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Biochemical Properties of Mitochondrial Membrane from Dry Pea Seeds and Changes in the Properties during Imbibition

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ABSTRACT

An attempt to isolate intact mitochondria from dry pea seeds (*Pisum sativum* var. Alaska) ended in failure. Cytochrome oxidase in crude mitochondrial fraction from dry seeds was separated into three fractions by sucrose density gradient centrifugation. Two of the fractions contained malate dehydrogenase, whereas the other did not. Equilibrium centrifugation of mitochondrial membrane on sucrose gradients revealed that the membrane from the fraction without malate dehydrogenase was lighter than that from the others. Differences were observed in relative content of phospholipid to protein and in polypeptide composition analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis among the membranes from three fractions and imbibed cotyledons. Membrane from the fraction without malate dehydrogenase was rich in phospholipid and lacking in polypeptides with relatively high molecular weights as compared with that from others. During imbibition, the fraction without malate dehydrogenase and one of the other two disappeared rapidly after a lag phase lasting for at least 1 hour. Concomitantly, active and stable mitochondria increased in the cotyledons. The results were interpreted to indicate that there were at least three types of mitochondria in dry seeds, the membranes of which differed in their biochemical properties, and that the mitochondria became active and stable through assembly of protein into the membranes during imbibition.

Seed germination is accompanied by a marked increase in respiratory activity of the cotyledons or endosperm. Biochemical and electron microscopic studies have revealed that the increase is linked either with the biogenesis of mitochondria or with the development of vesicular mitochondria with a few cristae to crista-rich ones (3). In peas, a rapid development of cotyledon mitochondria, *i.e.*, the formation of their membrane and an increase in their biological function, takes place in the imbibition stage (4, 7). The development does not require *de novo* synthesis of mitochondrial protein, and seems to result from transport of pre-existing cytoplasmic protein into immature mitochondria (5).

The purpose of this investigation was to isolate and characterize immature mitochondria in dry pea seeds and to elucidate how the mitochondria mature to become fully active during imbibition. This report describes the existence of at least three types of mitochondrial membranes in dry pea seeds, which differ in their biochemical properties, and a possible mechanism of mitochondrial development in the cotyledons during imbibition.

MATERIALS AND METHODS

Plant Material. Pea seeds (*Pisum sativum* var. Alaska) were purchased from Watanabe Seed Co., Kogota, Miyagi, Japan. The seeds were surface-sterilized with 1% (v/v) NaOCl, washed with deionized H_2O, and soaked in the dark at 28 C. The cotyledons were harvested at the required time, washed with deionized H_2O, and used as the source of mitochondria. When dry seeds were used as the source, the seeds were powdered with a motor pulverizer prior to homogenization.

Preparation and Sucrose Density Gradient Centrifugation of Crude Mitochondrial Fraction. Cell debris-free homogenates were prepared from dry seed powder or imbibed cotyledons as reported previously (4). The grinding medium was composed of 10 mM potassium phosphate, pH 7.2, 0.7-M mannitol, 1 mM-EDTA, 0.1% (w/v) BSA, and 0.05% (w/v) cysteine. In some experiments, the cell debris-free homogenates were centrifuged at 25,000*g* for 30 min, and the resulting pellets were suspended in a mannitol medium after being washed once with the medium. The ingredients of the suspending medium were the same as those of the medium used for grinding except that cysteine was omitted. In most cases, the cell debris-free homogenates were centrifuged for 80,000*g* for 1 hr, and the crude particulate pellets were suspended in the suspending medium as described for 25,000*g* pellets.

A 1.5-ml aliquot of the suspension was layered on 16 ml of a linear sucrose density gradient from 32.5 to 65% (w/v) and centrifuged at 77,000*g* for 3 hr in a Hitachi RPS 25-3A rotor. After centrifugation, the gradient was divided into 0.5-ml fractions.

All the above procedures were carried out at 0 to 4 C.

