Cyanide-insensitive Respiration in Pea Cotyledons

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ABSTRACT
Mitochondria isolated by a zonal procedure from the cotyledons of germinating peas possessed a cyanide-resistant respiration. This respiration was virtually absent in mitochondria isolated during the first 24 hours of germination but thereafter increased gradually until the 6th or 7th day of seedling development. At this time between 15 and 20% of the succinate oxidation was not inhibited by cyanide. The activity of the cyanide-resistant respiration was also determined in the absence of cyanide. Relationships among mitochondrial structure, cyanide-resistant respiration, and seedling development are discussed.

MATERIALS AND METHODS

Plant Material. Pea seeds (Pisum sativum L. var. Homesteader) were soaked in tap water for 6 h. Following this, damaged and nonimbibed seeds were discarded. The remaining seeds were planted in horticultural grade Vermiculite and were germinated at 27 C in the dark for various periods. Harvesting was done at 24-h intervals following the onset of imbibition.

Isolation of Mitochondria. The isolation procedure was basically that of Hamman and Spencer (7). Washed cotyledons (about 250 g) were ground with a mortar and pestle for 8 min in 300 ml of ice-cold extraction medium of the following composition: 0.5 M mannitol, 5 mM EDTA, 0.5% BSA, 0.05% cysteine, and 0.050 mM Tris, with pH adjusted to 7.4 at 20 C with Tris. The brei was filtered through one layer of Miracloth and the filtrate was then centrifuged at 700g for 7 min. The supernatant layer was edge-loaded into a Ti-XIV zonal rotor spinning at 1,500 rpm at 2 C in a Beckman Spinc L2-65B centrifuge. Then a sucrose-step gradient was loaded into the rotor. The gradient consisted of 100 ml each of 33, 37.5, and 43% (w/w) sucrose, and sufficient 48% (w/w) sucrose to fill the rotor. The gradient also contained 0.1% (w/w) BSA and 1% (w/w) Tris, with pH adjusted to 7.4 at 20 C with Tris. Centrifugation was for 1 h at 37,000 rpm. Following centrifugation, the rotor was center-unloaded by use of a 50% (w/w) sucrose solution. Protein peaks were monitored with a Pharmacia UV Duo Monitor (model 200). The protein peak at the interface of the 37.5 and 43% steps was saved. This fraction was diluted slowly with a buffered solution (0.1% in BSA, 25 mM Tris, with pH brought to 7.2 at 20 C by the addition of Tris). The suspension was centrifuged for 10 min at 19,000g. The mitochondria were then resuspended in a medium consisting of 0.3 M mannitol, 4 mM MgCl₂ and 25 mM Tris, with the pH adjusted to 7.2 at 2 C with Tris.

The procedure for the "washed" mitochondria was the same up to and including the 700g centrifugation. Following this, the supernatant layer was recentrifuged at 21,000g for 5 min. The mitochondria were then resuspended in wash medium (0.3 M mannitol, 0.3% BSA, and 25 mM Tris, brought to pH 7.2 at 20 C with Tris). The centrifugation steps were repeated, and the mitochondria were resuspended in the same suspending medium and assay mixture as the zonally isolated mitochondria.

Respiratory Measurements. Respiratory measurements were made with a Yellow Springs Instrument Company model 53 O₂ monitor connected to a Beckman 100-mv potentiometric recorder. The procedures and media were those of Solomos et al. (20). When cyanide was used, it was added to mitochondria in state 4, following two or three cycles of ADP addition. After an O₂ uptake rate was established, ADP (approximately 150 nmol) was again added. The cyanide-resistant rate was taken to be the rate following this ADP addition. (As anticipated, with succinate, ADP added after cyanide had no effect on the rate.) The relative contribution of the cyanide-insensitive pathway was determined by the method of Bahr and Bonner (1) with minor modifications. (Succinate was 8.0 mM, ADP and ATP were 100 μM, and SHAM

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2 Abbreviation: SHAM: salicylhydroxamic acid.
was used as inhibitor of the cyanide-resistant pathway.) Washed mitochondria from 2-, 4-, 6-, and 8-day-old cotyledons were used at approximately 0.2 to 0.5 mg/ml. Cyanide concentration was 0.5 mM. The O₂ monitor chambers were washed thoroughly with 50% aqueous dimethylformamide to remove the hydroxamic acid between experiments.

