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Isolation of Mitochondria from Leaf Tissue of *Panicum miliaceum*, a NAD-Malic Enzyme Type C_4 Plant[1]

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ABSTRACT

A mechanical isolation procedure was developed to study the respiratory properties of mitochondria from the mesophyll and bundle sheath tissue of *Panicum miliaceum*, a NAD-malic enzyme C_4 plant. A mesophyll fraction and a bundle sheath fraction were obtained from young leaves by differential mechanical treatment. The purity of both fractions was about 80%, based on analysis of the cross-contamination of ribulose bisphosphate carboxylase activity and phosphoenolpyruvate carboxylase activity.

Mitochondria were isolated from the two fractions by differential centrifugation and Percoll density gradient centrifugation. The enrichment of mitochondria relative to chloroplast material was about 75-fold in both preparations.

Both types of mitochondria oxidized NADH and succinate with respiratory control. Malate oxidation in mesophyll mitochondria was sensitive to KCN and showed good respiratory control. In bundle sheath mitochondria, malate oxidation was largely insensitive to KCN and showed no respiratory control. The oxidation was strongly inhibited by salicylhydroxamic acid, showing that the alternative oxidase was involved. The bundle sheath mitochondria of this type of C_4 species contribute to C_4 photosynthesis through decarboxylation of malate. Malate oxidation linked to an uncoupled, alternative pathway may allow decarboxylation to proceed without the restraints which might occur via coupled electron flow through the cytochrome chain.

In recent years, the role of mitochondria in the metabolism of photosynthetic tissues has gained increasing interest. In 1977 Douce *et al.* (5) described a method to isolate functionally intact mitochondria from the C_3 plant, spinach. Since then, several methods have been described to purify spinach leaf mitochondria, including phase partition (2, 10), Percoll density gradient centrifugation (2, 13), and use of protoplasts (9, 20). Mitochondria from the CAM-plant *Sedum praeltum* have been successfully purified using sucrose density gradient centrifugation (1).

A particular difficulty in the study of C_4 plant mitochondria is that two distinctly different cell types, mesophyll and bundle sheath cells, exist within the plant. The two cell types have different metabolic functions and the properties of the mitochondria in the two cell types are also different. Therefore, mitochondria must be isolated from both of these cell types with sufficiently low cross-contamination.

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In C_4 plants, CO_2 is fixed initially by PEP[2] carboxylase in the mesophyll cells, with subsequent synthesis of malate and aspartate. These C_4 acids are transferred into the bundle sheath cells where they are decarboxylated, supplying CO_2 to the Calvin cycle via RuBP carboxylase. The decarboxylation phase of the C_4 cycle can proceed via three different routes, giving rise to the subdivision of C_4 plants into NADP-malic enzyme type, PEP carboxykinase type, and NAD-malic enzyme type, depending on their main decarboxylating enzyme (6). In NAD-malic enzyme type C_4 plants, the decarboxylating enzyme is NAD-malic enzyme, which is found in high activity in the mitochondria of bundle sheath cells (14). Thus, in this C_4 type, the mitochondria are directly involved in the carbon fixation cycle.

In this paper we describe a procedure to purify mitochondria from a mesophyll fraction and a bundle sheath fraction of *Panicum miliaceum*, a NAD-malic enzyme type C_4 plant. We also compare the properties of the two types of mitochondria.

MATERIALS AND METHODS

Plant Material. *Panicum miliaceum* was grown in a soil mixture in a growth chamber at 27°C/20°C day/night temperature. The photosynthetic photon flux density was 400 $\mu E\ m^{-2}\ s^{-1}$ with a 14-h/10-h light/dark period. Mitochondria were isolated from about 2.5 week-old plants (leaves about 10–15 cm in length).

Preparation of Mesophyll and Bundle Sheath Fractions. Using a sharp razor blade, 50 g of leaves were cut perpendicular to the veins into 2- to 3-mm pieces. The leaf pieces were washed in preparation medium and filtered, then supplied with 200 ml of preparation medium (0.3 M sucrose, 25 mM Hepes, 1 mM EDTA, 4 mM cysteine, 0.2% BSA, pH 7.8), and homogenized in a Polytron homogenizer at setting 7 for 4 s. The homogenate was filtered through a plastic strainer (approximately 1 mm^2 holes) and an 80-μm nylon net, and the filtrate was taken as the mesophyll fraction.