Preparation and Equilibrium Centrifugation of Mitochondrial Membrane Fraction. After fractionation of 80,000*g* pellets on the sucrose gradient, active fractions with respect to Cyt oxidase activity were pooled, mixed with twice the volume of 0.1 M KCl, and centrifuged at 80,000*g* for 1 hr. The pellets were suspended in 0.1 M KCl and subjected three times to sonic oscillation at 20 kilocycles for 20 sec. The fractured membranes were collected by centrifugation at 80,000*g* for 1 hr and suspended in 0.5 M sucrose.

A 1.5-ml aliquot of the suspension was layered on 11.5 ml of a linear sucrose density gradient from 32.5 to 65% (w/v) and centrifuged at 187,000*g* for more than 10 hr in a Hitachi RPS 40T rotor. After centrifugation, the gradient was divided into 0.5-ml fractions.

All the above procedures were performed at 0 to 4 C.

Assays. Respiratory activity, (RCR),[3] ADP/O ratio, and Cyt

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[3] Abbreviations: RCR: respiratory control ratio; MDH: malate dehydrogenase.

oxidase and MDH activities were assayed as described previously (4), except that the total volume of the reaction mixture for the enzyme activities was 0.3 ml instead of 3 ml. Succinate-Cyt *c* reductase activity was determined by the method of Tisdale (8), except that the total volume of the reaction mixture was 0.3 ml. One unit of each enzyme activity was defined as the amount of enzyme which catalyzed an initial rate of oxidation of 1 μmole of reduced Cyt *c* or NADH or of reduction of 1 μmole of Cyt *c* per min.

Protein was determined by the method of Lowry *et al.* (2) with BSA as standard. The amount of phospholipid was estimated from phosphorus content in the lipid fraction extracted by the method of Folch *et al.* (1).

SDS-Polyacrylamide Gel Electorophoresis. Disc electrophoresis in a 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS was performed by the method of Weber and Osborn (9). Mitochondrial membrane suspension was mixed with an equal volume of 0.02-M phosphate buffer, pH 7.2, containing 4% (w/v) SDS and 40% (v/v) glycerol, and was boiled for 2 min in a sealed glass tube in the presence of 1% (v/v) mercaptoethanol. The solution containing 50 to 70 μg of solubilized membrane protein was applied on a gel. The staining of gels was performed with Coomassie blue after fixation with 20% (w/v) sulfosalicylic acid.

RESULTS

Activities of Crude Mitochondrial Fraction from Dry Seeds. Considerable amounts of mitochondrial MDH and Cyt oxidase in the cell debris-free homogenate prepared from dry seeds remained in the 25,000*g* supernatant (4). About half of Cyt oxidase but little MDH in the supernatant were recovered in the 80,000*g* pellet (Table I), suggesting that Cyt oxidase in the 25,000*g* supernatant is of fractured mitochondrial membranes but not of unbroken and very small or light mitochondria. There was no difference in the rate of O_2 uptake with succinate as substrate between the 25,000*g* and 80,000*g* pellets, neither of which responded to exogenous ADP (Table I).

Attempts were made to improve the isolation method in preparing crude mitochondrial fraction containing more MDH or responding to exogenous ADP from dry seeds. Media, which differed in pH or mannitol concentration, were used as grinding or suspending medium. In some experiments, HEPES buffer and sorbitol were substituted for phosphate buffer and mannitol, respectively. Moreover, $MgCl_2$ was added to some media to give a final concentration of 5 mM. However, these changes in the composition of grinding or suspending medium resulted in no increase in MDH activity or RCR of the 25,000*g* pellet. Coarsely powdered dry seeds and the seeds imbibed at 0 C under nitrogen atmosphere for several hr were used as the source of mitochondria. Mitochondria in these imbibed seeds are considered to be similar to those in dry seeds, since mitochondrial development does not occur at low temperatures (6). The 25,000*g* pellets showed the same MDH activity and RCR as those from finely powdered dry seeds. The respiratory activity of all preparations with succinate as substrate was 10 to 12 nmoles of O_2 consumed per min per g of dry seeds.

