Plant Physiol. (1982) 69, 916–920
0032-0889/82/69/0916/05/$00.50/0

Isolation and Characterization of Metabolically Competent Mitochondria from Spinach Leaf Protoplasts[1]

Received for publication September 10, 1980 and in revised form June 27, 1981

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ABSTRACT

Intact mitochondria were prepared from spinach (*Spinacia oleracea* L. var. Kyoho) leaf protoplasts and purified by Percoll discontinuous gradient centrifugation. Assays of several marker enzymes showed that the final mitochondrial preparations obtained are nearly free from other contaminating organelles, *e.g.* chloroplasts, peroxisomes, and endoplasmic reticulum. These mitochondria oxidized malate, glycine, succinate, and NADH, tightly coupled to oxidative phosphorylation with high values of ADP to O ratio as well as respiratory control ratio. The rate of NADH oxidation was 331 nmoles O_2 per milligram mitochondrial protein per minute, which is comparable to that obtained by highly purified potato or mung bean mitochondria. However, the activity of glutamine synthetase was barely detectable in the isolated mitochondrial fraction. This finding rules out a hypothetical scheme (Jackson, Dench, Morris, Lui, Hall, Moore 1971 Biochem Soc Trans 7: 1122) dealing with the role of the mitochondrial glutamine synthetase in the reassimilation of NH_3, which is released during the step of photorespiratory glycine decarboxylation in green leaf tissues, but it is consistent with the photosynthetic nitrogen cycle (Keys, Bird, Cornelius, Lea, Wallsgrove, Miflin 1978 Nature (Lond) 275: 741), in which NH_3 reassimilation occurs outside the mitochondria.

The technical advancement of isolating the metabolically competent mitochondria from plant sources, such as etiolated seedlings and storage tissues, has allowed us to study thoroughly the nature of respiratory oxidation and phosphorylation properties (7, 22, 23). Leaf mitochondria have not been prepared in a highly purified form, although it is believed that they carry out important functions related to the mechanism of photorespiration (12). It has been demonstrated (4, 5, 27) that the conversion of glycine to serine occurring in leaf mitochondria is coupled to the synthesis of 3 mol ATP (1, 5, 10, 15, 16). To clarify the roles of mitochondria in the overall mechanism of photorespiration, the isolation of the pure mitochondrial preparations free from other organelles is urgently necessary. Previously we have shown that protoplasts are the ideal starting materials for isolating pure and metabolically competent cell organelles, as they are surrounded only by the plasma membranes and are readily ruptured by gentle mechanical breakage (19–21).

In this communication, we report a method for preparing the highly purified and metabolically active mitochondria from spinach leaf protoplasts, and their oxidative and phosphorylative properties are examined. We also present experimental results concerning the absence of glutamine synthetase in mitochondria in relation to the reassimilation of NH_3 in the photorespiratory nitrogen cycle (25, 26).

After completion of the manuscript, our attention was drawn to the paper by Bergman *et al.* (3) describing the isolation of Chl-free spinach leaf mitochondria.

MATERIALS AND METHODS

Protoplasts. Protoplasts were isolated from freshly harvested spinach leaves (*Spinacia oleracea* L. var. Kyoho) after digestion of leaf strips (with lower epidermis removed), essentially following the method reported previously (18). The only modification was that the concentration of mannitol was lowered to 0.5 M to prevent osmotic shock, which has an adverse effect during the step of rupturing spinach protoplasts. The final protoplast preparation was shown to have photosynthetic activities of 35 to 70 μmol CO_2 incorporation/mg Chl·h at 25°C.

Disruption of Protoplasts and Isolation of Mitochondria by Density Gradient Centrifugation. All operations were carried out at 4°C. Spinach protoplasts (about 4 mg Chl) suspended in 40 ml of buffer—consisting of 0.3 M mannitol, 10 mM Mops[3]-KOH buffer (pH 7.2), 1 mM EDTA, 0.1% defatted BSA, and 0.6% Polyclar AT—were ruptured in a Teflon homogenizer (3-cm diameter). For the mechanical disruption of protoplasts, about 10 gentle strokes were found to be sufficient to break up plasma membranes from the measurements of the NADH-dependent O_2 uptake (experimental details given in "Results").

