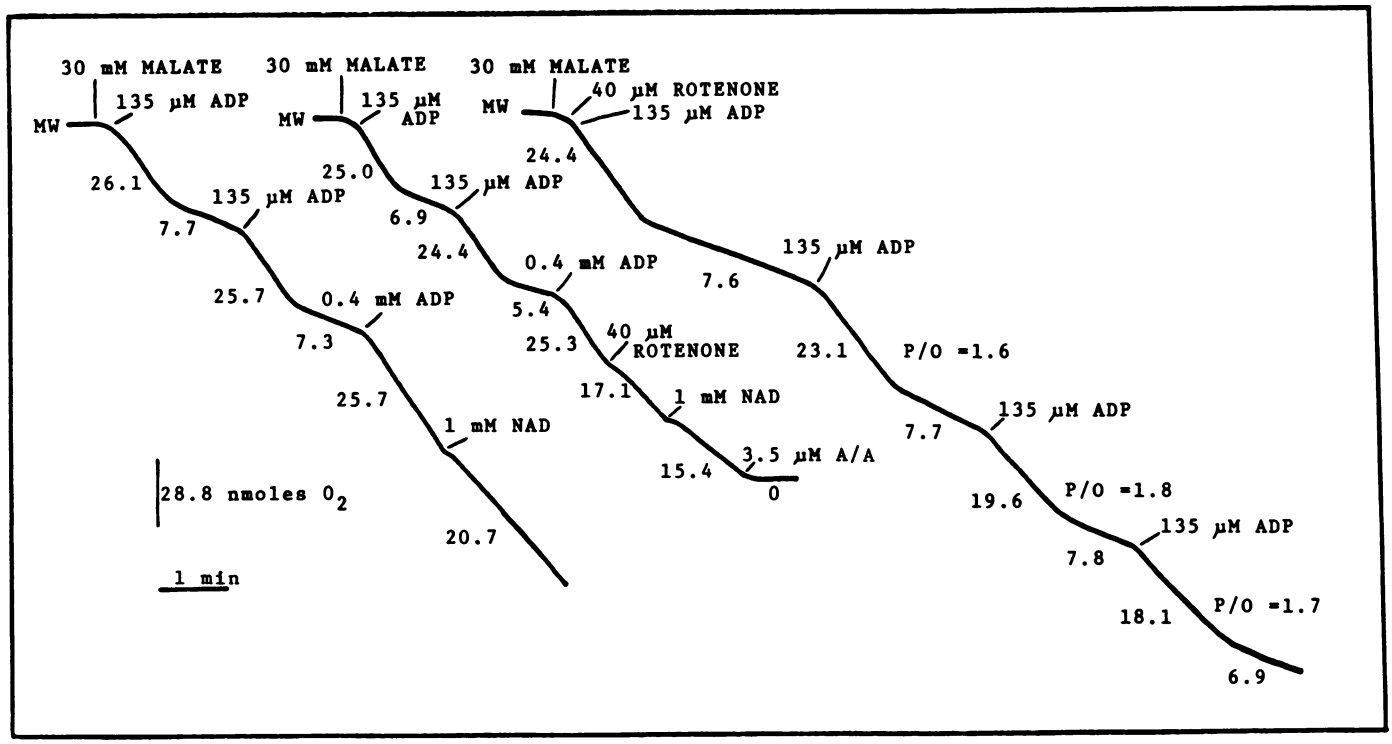

FIG. 3. Effect of rotenone and NAD on malate oxidation by *S. praealtum* leaf mitochondria. Concentrations given are final concentrations in reaction medium. Numbers on traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mw: washed mitochondria; A/A: antimycin A.

respiratory control and ADP/O ratios comparable to those observed with mitochondria from nongreen and green tissue (10, 12, 27).

S. praealtum leaf mitochondria possess a substantial rotenone-resistant bypass for the oxidation of endogenous NADH which is nonphosphorylating. This is in contrast to the position in spinach mitochondria where Douce *et al.* (10) found that glycine oxidation was inhibited 75% by the addition of rotenone. The bypass activity in spinach mitochondria was also nonphosphorylating.

S. praealtum leaf mitochondria oxidize NADPH with good respiratory control and ADP/O ratios similar to those obtained with NADH as substrate. *S. praealtum* possesses an NADP-malic enzyme apparently located in the cytoplasm (26) and the NADPH produced by this enzyme must be recycled during deacidification. Some of this NADPH may be oxidized by leaf mitochondria.

Spalding *et al.* found that NAD-malic enzyme activity is asso-

ciated with the mitochondria of *S. praealtum*. It was proposed that some malate is decarboxylated in the mitochondrion by malic enzyme and the pyruvate so formed is transported out of the organelle and used during a reversal of glycolysis in the chloroplast and cytoplasm rather than being metabolized to CO_2 and ATP within the mitochondria. The rate of pyruvate oxidation is low in *S. praealtum* leaf mitochondria (Fig. 2), as it is in C_3 plants (10). This may be due to a limitation of pyruvate transport across the inner mitochondrial membrane, as has been reported for corn mitochondria (8), or the tricarboxylic acid cycle may be unable to accommodate the high flux of carbon involved (23). Some CAM plants show O_2 inhibition of photosynthesis during phase 4 of gas exchange (late afternoon, stomata open) (23). There is also ultrastructural evidence of leaf peroxisomes (14) and evidence for a glycine decarboxylating system in CAM leaf mitochondria (K. C. Woo, unpublished). The capacity of isolated mitochondria from *S. praealtum* to respire glycine in the presence of ADP is low, although the *in vivo* capacity of the glycolate pathway in this species is uncertain.

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