Fractionation of Mitochondrial Particles from Dry Seeds. When the 80,000*g* pellet from dry seeds was centrifuged on the sucrose gradient, Cyt oxidase was separated into three fractions; a fraction carrying MDH at a lower region of the gradient at mean density 1.215 g/cm³ (fraction A), a major one containing little or no MDH at a middle region of the gradient at mean density 1.162 g/cm³ (fraction B), and the third coinciding with the distribution of MDH on the top of the gradient (fraction C) (Fig. 1A). Judging from its green color, fraction B was contaminated by chloroplast fragments. Similar results have been observed with the 25,000*g* pellet (4), but greater amounts of fractions B and C were obtained from the 80,000*g* pellet than from the 25,000*g* pellet.

Particles in fraction C from the 80,000*g* pellet were again centrifuged on the sucrose gradient (Fig. 2). Cyt oxidase was present in the gradient in two sharp and two broad peaks. A considerable activity of MDH was recovered within the gradient in a peak at density 1.165 g/cm³ (fraction C′), coinciding in distribution with one of Cyt oxidase activity peaks. The enzyme was also present in the gradient in a tailing shoulder and in the soluble fraction on the gradient.

Changes in Properties of Mitochondrial Particles during Imbibition. As shown in Table I, respiratory and enzyme activities of cotyledon 80,000*g* pellet increased after a lag phase lasting for at least 1 hr during imbibition. The pellet responded to exogenous ADP as the activities rose.

There was no significant difference in the distribution of Cyt oxidase and MDH activities after sucrose density gradient centrifugation between the 80,000*g* pellets from dry seeds and those from 1-hr cotyledons. As shown in Figure 1B, fraction A increased during the 2nd hr of imbibition with a concomitant decrease in fraction B. Cyt oxidase in fraction C increased during this period, whereas the MDH decreased. During imbibition for 6 hr, fractions B and C disappeared almost completely, and fraction A became very active in both Cyt oxidase and MDH activities (4).

Properties of Mitochondrial Membrane from Dry Seeds. Membranes in fractions A, B, and C′ from dry seeds were fractured by sonic oscillation and centrifuged on the sucrose gradient until the equilibrium density was reached. Cyt oxidase in fraction A was recovered in lower regions of the gradient at densities 1.242 and 1.209 g/cm³ (Fig. 3A). The lighter peak resembled the activity peak from 6-hr cotyledons in the density and the specific activity (Fig. 3, A and D). Most of the Cyt oxidase in fraction B was present in a middle region of the gradient in a broad peak at mean density 1.164 g/cm³, coinciding with protein distribution

Table I. *Increases in Activities of Crude Mitochondrial Fraction from Pea Cotyledons during Imbibition*

Activities are expressed as units or nmoles O_2/min per pellet or supernatant obtained from 1 g of dry seed powder or from the cotyledons eq to 1 g of dry seeds.

Imbibition Time	Centrifugation Condition	Cyt Oxidase		MDH	Succinate-Cyt *c* Reductase	Respiration		
		25,000*g* or 80,000*g* pellet	25,000*g* or 80,000*g* supernatant			Activity	RCR	ADP/O
hr	*g* × *hr*		*units*		*units* × *10*²			
0	25,000 × 0.5	0.88	0.47	0.19		11	1.0	
0	80,000 × 1	1.09	0.28	0.19	2.3	12	1.0	
1	80,000 × 1	0.86	0.24	0.18	2.6	11	1.0	
2	80,000 × 1	1.78	0.41	0.31	5.2	71	1.1	0.40
6	80,000 × 1	2.72	0.28	0.35	10.0	152	1.6	0.81