**Succinate-Cyt c Reductase.** Succinate-Cyt c reductase activity was measured by the method of Douce et al. (6). The mitochondria were added to 3 ml of the usual succinate assay medium, which in this case also contained 0.02 μmol of Cyt c, and was 0.5 mM in KCN. The reaction was initiated by the addition of succinate to a final concentration of 8 mM and was followed by observing the increase in A at 550 nm on a Cary 15 recording spectrophotometer.

Osmotically burst mitochondria were obtained by assaying in the absence of mannitol. A 10 mM phosphate buffer was substituted for the regular assay medium.

**Protein.** Protein was first precipitated by addition of 0.5 ml of 20% trichloroacetic acid to 5 to 25 μl of the mitochondrial suspension. The pellet was redissolved in 0.5 N NaOH and brought to a final volume of 1 ml with distilled H₂O. The protein was then measured by the method of Lowry et al. (11), with human serum albumin as the standard.

**Chemicals.** All chemicals were of reagent grade and with the exception of the following were from Fisher Scientific Co. Cyt c (type III), NADPH, ADP, Tes, and cysteine were from Sigma Chemical Co., BSA and β-ketoglutaric acid were from Calbiochem, and SHAM was from Aldrich Chemical Co.

**RESULTS**

**Mitochondrial Integritiy and Purity.** A comparison between zonally isolated and washed mitochondria is given in Table I. The former mitochondrial preparation was buff in color, while the latter was always distinctly brown. Zonally isolated mitochondria consistently showed higher specific rates of O₂ uptake, and slightly better respiratory control ratios. However ADP/O ratios and the percentage of respiration inhibited by cyanide were similar in both preparations. Also shown in Table I are the succinate-Cyt c reductase activities. The succinate-Cyt c reductase activity with mannitol was less than 10% of the activity without mannitol. In mannitol this activity was somewhat lower with zonally isolated mitochondria than for washed mitochondria.

**Effect of Cyanide on Mitochondrial Respiration.** The effect of cyanide on mitochondria isolated from 6-day-old cotyledons is shown in Figure 1. Half-maximal inhibition occurs between 4 and 10 μM, but complete inhibition is not obtained even with levels of cyanide of 5 mM. The cyanide-insensitive respiration is inhibited by 1 mM SHAM, indicating that a cyanide-insensitive terminal oxidase similar to that described for other tissues (1, 4–6, 8, 9, 19) is present in the pea cotyledon mitochondria. Residual respiration was usually 1 to 2% of the state 3 respiration in the washed mitochondria, but was normally absent in the zonally isolated mitochondrial.

The amount of cyanide-insensitive respiration at various stages during the germination of the cotyledons, as well as other respiratory parameters, is shown in Table II. Mitochondria from 1-day-old cotyledons show virtually no cyanide resistance. By the 2nd day, a cyanide-resistant respiration has appeared. The amount of this cyanide-insensitive respiration increases gradually until the 6th or 7th day. Thereafter, it declines somewhat. At 6 to 7 days, approximately 30% of the malate and β-ketoglutarate O₂ uptake, and between 15 and 20% of the succinate oxidation is not inhibited by cyanide.

**Effect of SHAM on Succinate Oxidation.** The following results were obtained by the method of Bahr and Bonner (1). A plot of the reciprocal of the respiration rate against SHAM concentration in the presence of 0.5 mM KCN yielded a Kₛₜ of 170 μM. Plotting total respiration against the KCN-resistant respiration at various SHAM concentrations gave straight lines. The slope of these lines gave the portion of the cyanide-resistant respiration that was operating normally (without KCN present). This respiration was 20% of the maximum in the presence of cyanide, in state 3, and 50% in state 4 mitochondria. The age of the cotyledons from which the mitochondria were isolated did not influence the percentage of the cyanide-resistant pathway actually operating under state 3 or state 4 conditions. Because of the much larger capacity of the cyanide-resistant pathway at the later stages of germination, the cyanide-resistant pathway made up a larger part of the state 3 and state 4 rates at this time.