The whole homogenate was then centrifuged at 1,000*g* for 10 min, the sedimented pellet containing chloroplasts and aggregated organelles (see "Results"). The supernatant fraction was centrifuged at 10,000*g* for 10 min; the pellet obtained was gently dispersed in 1 ml of suspending buffer containing 0.3 M mannitol, 10 mM Mops-KOH buffer (pH 7.2), and 0.1% defatted BSA and was layered on top of a discontinuous or linear Percoll density gradient. The discontinuous gradient was composed of the following: A, 3 ml 60% (v/v) Percoll; B, 4 ml 45% (v/v) Percoll; C, 4 ml 28% (v/v) Percoll; and D, 4 ml 5% (v/v) Percoll containing 0.25 M sucrose, 20 mM Mops-KOH buffer (pH 7.2), and 0.2% defatted BSA. The linear gradient was 5 to 60% (v/v) Percoll containing the same ingredients as above. Centrifugation was carried out at 30,000*g* for 30 min in a Beckman model L2-65B ultracentrifuge equipped with an SW 25-3 rotor. The mitochondrial fraction

[1] This is paper No. 55 in the series 'Structure and Function of Chloroplast Proteins,' and the research was supported partly by grants from the Ministry of Education of Japan (No. 576049 to [M. N.] and No. 511007 and 376039 to [T. A.]).

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[3] Abbreviation: Mops, 3-(*N*-morpholino) propanesulfonic acid.

appeared at the interface between C and B. For the polarographic analysis of O_2 uptake, the mitochondrial fraction was collected and washed with 20 ml 10 mM Mops-KOH buffer (pH 7.2) containing 0.25 M sucrose and 0.2% defatted BSA. The final mitochondrial fraction was suspended in 2 to 3 drops of suspending buffer.

For assaying several enzyme activities of the fractionated samples, 0.4 ml of each fraction was collected using an ISCO density gradient fractionator (model 640).

Enzyme Assays and Analytical Methods. The following assay methods were employed: catalase (14); fumarase (6); NADPH-Cyt *c* reductase (20); Chl (2); and protein (13). Activity of glutamine synthetase was measured by the biosynthetic assay method (24), except for the density gradient experiments in which the enzyme coupling method (24) was employed.

Measurement of O_2 Consumption by Mitochondria. O_2 uptake was followed polarographically at 25°C using a Clark-type electrode system purchased from Hansatech Ltd. (Hardwick Industrial Estate, Kings Lynn, Norfolk, UK). The reaction medium contained 0.3 M mannitol, 10 mM Na-phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 10 mM KCl, 0.1% defatted BSA, and a known amount of mitochondrial fraction, in a total volume of 1 ml.

RESULTS

Disruption of Protoplasts. To get a satisfactory disruption of protoplasts, several methods—including osmotic shock, passage through syringe, and mechanical breakage by a Teflon homogenizer—were tested. In each case, we measured the magnitude of unmasking of the NADH oxidation reaction as an index of the protoplast disruption, because plasma membranes are impermeable to NADH, and their disruption causes an enhancement of NADH oxidation. During osmotic shock, the rupture of plasma membranes starts from 400 mosM and reaches maximum at around 250 to 300 mosM. However, we found that the breakage of the protoplast plasma membranes by a single hypotonic shock is insufficient to release the mitochondria from the cytoplasmic network. Under such treatment, mitochondria tend to stick to the outer surface of the vacuolar system, and, upon centrifugation at 1,000*g* for 10 min, they sediment together with other organelles. Therefore, we examined the effectiveness of the mechanical breakage to disperse each organelle component. The passage of protoplasts through a syringe (21) caused a complete dispersion of organelles, and it was found that less than 10% of the fumarase activity (marker of mitochondria) was detectable in the 1,000*g* precipitate, whereas 73% remained in the 10,000*g* precipitate (crude mitochondria). However, the crude mitochondrial fraction did not show high ADP to O and respiratory control ratios, indicating that the mitochondria were damaged during passage through syringe. The third method of the mechanical breakage using a Teflon homogenizer was less effective for the organelle dispersion. We found that 57% of the fumarase activity is localized in the 1,000*g* precipitate and 26% in the 10,000*g* precipitate (Table I). However, mitochondrial fractions separated by this disruption method proved to be metabolically competent, sustaining the high ADP to O and respiratory control ratios (Table II; Fig. 3). Therefore, we decided to use the mechanical breakage by a Teflon homogenizer throughout the present investigation.