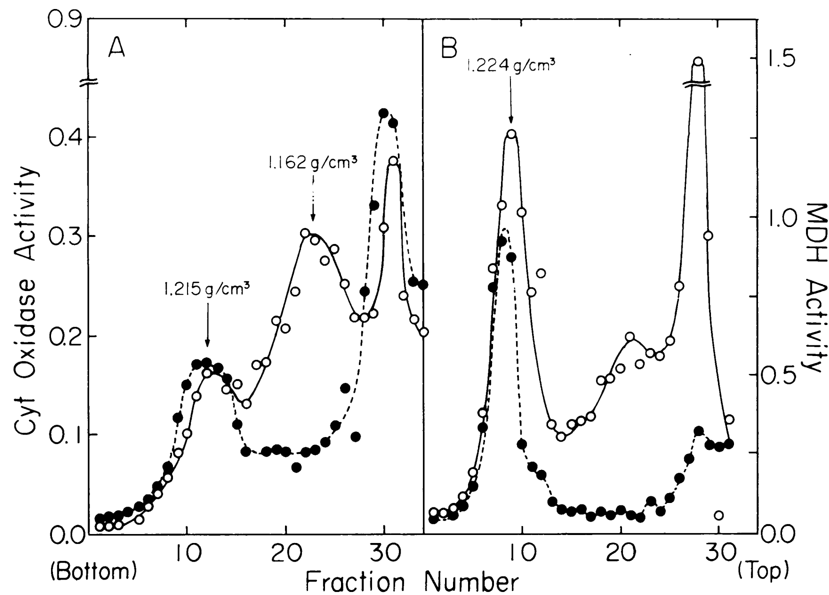

FIG. 1. Enzyme distribution after separation of 80,000g pellets from dry seeds and imbibed cotyledons on a sucrose density gradient. A: Dry eeds; B: cotyledons of the seeds imbibed for 2 hr. The suspension prepared from 5 g of dry seed powder or the cotyledons equal to 5 g of dry eeds was layered on a gradient. Cyt oxidase activity expressed as units per fraction (○); MDH activity expressed as units per fraction (●).

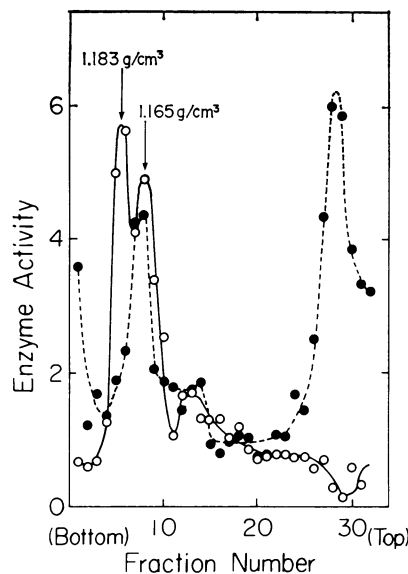

FIG. 2. Enzyme distribution after separation of fraction C from dry seeds on a sucrose density gradient. Fraction C from 5.5 g of dry seed powder was diluted with 3 times the volume of deionized H_2O and centrifuged at 80,000g for 1 hr. The pellet was suspended in 10% (w/v) sucrose and layered on 16 ml of a linear sucrose density gradient from 15 to 50% (w/v). After centrifugation at 72,000g for 3 hr, the gradient was divided into 0.5-ml fractions. Cyt oxidase activity expressed as units $\times$ 10^2 per fraction (○); MDH activity expressed as units $\times$ 40 per fraction (●).

(Fig. 3B). There was an additional minor peak of Cyt oxidase activity at a denser position, the density of which (1.214 g/cm³) was very similar to that of mitochondrial membrane from 6-hr cotyledons. Most of the contaminating chloroplast fragments in fraction B were sedimented at this position. Cyt oxidase in fraction C′ was recovered in a lower region of the gradient in a peak at density 1.209 g/cm³ (Fig. 3C). The specific activity of the peaks from fractions B and C′ was the same as that of the denser peak from fraction A.

