Appearance and Disappearance of Cyanide-Resistant Respiration in Vigna mungo Cotyledons during and following Germination of the Axis

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ABSTRACT

Mitochondrial preparations isolated from black gram (Vigna mungo L.) cotyledons exhibited cyanide-resistant respiration which was of mitochondrial origin. The appearance and the disappearance of this alternative respiration took place during and following imbibition. During the first 6 hours of imbibition, the respiration was completely inhibited by cyanide, but after this time the alternative respiration markedly developed, reaching a maximal cyanide-resistance 12 to 16 hours after the start of imbibition. Subsequently, the alternative respiration gradually disappeared. The actions of cycloheximide and chloramphenicol indicated that the appearance was dependent on cytoplasmic protein synthesis and that the disappearance depended on both cytoplasmic and mitochondrial protein synthesis. The alternative pathway contributed to state 4 respiration, but not to state 3 respiration, in mitochondria from 1-day-old cotyledons. On day 3, it contributed to neither state 3 nor state 4.

It is well known that CN'-resistant, alternative respiration is a widespread phenomenon in higher plants. Nevertheless, the studies on the alternative respiration in germinating seeds are scarce and have given inconsistent results (2, 13, 21).

In this study, we have sought to demonstrate that CN-insensitive respiration observed in black gram was of mitochondrial origin, and have examined in detail the appearance and the disappearance of the alternative respiration during and following germination.

MATERIALS AND METHODS

Plant Material. Seeds of black gram, Vigna mungo L. (formerly called Phaseolus mungo L.), treated with H₂SO₄ (16) were soaked in water at 28°C for 6 h in the dark. After imbibition, the seeds were husked and the two cotyledons of each seed were separated, the embryonic axis remaining attached to one of them. The cotyledons with the axis were placed on filter paper (in Petri dishes) moistened with water and incubated in the dark at 28°C. In some experiments, the cotyledons were incubated in N₂.

Isolation of Mitochondria. Cotyledons were disrupted in a Polytron homogenizer (Kinematica) for 7 s at setting 4.5 in 0.5 M mannitol, 1 mM EDTA, 0.05% (w/v) cysteine, and 0.1% (w/v) BSA in 30 mM phosphate buffer (pH 7.2; 0.5 ml/cotyledon).

Abbreviations: CN, cyanide; BHAM, benzohydroxamic acid; AP, alternative pathway; CH, cycloheximide; CP, chloramphenicol; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

After adjusting to pH 7.2, the homogenate was centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 20,000 g for 10 min and resultant pellet was washed once with 0.4 M mannitol, 1 mM EDTA, and 0.1% (w/v) BSA in 10 mM phosphate buffer (pH 7.2). The resulting pellet was suspended in a small volume of wash medium (washed mitochondria preparation). Further purification of the mitochondria was carried out by a slight modification of the method of Jackson et al. (11). The washed mitochondria fraction was loaded onto a discontinuous Percoll gradient prepared with final concentrations of 11.8% (v/v), 21% (v/v), and 45% (v/v) Percoll, each containing 0.25 M sucrose and 2 mM K₂HPO₄. The final pH was adjusted to 7.2 with HCl. Each gradient comprised 2 ml of 45%, 4.25 ml of 21%, and 4.25 ml of 11.8% Percoll mixtures. Centrifugation conditions were 20 min at 8,000g, utilizing an angle rotor. After separation and collection of the mitochondria fraction, it was suspended in wash medium, pelleted, and resuspended in wash medium (purified mitochondria preparation).

Mitochondrial Respiration. Mitochondrial O₂ consumption was measured polarographically with succinate as substrate at 30°C in 2.5 ml of reaction medium comprising 0.4 M mannitol, 4 mM MgCl₂, and 0.1% (w/v) BSA in 10 mM phosphate buffer (pH 7.2) (15).

Lipoxygenase Activity. The activity in mitochondrial preparations was determined polarographically in 2.5 ml of the same reaction medium as used for the assay of mitochondrial respiration. The reaction was initiated by the addition of linoleic acid (Wako Chemicals, final concentration 1.1 mM, added as 10 μl ethanolic solution).

Analysis of Respiration Pathways. The analysis was done following the method of Bahr and Bonner (1), where the total respiration rate (Vₜ) is

\[ Vₜ = p \cdot g(i) + V_{cya} + V_{res}. \]

In this equation, g(i) is the flux through the AP in the presence of CN at a given concentration of BHAM, and p is the proportion of the pathway which is actually operating. When BHAM is absent, g(i) will equal the maximal capacity of the AP, Vₚₚₚ, and is the flux through Cyta pathway. Vₚₚ is residual O₂ consumption which is insensitive to the combination of CN and BHAM. Since, in mitochondria from black gram cotyledons, O₂ uptake was completely inhibited by CN plus BHAM, Vₚₚ was zero.

RESULTS

Respiration of Washed Mitochondria. Respiratory activities of the washed mitochondria are shown in Figure 1. Respiratory rate, based on mitochondria obtained per cotyledon, increased during the first 2 d and declined on day 4. ADP/O ratios...
Fig. 1. Changes in activities of washed mitochondria from black gram cotyledons. A, State 3 respiration rate. B, RC ratio. C, ADP/O ratio. The means and se (vertical bars) of three to four replicates are shown. Respiration rate per protein weight of washed mitochondria from 3-d-old cotyledons was 102 nmol O₂/min·mg protein.