The remaining material in the strainer was divided in two parts, each supplied with about 200 ml preparation medium and each blended with the Polytron homogenizer for 60 s at setting 6 and 30 s at setting 8. The homogenate was filtered through the plastic strainer and a 211-μm net. The material collected on the filter was pooled, blended again with 200 ml medium for 60 s at setting 6 and 15 s at setting 8, filtered through the strainer and 211-μm net, and washed with preparation medium. Examination with a light microscope showed that most of the strands collected on the filters were free of mesophyll cells following this treatment.

The bundle sheath extract was obtained by grinding the bundle sheath strands in a mortar with 25 ml preparation medium (with 2% BSA) for 40 s; 80 ml preparation medium were added and the slurry was filtered through a strainer and 80-μm net. The filtrate was used as the bundle sheath fraction. As described earlier, the

[2] Abbreviations: PEP, phosphoenolpyruvate; RuBP, ribulose bisphosphate; SHAM, salicylhydroxamic acid.

residue in the strainer was homogenized two more times to increase the yield of mitochondria.

Isolation of Mitochondria. The filtrate from the mesophyll fraction and the bundle sheath fraction was centrifuged at 5000*g* for 1.75 min. Mitochondria were collected from the supernatant by a centrifugation at 27,000*g* for 2.5 min. The mitochondrial pellet was suspended in 0.25 M sucrose using a glass homogenizer and diluted in a medium to give the final composition of 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM Hepes, 0.1% BSA, pH 7.2. The suspension was layered on top of a discontinuous Percoll gradient in a 35-ml centrifuge tube. The composition of the gradient was 5 ml 60% (v/v) Percoll, 10 ml 20% (v/v) Percoll, and 5 ml 15% (v/v) Percoll for bundle sheath mitochondria. For meosphyll mitochondria, the middle layer was 22% (v/v) Percoll, which removed considerably more Chl than a 20% (v/v) Percoll layer. In addition to Percoll, the gradient contained 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM Hepes, and 0.1% BSA, pH 7.2. The gradient was centrifuged at $10{,}000g_{max}$ for 30 min in a Beckman L8-70 ultracentrifuge using a 70 Ti rotor. The mitochondria were then collected from the 20% (v/v)/60% (v/v) and 22% (v/v)/60% (v/v) interfaces, respectively, by puncturing the tube with an injection needle. The mitochondrial fraction was diluted 10 to 15 times with preparation medium and centrifuged at 13,000*g* for 10 min. The (loose) pellets were suspended in preparation medium by shaking the tubes, and then centrifuged at 13,000*g* for 10 min. The pellets were resuspended in 0.3 M sucrose and after withdrawal of a sample for protein determination, supplied with medium to the final composition of 0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM K-phosphate, and 0.1% BSA, pH 7.2 (respiration medium).

Enzyme Assays. For RuBP carboxylase determinations, samples were solubilized in 1% Triton X-100, activated, and assayed essentially according to Lorimer *et al.* (16). Final composition of the assay mixture (0.5 ml) was 100 mM Tris-HCl (pH 8.2), 5 mM DTT, 20 mM $MgCl_2$, and 9.6 mM $NaH^{14}CO_3$ (about 2 Ci mol^{-1}); the reaction was started by adding 0.5 mM RuBP and stopped after 90 s by adding 100 μl of 2 N HCl.

PEP carboxylase was assayed at 340 nm by a coupled spectrophotometric method with malate dehydrogenase. Assay composition was 5 mM $MgCl_2$, 1 mM DTT, 10 mM $NaHCO_3$, 2 mM PEP, 0.2 mM NADH, 50 mM Tris-HCl, pH 8.5, and 1.5 units/ml malate dehydrogenase.

Cyt *c* oxidase was assayed in 10 mM of K-phosphate buffer, pH 7.0. Before assay, the sample was treated with 0.04% Triton X-100 in the cuvette for 0.5 to 1 min and the reaction was initiated by the addition of 50 μM reduced Cyt *c*. The initial rate of decrease of the *A* at 550 nm was used to calculate the activity.

NAD-malic enzyme was assayed essentially according to Hatch *et al.* (12) in 25 mM Tes-KOH, pH 7.2, 5 mM malate, 2 mM NAD^+, 5 mM DTT, 0.2 mM EDTA, 25 μM NADH, 1 unit/ml malate dehydrogenase, 4 mM $MnSO_4$, and 0.15 mM CoA with 0.04% Triton X-100. Higher activity was obtained by adding 0.05% (w/v) Triton X-100 to the assay than by solubilizing preparations with 0.5% (w/v) Triton X-100 and desalting on a G-25 column.