Purification of Mitochondria by Percoll Density Gradient Centrifugation. Figure 1 shows the results of the separation of mitochondria by the Percoll density gradient centrifugation using the 10,000*g* precipitate as a starting material (details given in "Materials and Methods"). Bands I (on top of D) and II (interface of D and C) are greenish, but Band III mitochondria (interface of C and B) are white. Results of the sedimentation profile of the broken protoplasts and Chl contents after the Percoll discontinuous density gradient centrifugation, as measured by marker enzyme activities, are presented in Figure 2. Approximately 60%

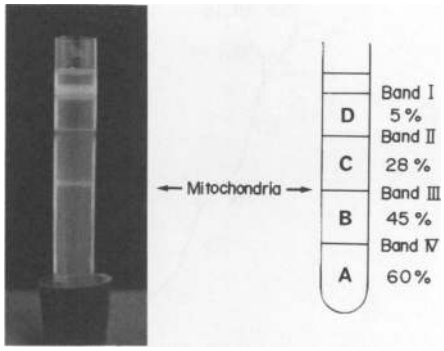

FIG. 1. Separation of mitochondria by Percoll discontinuous density gradient centrifugation.

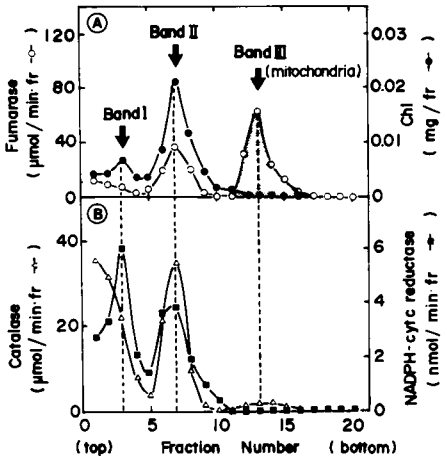

FIG. 2. Enzyme localization in mechanically ruptured spinach leaf protoplasts followed by Percoll discontinuous density gradient centrifugation. Experimental details of the mechanical breakage of protoplasts, sedimentation of crude mitochondrial fraction (10,000*g* precipitate), and subsequent fractionation by Percoll discontinuous gradient centrifugation are described in the text. Aliquots (0.4 ml) of the fractions were subjected to the enzyme assays (fumarase, catalase, and NADPH-Cyt *c* reductase) and Chl analysis.

of the fumarase activity was recovered in the Band III fraction, with the remainder being present in Band II. The activities of NADPH-Cyt *c* reductase (marker of ER) and Chl (marker of the thylakoid membranes) were barely detectable in Band III, and only small catalase activities (marker of peroxisomes) were detectable in Band III (less than 1% of the total catalase activities in the whole homogenate). On the other hand, Chl, catalase, and NADPH-Cyt *c* reductase activities were detected in Bands I and II. The density of peroxisomes is greater than that of mitochondria in the sucrose density gradient (19, 21), although it is lighter than the latter in the presently used Percoll density gradient experiment.

Overall results of the purification of mitochondria from spinach leaf protoplasts are summarized in Table I. The final recovery of the intact mitochondria was 15.6%, and it can be seen that the pure mitochondrial preparation obtained (Band III) was barely contaminated by the thylakoid membranes, peroxisomes, and ER.