There was no significant difference in the relative content of phospholipid to protein between the denser peak from fraction A and the peak from 6-hr cotyledons. The contents in the peak fractions of the former (fraction 4 in Fig. 3A) and of the latter (fraction 8 in Fig. 3D) were 0.306 to 0.345 and 0.262 to 0.364, respectively. The broad peak from fraction B (fractions 15 to 18 in Fig. 3B) was very rich in phospholipid (0.607–0.715), as expected from its density.

The polypeptide composition of mitochondrial membranes from fractions A, B, and C′ of dry seeds was compared by SDS-polyacrylamide gel electrophoresis of the solubilized membranes. Figure 4 shows the scans of polypeptide bands in the gels applied with these peak fractions and the mitochondrial peak from 6-hr cotyledons. There is a close correspondence between the bands of the membranes from fraction C′ and 6-hr cotyledons. There is also a similarity between the bands of the membranes from the denser peak of fraction A and from 6-hr cotyledons. There is an evident difference in that the former contained greater amounts of band numbers 8 and 10 (mol wt 33,800 and 20,000) than did the latter. The membrane from fraction B was quite different from the others in its composition. It contained much greater amounts of polypeptides with relatively low mol wt than did the others.

DISCUSSION

There seem to be two phases in mitochondrial development in pea cotyledons during the early stages of germination (4). In the first phase (the first several hr), marked increases in respiratory and enzyme activities of crude mitochondrial fraction are observed. During this period, immature mitochondria in dry seeds are considered to mature partially. Thereafter, there are marked increases in mitochondrial protein and phospholipid and some increase in the respiratory activity of isolated mitochondria. No increase in the activities of mitochondrial enzymes occurs in this period. In this study, some aspects of the development in the first phase have been presented. It is now evident that the development sets in after a lag phase lasting for at least 1 hr.

Our results indicate the existence of at least three types of mitochondrial membranes in dry pea seeds. The membrane of fraction B (B-type membrane) is very rich in phospholipid and lacking in

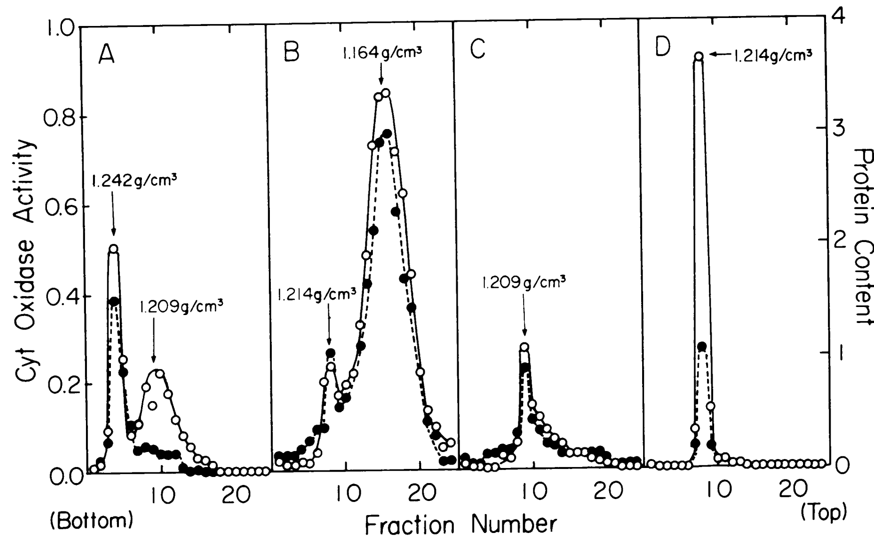

FIG. 3. Equilibrium centrifugation of mitochondrial membrane from dry seeds and imbibed cotyledons. A: Fraction A from dry seeds; B fraction B from dry seeds; C: fraction C′ from dry seeds; D: cotyledons of the seeds imbibed for 6 hr. Mitochondrial membrane fraction pre pared from 60 g of dry seed powder or from the cotyledons equal to 2 g of dry seeds was layered on a gradient. Cyt oxidase activity expressed as units per fraction (○); protein content in mg per fraction (●).