Succinate/Cyt *c* oxidoreductase was used to determine intactness as described earlier (10).

Respiration. Respiration was measured in 0.4 ml respiration medium at 25°C using a Clark-type O_2 electrode. Substrate concentrations were 1 mM for NADH and 10 mM for succinate, malate, and other substrates tested. An O_2 concentration of 250 μM in air-saturated water was used in the calibration of the electrode and ADP/O and RC ratios were measured according to Estabrook (8). Determination of P/O ratios was made essentially according to Keister and Minton (15) using ^{32}P.

Cross-contamination. The cross-contamination of mesophyll fraction and bundle sheath fraction was determined on samples which were frozen as soon as possible after extraction and kept at −20°C for 1 to 2 weeks. RuBP carboxylase was used as a marker for bundle sheath cells and PEP carboxylase as a marker for mesophyll cells. Control tests on unfrozen *versus* frozen samples indicated that the samples could be frozen without loss of activity. The contamination of the mesophyll fraction by bundle sheath material was estimated by calculating the activity of RuBP carboxylase in the mesophyll fraction as a percentage of the activity in the bundle sheath fraction on a Chl basis. The degree of contamination by mesophyll material in the bundle sheath fraction was estimated by determining the activity of PEP carboxylase in the bundle sheath fraction as a percentage of the activity in the mesophyll fraction on a Chl basis. This method gives a reasonable estimate of the degree of cross-contamination when the calculated values are 20% or less (7).

Isolation of Mesophyll Protoplasts and Bundle Sheath Strands. Mesophyll protoplasts and bundle sheath strands were used to determine the distribution of Cyt *c* oxidase and NAD-malic enzyme between mesophyll cells and bundle sheath cells on a Chl basis. Eight to 10 g leaves were cut into small segments (less than 1 mm) and incubated in room light for 3 h at 28°C in digestion medium (0.5 M sorbitol, 1 mM $CaCl_2$, 0.1% BSA, 2% cellulase (Onozuka 4S), and 0.3% pectinase (Macerozyme R-10) at pH 5.0. After incubation, the digestion medium was gently removed and the leaf segments were washed in wash medium (0.5 M sucrose, 1 mM $CaCl_2$, 5 mM Hepes-KOH, pH 7.0). Undigested material was collected on a plastic strainer, the strands were collected on a 80-μm net, and the filtrate was used for protoplast purification. The strands were washed in wash medium and kept on ice in wash medium until used. Under examination with a light microscope, the strands were found free of mesophyll cells. Protoplasts were purified from the filtrate by flotation. Two to three ml of 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM Hepes-KOH (pH 7.0) were layered on top of the wash medium containing mesophyll protoplasts in a centrifuge tube. After centrifugation at 500*g* for 5 min, intact mesophyll protoplasts at the sucrose/sorbitol interface were removed with a Pasteur pipette.

To determine the distribution of Chl between mesophyll and bundle sheath cells within a leaf, about 3 g leaves (3–4 weeks old) were cut and incubated in digestion medium as described above except digestion was for 5 h. The digestion medium and the leaf pieces were then poured onto an 80-μm net. The material on the net was washed with wash medium and taken as the bundle sheath fraction. The pooled wash media were centrifuged at 16,000*g* for 10 min and the pellet was taken as the mesophyll Chl fraction. From examination by light microscopy, the bundle sheath fraction was found to be free of mesophyll contamination.

Protein and Chl Determination. Chl in the different fractions collected from differential and Percoll gradient centrifugation was determined using 80% acetone (3). For estimation of Chl distribution within the leaf, the strands and the mesophyll pellet were extracted in absolute ethanol overnight and Chl was determined (22). Protein was determined according to Lowry *et al.* (17) using BSA as the standard.

Table I. *Activities of Enzymes in Mesophyll Protoplasts and Bundle Sheath Strands of P. miliaceum*

Values are the mean of two to four determinations.