Oxidative Activities of Mitochondria. The pure mitochondria prepared from spinach leaf protoplasts by employing the method described above were able to oxidize glycine, malate, succinate, and NADH, with the high respiratory control ratio as well as ADP-to-O ratio (Fig. 3). The rate of NADH oxidation by the mitochondria was 331 nmol/mg mitochondrial protein·min (Table II). This activity is comparable with that of the highly purified potato and mung bean mitochondria (17) and is 2- to 3-times higher than that reported previously for leaf mitochondria (3, 5,

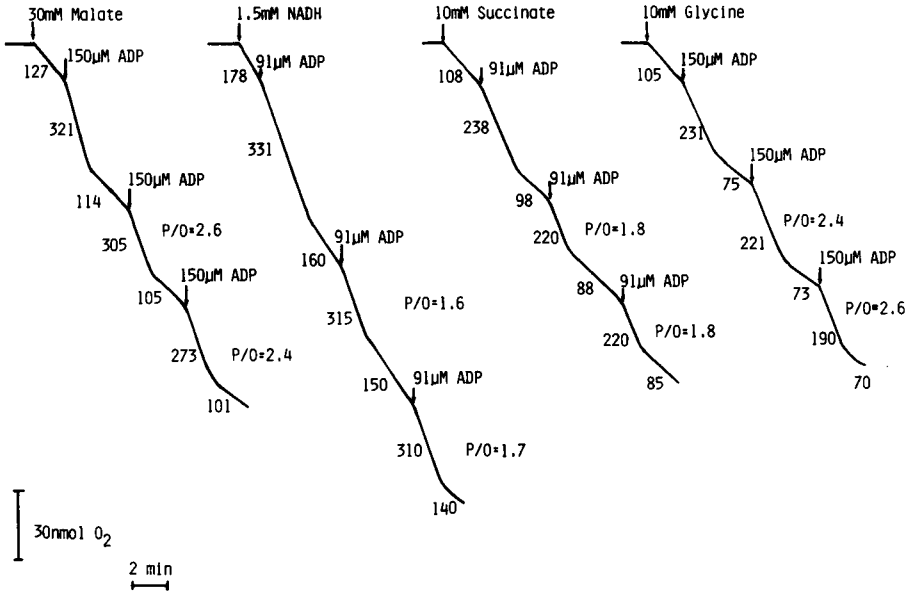

FIG. 3. Oxidation of malate, NADH, succinate, and glycine by purified mitochondria isolated from spinach leaf protoplasts. Purified mitochondria, Band II fraction, isolated by the method shown in Figure 2 were examined for their oxidative activities. Concentrations of substrates and nucleotides shown are the final concentrations in the reaction mixture applied to the electrode vessel. Values expressed on the traces refer to nmol O_2 taken up per mg mitochondrial protein per min.

Table I. *Subcellular Localization of Some Enzyme Activities in Mechanically Ruptured Spinach Leaf Protoplasts followed by Percoll Gradient Centrifugation*

In experiments 1 through 4, mechanically ruptured spinach leaf protoplasts (about 4 mg Chl) were subjected to the fractional centrifugation following the method as described in the text. In experiment 5, 10,000*g* precipitate was applied to the Percoll discontinuous (28–45%) gradient centrifugation. Mitochondrial fraction, separated as shown in Figure 1, was collected.

Experiment	Fractions	Chl	Fumarase	Catalase	NADPH-Cyt *c* Reductase
		mg	*mmol/min*		*μmol/min*
1	Whole homogenate	3.88	0.90	1.59	0.39
2	1,000*g* Precipitate	3.56	0.52	0.34	0.10
3	10,000*g* Precipitate	0.08	0.23	0.18	0.02
4	10,000*g* Supernatant	0.01	0.02	0.84	
5	Mitochondria (Percoll)	ND[a]	0.14	0.01	ND

[a] Not detectable.

Table II. *Oxidative and Phosphorylative Activities of Purified Spinach Leaf Mitochondria Isolated by Percoll Gradient Centrifugation*

Values are summaries of data shown in Figure 3. Experimental details for measurements using an O_2 electrode are described in the text.

Substrates	Specific Activity	Respiratory Control Ratio	ADP-to-O Ratio
	nmol/mg mitochondrial protein·min		
Glycine	231	2.7–3.1	2.4–2.6
Malate	321	2.7–2.9	1.4–2.6
Succinate	238	2.4–2.6	1.8
NADH	331	2.1–2.2	1.6–1.7

8, 10, 15). The glycine oxidation is tightly coupled to phosphorylation, giving an ADP to O ratio of 2.4–2.6, which indicates that the oxidative decarboxylation of glycine is connected with three coupling sites, as reported by Douce *et al.* (5) and Arron *et al.* (1). These results show that highly purified mitochondria from leaf protoplasts are metabolically competent.