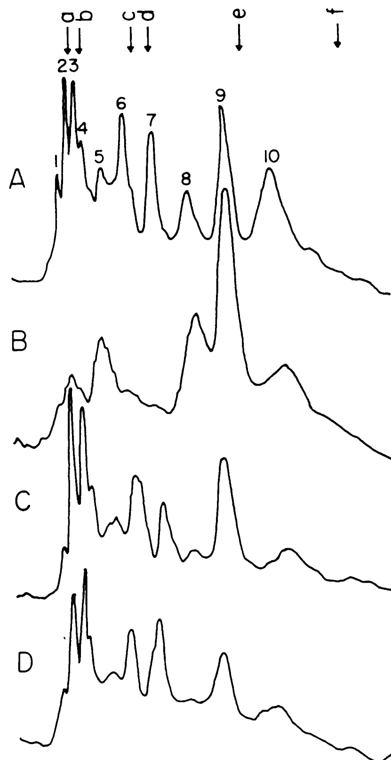

FIG. 4. Scans of SDS-polyacrylamide gels applied with solubilized mitochondrial membrane from dry seeds and imbibed cotyledons. Gels were scanned at 570 nm using a Joyce Loeble Chromatoscan. A: Denser peak from fraction A (fraction No. 4 in Fig. 3A); B: broad peak from fraction B (fraction No. 16 in Fig. 3B); C: fraction C′ (fraction No. 9 in Fig. 3C); D: cotyledons of the seeds imbibed for 6 hr (fraction No. 8 in Fig. 3D). Arrows indicate positions of marker proteins. a: BSA (mol wt 68,000); b: catalase (mol wt 60,000); c: ovalbumin (mol wt 45,000); d: aldolase (mol wt 40,000); e: trypsin (mol wt 23,300); f: Cyt *c* (mol wt 11,700).

polypeptides with relatively high mol wt, as compared with that of cotyledon mitochondria from the seeds imbibed for 6 hr (partially mature membrane). The B-type membrane must be of very fragile mitochondria, because fraction B did not carry MDH (Fig. 1A). The abnormal composition of the membrane may cause the susceptibility of the mitochondria to destruction through isolation. The data presented in Figure 1 suggest that the B-type membrane is transformed into the partially mature one within 6 hr after initiation of imbibition. The transformation must be brought about by assembly of polypeptides with relatively high mol wt into the phospholipid-rich membrane. Nawa and Asahi (5) showed that mitochondrial development in pea cotyledons during the early stage of germination did not require *de novo* synthesis of mitochondrial protein. We propose that the polypeptides assembled pre-exist in a soluble form in the fragile mitochondria or in the cytoplasm.

The other two types of mitochondrial membranes also differ from the partially mature membrane. Mitochondrial particles in fraction A resemble the partially mature mitochondria in sedimentation behavior through the sucrose gradient (Fig. 1) but not in polypeptide composition of the membrane (Fig. 4). We feel that this type (A-type) of mitochondrial membrane is not as stable as the partially mature one. During isolation, some of the A-type membrane seems to be broken into membrane fragments, which are not sedimented through the sucrose gradient. After centrifugation of the crude mitochondrial fraction from 2-hr cotyledons on the sucrose gradient, a considerable part of Cyt oxidase and little MDH were present on the top of the gradient (Fig. 1B). This may be interpreted to mean the existence of a large amount of the A-type membrane in the cotyledons. If that is the case, the B-type membrane is assumed to be transformed first into the A-type one, then into the partially mature one.

Mitochondrial particles in fraction C′ were difficult to sediment through the sucrose gradient (Figs. 1A and 2). The mitochondrial membrane disappears during the 2nd hr of imbibition, probably to be transformed into the partially mature one.

We infer that there are at least three types of mitochondria in dry pea seeds, the membranes of which differ in their biochemical properties as mentioned above. They may develop into mature mitochondria through assembly of protein pre-existing in the seeds into the membranes during the very early stage of germination. The mitochondria in dry seeds are very fragile and become stable as the protein is assembled in their membranes.

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