	Mesophyll Protoplasts	Bundle Sheath Strands
	μmol mg^{-1} Chl min^{-1}	
PEP carboxylase	13.0	0.10
RuBP carboxylase	0.24	30.1
Cyt *c* oxidase	0.99	0.86
NAD-malic enzyme	0.23	3.5

Table II. *Recovery of Chl and Cyt c Oxidase Activity in Differential Centrifugations of Mesophyll and Bundle Sheath Fractions of P. miliaceum*

After the filtrates were centrifuged at 1000*g* for 1.75 min, the supernatant was transferred to a new tube and centrifuged at 4000*g* for 1.75 min, after which the supernatant was decanted again and then centrifuged at 27,000*g* for 2.5 min. Pellets were suspended in the respiration medium (see "Materials and Methods"). Chl is expressed as a percentage of the amount in the filtrate. The Cyt *c* oxidase in each fraction is expressed as a percentage of activity in the pellet from a centrifugation of the filtrate at 200,000*g* for 90 min. Activity in the 27,000*g* supernatant fraction was determined by centrifugation at 200,000*g* and assay of activity in the pellet. Values are the mean of three experiments.

Fraction	Mesophyll		Bundle Sheath	
	Cyt *c* oxidase	Chl	Cyt *c* oxidase	Chl
	%		%	
1,000*g* Pellet	9	61	6	31
4,000*g* Pellet	10	27	10	22
27,000*g* Pellet	37	9	25	19
27,000*g* Supernatant	49	5	60	28

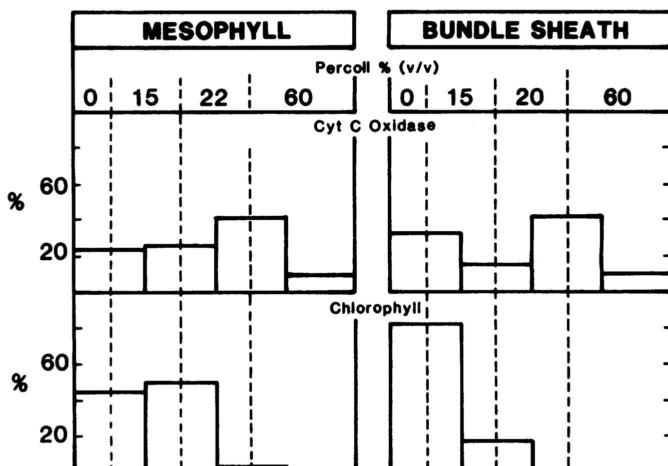

FIG. 1. Recovery of Chl and Cyt *c* oxidase on discontinuous Percoll gradients. Dashed lines indicate interfaces. The values are expressed as per cent recovered on the gradient and are the mean of two experiments.

Table III. *Enrichment of Mitochondria Isolated from Mesophyll and Bundle Sheath Fractions of P. miliaceum*

Recoveries of Chl and Cyt *c* oxidase activity (activity in the filtrate determined as in Table I) after differential centrifugation and Percoll density gradient centrifugation. Amount of Chl and protein refers to a preparation from 50 g leaves. Values are the mean of four experiments, and the highest and lowest values obtained are in parentheses.

	Mesophyll	Bundle Sheath
% Cyt *c* oxidase	11.2 (8.7–13)	5.9 (4.5–7.5)
% Chl	0.19 (0.10–0.30)	0.088 (0.04–0.14)
% Cyt *c* oxidase/% Chl	75 (29–130)	79 (48–130)
μg Chl	17 (0–26)	38 (1.8–5.9)
mg Protein	1.2 (0.8–1.7)	0.77 (0.54–0.98)
mg Protein/mg Chl	77 (30–138)	223 (123–322)

RESULTS

In the plants used in this study, 45% of the Chl was found in the mesophyll fraction and 55% in the bundle sheath fraction (mean of two determinations) when estimated as described in "Materials and Methods." Because of this even distribution, activities expressed on a Chl basis in the mesophyll and the bundle sheath fractions give a good estimation of the distribution of the activity within the leaf. Table I shows the distribution of activities between mesophyll protoplasts and bundle sheath strands. The extreme distribution of PEP carboxylase and RuBP carboxylase shows that very little cross-contamination occurs between the two fractions. The Cyt *c* oxidase activity is evenly distributed in both fractions, whereas the NAD-malic enzyme activity is considerably higher in the bundle sheath fraction than in the mesophyll fraction.

Table IV. *Cyt c Oxidase and NAD-Malic Enzyme Activities in Purified Mesophyll and Bundle Sheath Mitochondria of P. miliaceum*

Mean and SD from five experiments.