**DISCUSSION**

**Mitochondrial Integritiy and Purity.** The tests used here to assay for mitochondrial "purity" and "intactness" have been used by several authors (6, 9, 10, 18). The reduction of externally added Cyt c by succinate has been recommended as a measure of the damage to the outer mitochondrial membrane (6, 18). The low rates of succinate-Cyt c reductase show that mitochondria from both preparations had relatively little damage of the outer mitochondrial membrane. (Mitochondria from isolated peanut embryos have been shown to be deficient in Cyt c up to 16 h...
following a 40-min imbibition [21]. This implies that if high rates of succinate-Cyt c reductase are obtained at this stage it does not necessarily indicate mitochondrial damage. However, none of our preparations had high rates, suggesting that the Cyt c-deficient condition did not exist in this tissue at the stages tested. The zonally isolated mitochondria had higher maximal rates of O2 uptake, which probably indicates that there were fewer contaminating proteins in the zonal preparation (6). Both enzymic examination and electron microscopy have shown that pea cotyledon mitochondria from a zonal preparation similar to the one used here have a high degree of purity and intactness (7). They are fully developed mitochondria, in contrast to particles of various kinds obtained from fractions of other densities.

**Pattern of Development of Cyanide-resistant Respiration.** Pea cotyledons provide an interesting system to study since they undergo a number of striking developmental changes in a relatively short period of time. These developmental changes extend to the mitochondria. The mitochondria present in the dry seed are relatively disorganized, but develop rapidly to supply the energy needs of the cotyledons (2, 12–18, 23).

Mitochondria isolated after 4 h of imbibition have slow rates of O2 uptake and relatively poor respiratory control (Table II). After 24 h of imbibition the succinate-oxidizing system is well developed, and good respiratory control is observed. The respiratory activity with a-ketoglutarate as the substrate develops somewhat more slowly than does the activity with succinate (Table II). Apart from cyanide resistance, activity with a-ketoglutarate is similar for mitochondria from 0.2- and 1-day-old cotyledons. Between the 1st and 2nd days, cyanide resistance increased more than 2-fold. Malate oxidation is very low until the 2nd day of germination (Table II).

Increases in respiration rates continue until approximately the 5th or 6th day of germination. Thereafter, O2 uptake values tend to drop off slowly as do phosphorylative activities.

The development of cyanide resistance roughly parallels that of other respiratory activities. Figure 2 compares the development of two enzymic activities as determined by Solomos et al. (20), and the development of cyanide-resistant respiration.

The peak in cyanide-resistant respiration (at 6 days) coincides with the maximum O2 uptake by the mitochondria. At this time the respiratory control ratios and ADP/O values have decreased from the maximum values achieved earlier in germination. This is consistent with an increase in the proportion of succinate oxidation through the (nonphosphorylating) alternative pathway. Earlier work (12, 13, 20) with pea cotyledons in vivo and in vitro has shown that mitochondria are fully developed by day 4 of germination. This work also showed that Cyt oxidase activity was maximum near the 4th day. This is consistent with a maximum in alternate path contribution at 6 days. There is no cyanide-resistant respiration at the early stages of germination (Table II). This situation extends until after the first 24 h of germination. However, mitochondria from 1-day-old cotyledons are able to oxidize succinate rapidly. Earlier work (2, 12, 20) indicated that mitochondria...
present in dormant pea seeds have few membranes, but by day 4 to 5 they are fully developed. Both structure (2) and certain enzymic activities (Fig. 2 and ref. 20) deteriorate as the cotyledons age.

Low rates of cyanide-resistant respiration during the early stages of germination have been found in a similar type study done on chick peas (5) but are in contradiction to a view put forward recently (22) which held that a cyanide-resistant respiration operated very early in the germination of several seeds. The latter study did not utilize isolated mitochondria, and the results may reflect the involvement of nonmitochondrial oxidative pathways.

It is also interesting to compare Figure 2 with the three phases of germination defined by Bain and Mercer (2, 3). According to their studies phase 1 is a period in which the developing axis is not dependent on the storage reserves of the cotyledons. It ends when the radicle is approximately 40 mm long, about 2 days in our system. We have shown that this corresponds to the period when new mitochondria are developing in the cotyledons. During phase 2, from 2 to 4 days in our system, the axis is completely dependent on the reserves in the cotyledon. During this period the mitochondria in our system show good oxidative and phosphorylative activities. However, the greatest loss of reserve material from the cotyledons does not occur until phase 3. This is the period in which the mitochondria have the greatest O₂ uptake values, and also the largest amount of cyanide resistance. Palmer (18) has suggested that the cyanide-resistant pathway allows for the interconversion of the organic acids of the Kreb's cycle, even in the presence of a high energy charge, a process which may be occurring at this time.

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LITERATURE CITED