Localization of Glutamine Synthetase. Glutamine synthetase and glutamate dehydrogenase, which catalyze NH_3 assimilation, are present in green leaves. Glutamine synthetase appears to play a role in reassimilating NH_3, which is liberated during the glycine-serine conversion reaction in the photorespiratory process (11). Jackson *et al.* (9) reported that glutamine synthetase is localized in mitochondria isolated from spinach leaf and is responsible for the reassimilation of NH_3. However, Wallsgrove *et al.* (25, 26) reported that the enzyme activity was not detectable in the mitochondrial fraction separated by the sucrose density gradient centrifugation. To examine the subcellular localization of glutamine synthetase activities more rigorously, the whole homogenate pre-

Table III. *Subcellular Localization of Glutamine Synthetase Activities in Mechanically Ruptured Spinach Leaf Protoplasts followed by Percoll (Discontinuous or Linear) Gradient Centrifugation*

In experiments 1 through 4, basic experimental procedures were the same as those shown in Table I. In experiments 5 and 6, 10,000*g* precipitate was applied to either discontinuous or linear Percoll gradient centrifugation, and mitochondrial fractions were isolated. Glutamine synthetase activity was measured by the biosynthetic method in experiments 1 through 4 and by the enzyme coupling method in experiments 5 and 6. By taking the total enzyme activity in the whole homogenate as 100%, percentage distribution of the activity in each fraction was calculated.

Experiment	Fractions	Glutamine Synthetase	
		nmol/min	*%*
1	Whole homogenate	6,635	100
2	1,000*g* Precipitate	4,906	73.9
3	10,000*g* Precipitate	603	9.1
4	10,000*g* Supernatant	2,035	30.7
5	Mitochondria (Percoll discontinuous, 28–45% interface)	68	1.0
6	Mitochondria (Percoll linear, fraction 19–25)	ND[a]	

[a] Not detectable.

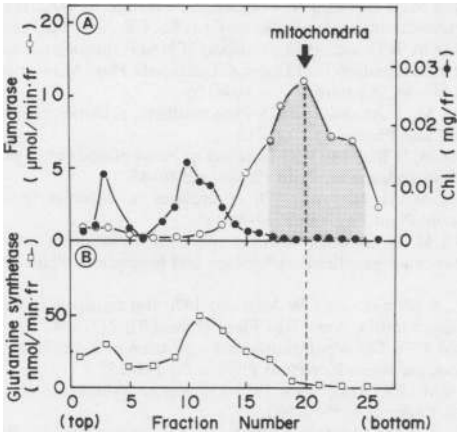

FIG. 4. Subcellular localization of some enzyme activities in mechanically ruptured spinach leaf protoplasts followed by Percoll linear gradient centrifugation. Experimental details of Percoll linear gradient (5–60%, v/v) centrifugation are described in the text, and other basic experimental procedures were the same as those in Figure 2. Aliquots were subjected to the enzyme assays (fumarase and glutamine synthetase) and Chl analysis.

pared from the disrupted protoplasts was fractionated, followed by the Percoll gradient centrifugation. As shown in Table III, a small enzyme activity (9.1%) is detectable in the crude mitochondrial fraction (10,000*g* precipitate), whereas 74% of the original activity is localized in the 1,000*g* precipitate and 30% in the 10,000*g* supernatant, respectively, suggesting that glutamine synthetase is localized in cytosol and chloroplasts. It was found that the purified mitochondria (Fig. 1, Band III) separated by the Percoll discontinuous gradient centrifugation contain only 1% of the total glutamine synthetase activity. The specific enzyme activity of 20 nmol/mg mitochondrial protein·min was much lower than that reported by Jackson *et al.* (9). As the second means of examining the possible presence of glutamine synthetase in mitochondria, we employed the Percoll linear (5–60%) gradient centrifugation technique. Although the method used was not perfectly satisfactory for the purpose, the enzyme distribution profile shown in Figure 4 clearly indicates that the glutamine synthetase activities were not present in fractions 19 through 25, in which potent fumarase activities are localized. From the overall results, we conclude that glutamine synthetase is not present in mitochondria and that the enzyme activity detectable in the crude mitochondrial fraction is likely to be ascribed to the contaminated thylakoid membranes.