	Mesophyll	Bundle Sheath
	μmol mg^{-1} *protein min*$^{-1}$	
Cyt *c* oxidase	0.59 ± 0.13	0.27 ± 0.05
NAD-malic enzyme	2.0 ± 0.7	4.5 ± 1.1
NAD-malic enzyme/Cyt *c* oxidase	3.2 ± 0.8	17.7 ± 5.7

The ratio of RuBP carboxylase activity between the bundle sheath and the mesophyll fractions obtained mechanically (as described in "Materials and Methods") was 5.7 ± 1.2 ($n = 7$) and the ratio of PEP carboxylase between the two fractions was 4.5 ± 0.7 ($n = 9$). These ratios are close to the ratios expected for 15% bundle sheath contamination in the mesophyll fraction and 20% mesophyll contamination in the bundle sheath fraction.

Table II shows the recovery of Cyt *c* oxidase and Chl by differential centrifugation of the preparations. A substantial amount of the Cyt *c* oxidase activity remained in the supernatant after a centrifugation at 27,000*g* for 2.5 min. This activity seemed, however, to be associated with broken organelles, since a centrifugation at 27,000*g* for 4.5 min decreased the intactness of the sedimented mitochondria as measured by the succinate-Cyt *c* oxidoreductase assay. Centrifugation at 4000*g* for 1.75 min compared to 100*g* for 1.75 min reduced the Chl contamination in the mitochondrial pellet considerably.

The enrichment of mitochondria relative to Chl in the 27,000*g* pellet was about 4 times in the mesophyll fraction and only 1.3 times in the bundle sheath fraction. This is considerably less than obtained for spinach (2). The grinding procedure to release bundle sheath mitochondria produces a lot of thylakoid membrane fragments co-sedimenting with the mitochondria, and apparently a lot of fragmented mitochondria as shown by the high activity of Cyt *c* oxidase in the 27,000*g* supernatant. For the following studies, the material sedimenting between 4000*g* at 1.75 min and 27,000*g* for 2.5 min was used. The intactness of the mitochondria in this fraction was 82% to 86% for the mesophyll fraction and 66% to 72% for the bundle sheath fraction as estimated by the succinate-Cyt *c* oxidoreductase assay.

Mitochondria were purified from the crude mitochondrial preparation on a discontinuous Percoll gradient. Figure 1 shows the distribution of Chl and Cyt *c* oxidase activity on the gradient. Most of the thylakoid contamination of the mitochondria was removed as the mitochondria had a higher density than the chloroplast material. The Percoll gradient was very efficient in removing thylakoid material from the bundle sheath fraction. Only 0.5% of the Chl applied to the gradient was recovered on the 20%/60% interface, compared to 2.5% for the mesophyll fraction. The intactness after Percoll gradient centrifugation increased slightly to 84% to 87% for mesophyll mitochondria and 67 to 76% for bundle sheath mitochondria.

Table III summarizes the recovery and the enrichment of mitochondria relative to chloroplast material following differential centrifugation and Percoll density gradient centrifugation. The average recovery of mesophyll mitochondria was higher than the average recovery of bundle sheath mitochondria. The difference is mainly due to the lower recovery of bundle sheath mitochondria

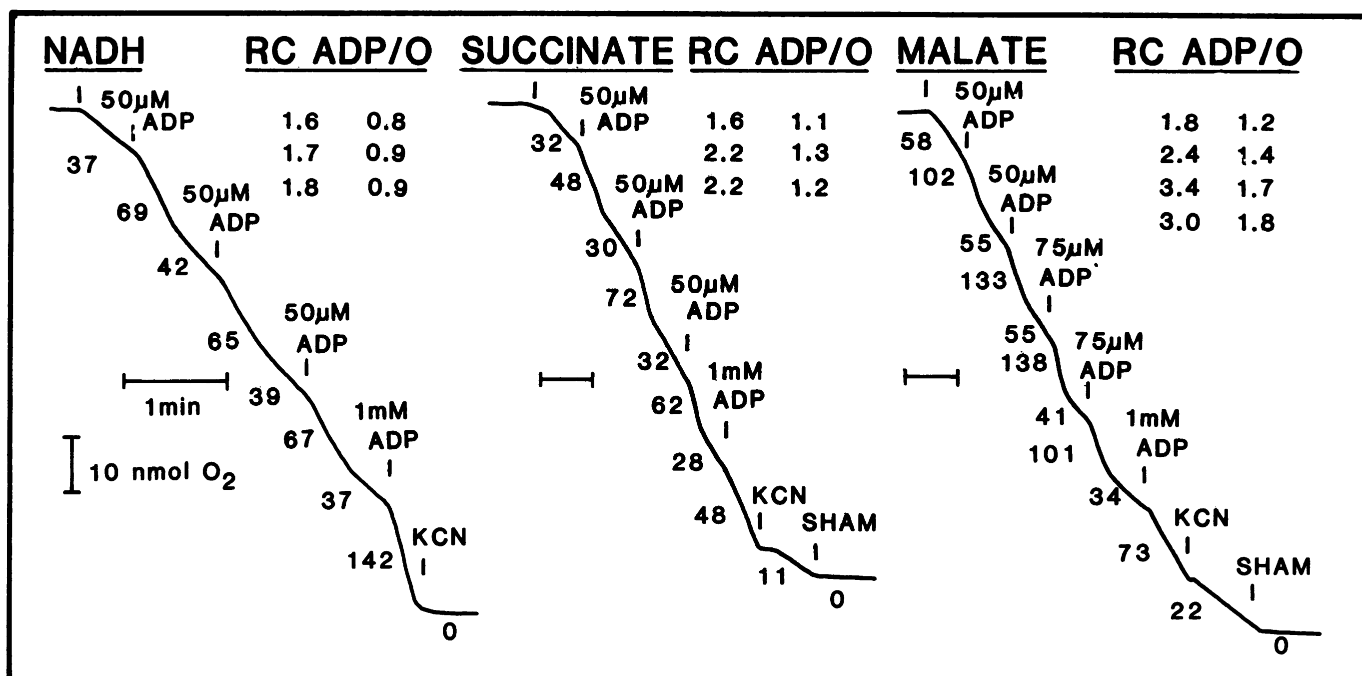

FIG. 2. Oxidation of NADH, succinate, and malate by *P. miliaceum* mesophyll mitochondria. Numbers on the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. The results are typical of those obtained with a number of preparations.

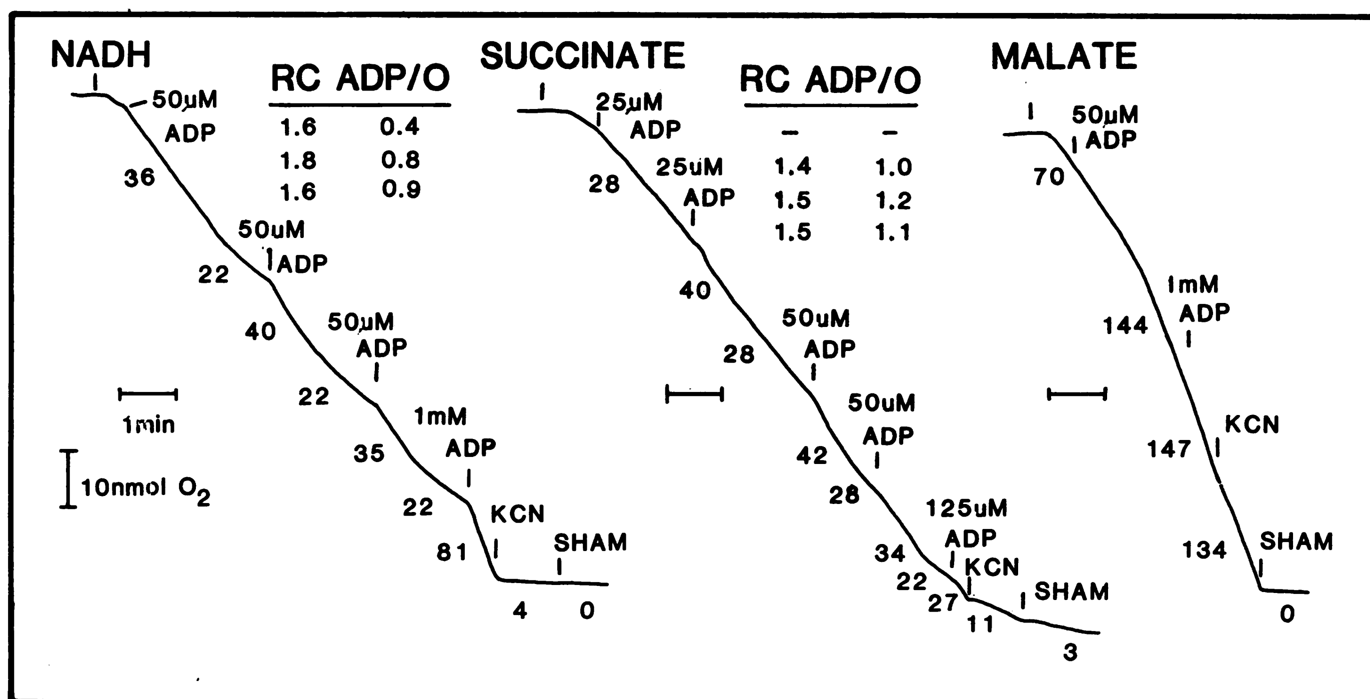

FIG. 3. Oxidation of NADH, succinate, and malate by *P. miliaceum* bundle sheath mitochondria. Numbers on the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. The results are typical of those obtained with a number of preparations.