DISCUSSION

The isolation of leaf mitochondria by differential centrifugation was reported by Douce *et al.* (5), who fully examined their oxidative properties. Subsequently, Jackson *et al.* (8) purified spinach leaf mitochondria by using the Percoll discontinuous gradient centrifugation technique. There are some notable differences between the experimental methods of the latter workers and ours reported herein. In our present study, to avoid Chl contamination in the mitochondrial fraction, we have chosen a composition of the Percoll gradient which is slightly different from the one used by Jackson *et al.* (*cf.* Fig. 1). We have observed that our mitochondrial fraction does not sediment through the Percoll layer using their experimental conditions, *i.e.* centrifugation at 7,500*g* for 30 min. Although the reason for discrepancies between these results is not clear, it is likely that their mitochondrial fractions associated with the thylakoid membranes as well as other organelles can pass through the Percoll layer. In fact, we have found that the mitochondria highly contaminated by the thylakoid membranes can pass through the Percoll gradient. Recently Bergman *et al.* (3) isolated Chl-free mitochondria from spinach leaves by using the phase partition and the Percoll discontinuous gradient centrifugation technique, the Percoll composition being similar to that used in our present investigation.

The rate of NADH oxidation of the mitochondrial preparation reported by the above workers is about one-third of ours (3). Our results show that mitochondria isolated from spinach leaf protoplasts and purified by the Percoll gradient centrifugation rapidly oxidize glycine, malate, succinate, and NADH, accompanying the good respiratory control and ADP-to-O ratios, indicating that the organelle isolated sustains a high level of structural and functional integrity. However, the addition of Cyt *c* to the preparation was found to enhance the O_2 consumption due to NADH oxidation (results not shown). Because it is generally recognized that the outer membrane of intact mitochondria prevents externally added Cyt *c* from interacting with Cyt *c* oxidase in the respiratory chain, which is located on the outer surface of the mitochondrial inner membranes, the observed Cyt *c* effect in the leaf mitochondria might probably be ascribed to the structural damage of the outer membranes. Reduced Cyt *c*, produced by NADH-Cyt *c* reductase localized in the mitochondrial outer membranes as well as in the ER, can penetrate into inter-membrane space and is eventually oxidized by Cyt *c* oxidase. This implies that, during the course of the protoplast breakage, the outer membranes of the mitochondria are ruptured to a certain extent. By carefully controlling the final osmolarity of the suspending medium as well as by improving the procedures of mechanical disruption, the isolation of the truly intact mitochondria in a better yield can be attained.

It was reported that the activity of glutamine synthetase is detectable in the mitochondrial fraction which was purified by the Percoll gradient centrifugation (9) and that the specific enzyme activity determined (72 nmol/mg mitochondrial protein·min) can account for the photorespiratory release of NH_3 by glycine decarboxylase (about 70 nmol/mg mitochondrial protein·min) (15). The specific activity of glutamine synthetase in the mitochondrial fraction 10 nmol/mg protein·min determined in the present investigation is much lower than that needed to account for the reassimilation of NH_3. Furthermore, glutamine synthetase could be clearly separable from the mitochondrial fraction by the Percoll linear density gradient centrifugation.

Recently, Wallsgrove *et al.* (25) reached the same conclusion from the polarographic analysis of the isolated leaf mitochondria. If the tightly coupled mitochondria oxidizing glycine are provided with glutamate, all the substrates needed for the glutamine synthesis are present, and the consequent production of ADP should release the respiratory control, accompanying the increase of the oxidation rate to that obtained in state 3. However, no such increase in the respiratory rate was observed in their experiments, and respiratory control was maintained in the presence of glutamate. Taken together, overall findings indicate that glutamine synthetase is not present in mitochondria and that the reassimilation of NH_3 occurs outside the mitochondria, presumably in the cytosol and/or chloroplasts. The definite answer for the possible presence of two isozymic forms of glutamine synthetase between chloroplasts and cytosol must await future investigations, and their comparisons may shed light on the nature of photorespiratory nitrogen metabolism.

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