in the centrifugation step (Table II). The enrichment of mitochondria relative to chloroplast material was similar in the two fractions. The protein to Chl ratio was considerably higher in the purified bundle sheath mitochondrial preparation compared to the purified mesophyll mitochondrial preparation.

In Table IV, the specific activities of Cyt *c* oxidase and NAD-malic enzyme with the purified mitochondria are compared. The specific activity of Cyt *c* oxidase is more than 2 times higher in mesophyll mitochondria than in bundle sheath mitochondria. The specific activity of NAD-malic enzyme is, on the other hand, higher in mitochondria isolated from the bundle sheath fraction.

Mitochondria isolated from both the mesophyll and bundle sheath fractions oxidized NADH, malate, and succinate (Figs. 2 and 3). Mesophyll mitochondria oxidized all three substrates with RC and in all cases the oxidation was rather sensitive to KCN inhibition. Mitochondria isolated from the bundle sheath fraction oxidized NADH and succinate at relatively slow rates with some RC, whereas malate was oxidized at a higher rate with no RC. The oxidation of malate was inhibited very little by 1 mM KCN in contrast to NADH and succinate oxidation. For all three

substrates, the residual activity after KCN inhibition was SHAM sensitive. The KCN inhibition of malate oxidation varied between 5% to 20% in different preparations. When 1 mM SHAM was added to bundle sheath mitochondria oxidizing malate, the inhibition was 75% to 90%, with the residual rate being KCN sensitive. In addition, mechanically isolated bundle sheath strands supplied with 10 mM malate gave an O_2 consumption of 405 nmol mg^{-1} Chl min^{-1}, which was inhibited 75% by 1 mM SHAM and 27% by 1 mM KCN in separate assays. The rate of oxidation of malate on a Chl basis, calculated from the recovery of mitochondria and total Chl in the filtrate, was 380 nmol O_2/mg Chl·min for the bundle sheath fraction and 140 nmol O_2/mg Chl·min for the mesophyll fraction.

NADH oxidation was about 2 times greater at high ADP (1 mM) than at low ADP (50 μM) concentration in both mesophyll and bundle sheath mitochondria. High ADP did not stimulate malate and succinate oxidation relative to low ADP. The P/O ratio during NADH oxidation, measured by ^{32}P incorporation at 1 mM ADP, was 1.4 for mesophyll mitochondria and 1.2 for bundle sheath mitochondria (mean of two experiments). For malate oxidation, P/O ratios of 2.0 and 1.2 were obtained for mesophyll and bundle sheath mitochondria, respectively, using the ^{32}P assay. Both types of mitochondria also oxidized pyruvate, isocitrate, and α-ketoglutarate, but at a considerably slower rate.

DISCUSSION

Bundle sheath mitochondria of NAD-malic enzyme species have high levels of NAD-malic enzyme, which has an important role in malate decarboxylation in the C_4 pathway. Also, the bundle sheath mitochondria of this C_4 subgroup are larger and have a greater development of cristae than mesophyll cell mitochondria (11). It is therefore of interest to isolate these mitochondria in an intact and relatively pure form in order to compare their properties, including their degree of differentiation in function.

In the present study with *P. miliaceum*, an NAD-malic enzyme species, most of the NAD-malic enzyme (approximately 95%) was found in the bundle sheath tissue (Table I). The activity in the mesophyll fraction cannot be accounted for by bundle sheath contamination. Less than 1% of the RuBP carboxylase activity was in the mesophyll preparation, indicating a high degree of purity of the mesophyll protoplasts. Thus, the results show that mesophyll mitochondria have NAD-malic enzyme activity which is similar to or slightly lower than that reported for C_3 mitochondria (12). Chl and Cyt *c* oxidase were approximately equally distributed between mesophyll and bundle sheath tissue.

A mechanical method was developed for isolation of mitochondria from the mesophyll and bundle sheath cells of *P. miliaceum*. The method gives considerably higher yields, which are needed for respiratory studies, than can be routinely obtained using enzymic techniques. The cross-contamination between the mesophyll and bundle sheath fraction isolated mechanically was about 20% as measured by the activities of RuBP carboxylase and PEP carboxylase.

The ratio of Cyt *c* oxidase activity to NAD-malic enzyme activity in the mitochondrial preparations of the mesophyll fraction was 5.6 ± 0.9 (n = 5) times higher than that of the mitochondria of bundle sheath cells. This is in agreement with a cross-contamination of about 20% when the difference in specific activity of Cyt *c* oxidase and the distribution of NAD-malic enzyme are taken into account. Thus, the purity of the two fractions is about 80% which is considered sufficient to make meaningful studies on the two mitochondrial types.

The properties of malate oxidation were found to be very different between mitochondria isolated from the mesophyll and bundle sheath fractions, respectively. With mesophyll mitochondria, malate oxidation was sensitive to KCN and coupled to phosphorylation (Fig. 2) as has been reported for leaf mitochondria from C_3 (5) and CAM plants (1) and mesophyll mitochondria from the C_4 plant maize (18). Malate oxidation in bundle sheath mitochondria of *P. miliaceum* was, on the other hand, largely insensitive to KCN (Fig. 3). The oxidation was strongly inhibited by SHAM, which shows that electron transport proceeds via the alternate oxidase pathway. The alternative pathway is considered to be nonphosphorylating (4). The P/O ratio of 1.2 that we obtained can be explained by the phosphorylation by contaminating mesophyll mitochondria and phosphorylation at site 1 in the bundle sheath mitochondria. The occurrence of high NAD-malic enzyme activity and respiration via the alternative pathway in the bundle sheath mitochondria is interesting in relation to a recent suggestion by Rustin *et al.* (21) that oxidation through NAD-malic enzyme, in contrast to malate dehydrogenase, was directly connected with the alternative pathway. However, other results indicate that malate dehydrogenase and NAD-malic enzyme compete at the level of the pyridine nucleotide pool in the mitochondrial matrix (19). Bundle sheath mitochondria of *P. miliaceum*, with their high activity of the alternative pathway, may be useful for studies on regulation of the activity of the alternative oxidase.

The capacity of malate oxidation via the respiratory chain in bundle sheath cells was calculated to be about 0.8 μmol mg^{-1} Chl min^{-1} (considering the original Chl content and the recovery of Cyt *c* oxidase activity). This is similar to that of an earlier report with mitochondria from *Atriplex spongiosa*, another NAD-malic enzyme C_4 plant (14). The rate of C_4 acid decarboxylation in bundle sheath cells is considerably higher than the rate of malate oxidation (14). Thus, the malate oxidation via the respiratory chain can only make a small contribution to the total decarboxylation. However, the present results suggest that the malate oxidation in bundle sheath mitochondria can continue in the light even if a high energy charge restricts respiration via Cyt *c* oxidase. This may allow malate, as well as aspartate, to contribute as donors of carbon to the C_3 pathway via decarboxylation through NAD-malic enzyme. Decarboxylation with aspartate as a donor need not be linked to the electron transport chain since the pyridine nucleotide would be recycled through malate dehydrogenase and NAD-malic enzyme (14).

In the present study, we find the Cyt *c* oxidase activity is about equally distributed between the two cell types (Table I). Therefore, at least with certain substrates, both mitochondrial types may perform oxidative phosphorylation through the Cyt-linked electron transport chain. However, the specific activity of the Cyt *c* oxidase was more than 2 times higher in purified mesophyll mitochondria than bundle sheath mitochondria (Table IV). Taken together, this suggests that there is more mitochondrial protein in bundle sheath cells than in mesophyll cells. Furthermore, the purified mitochondria of the bundle sheath fraction had a higher ratio of mitochondrial protein to Chl than did the purified mitochondria of mesophyll fraction, but the enrichment of Cyt *c* oxidase activity to Chl was similar in the two preparations (Table II). This also suggests that there is more mitochondrial protein in bundle sheath cells than in mesophyll cells, which is in agreement with suggestions from electron microscopy studies (11). Inasmuch as the mitochondria of bundle sheath cells have a role in photosynthesis, it is reasonable that there is a relatively large amount of mitochondria in these cells. The bundle sheath mitochondria have a high capacity for C_4 acid decarboxylation which is mediated through several enzymes and may, in part, be linked to the alternative electron transport pathway. This important function of these mitochondria may account for their relatively high protein to Cyt *c* oxidase ratio in comparison to the mitochondria of the mesophyll cells.

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