Fig. 2. Changes in CN sensitivity of respiration of washed mitochondria. KCN: 0.5 mM. The means of, and differences between (vertical bars), two replicates are shown.

remained at almost the same level during the experimental period. Changes in RC ratios were very characteristic. The ratios dropped markedly on day 1 and restored a high value on day 2. The effects of CN on the respiration are shown in Figure 2. The respiration of washed mitochondria from 3- and 6-h-old cotyledons was completely inhibited by CN. Even though the seeds were imbibed for 6 h in water into which the air was vigorously bubbled, no alteration of CN sensitivity was observed. After 6 h, CN resistance rapidly increased, reaching a maximum at 12 to 16 h of imbibition. After this time, CN sensitivity was gradually recovered. The addition of BHAM completely inhibited CN-insensitive O₂ uptake of the mitochondria from cotyledons of any age (data not shown). These results seem to show that the increase in CN insensitivity is due to the appearance of the AP. However, it has been claimed that hydroxamate sensitivity was not a sufficient criterion to classify a given O₂ uptake as being due to the mitochondrial AP, because, in some cases, hydroxamate-sensitive, CN-insensitive O₂ uptake in crude mitochondrial preparations has been attributed to activity of lipoxygenase contaminated in the preparations (8, 17). Miller and Obendorf (14) demonstrated that disulfiram could be used to distinguish between the alternative respiration and lipoxygenase O₂ uptake; that is, disulfiram inhibits the AP, whereas it has no effect on lipoxygenase activity. In washed mitochondria from 1-d-old cotyledons, disulfiram (90 μM) strongly inhibited the CN-resistant respiration (approximately 95% inhibition). This result indicates that the AP is responsible for the CN-insensitive respiration in black gram cotyledons.

Lipoxygenase Activity and CN Sensitivity in Purified Mitochondria. Goldstein et al. (8) demonstrated that the activity of lipoxygenase associated with the crude mitochondria fractions

Table I. Lipoxygenase Activity and Cyanide Sensitivity in Washed and Purified Mitochondria Isolated from 1-Day-Old Black Gram Cotyledons

<table>
<thead>
<tr>
<th>Lipoxygenase Activity</th>
<th>Inhibition by CN&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
</tr>
<tr>
<td>nmol O₂/min·cotyledon</td>
<td></td>
</tr>
<tr>
<td>Washed mitochondria</td>
<td>1.5</td>
</tr>
<tr>
<td>Purified mitochondria</td>
<td></td>
</tr>
<tr>
<td>Band 1</td>
<td>0.05</td>
</tr>
<tr>
<td>Band 2</td>
<td>0.17</td>
</tr>
</tbody>
</table>

<sup>*</sup> KCN concentration: 0.5 mM.

Table II. Effects of the Addition of BHAM on RC Ratios in Washed Mitochondria

<table>
<thead>
<tr>
<th>Cotyledon Age</th>
<th>H₂O</th>
<th>1 mM BHAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-h-old</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>1-d-old</td>
<td>2.2</td>
<td>2.8</td>
</tr>
<tr>
<td>3-d-old</td>
<td>3.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Table III. Respiratory Rates and RC Ratios in Washed Mitochondria from Black Gram Cotyledons Treated with CH and CP

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Day 1 O₂ uptake (State 3)</th>
<th>Day 2 O₂ uptake (State 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min-cotyledon</td>
<td>nmol/min-cotyledon</td>
</tr>
<tr>
<td>None</td>
<td>3.5</td>
<td>4.2</td>
</tr>
<tr>
<td>0.01 mM CH</td>
<td>4.5</td>
<td>2.1</td>
</tr>
<tr>
<td>0.3 mM CH</td>
<td>4.6</td>
<td>3.3</td>
</tr>
<tr>
<td>3 mM CP</td>
<td>3.7</td>
<td>1.9</td>
</tr>
<tr>
<td>3 mM CP + 0.3 mM CH</td>
<td>4.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Not determined.

Fig. 4. Effects of the presence of CH and CP during incubation of black gram cotyledons on CN sensitivity of state 3 respiration of washed mitochondria.

Table IV. Respiratory Activities and CN Sensitivity in Washed Mitochondria from 12-Hour-Old Black Gram Cotyledons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O₂ Uptake (State 3)</th>
<th>ADP/O</th>
<th>Inhibition by KCN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min-cotyledon</td>
<td></td>
<td>State 3</td>
</tr>
<tr>
<td>None</td>
<td>4.1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>N₂</td>
<td>3.5</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>KCN</td>
<td>2.0</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>CCCP</td>
<td>2.9</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

could be removed from them through purification of the organelle. We tried to purify mitochondria from 1-d-old cotyledons by Percoll density gradient centrifugation. Mitochondria aggregated at the interface of the 45% and 21% layers (Band 1), and at the interface of the 21% and 11.8% layers (Band 2). Examination with the electron microscope revealed that the mitochondria of both bands were essentially free of contamination of other organelles. The rates of succinate oxidation by mitochondria of Band 1 and Band 2 were 240 and 155 nmol O₂/min·mg protein, respectively. RC (2.3–2.4) and ADP/O ratios (1.4) (with succinate as substrate) were almost the same between the two populations of mitochondria. Purification of the mitochondria resulted in most of lipoxygenase activity being removed (Table I). However, the large loss of the enzyme activity was accompanied by little change in CN sensitivity (Table I). This observation clearly shows that the CN-resistant respirator observed in the crude mitochondria from black gram cotyledons is the alternative one of mitochondrial origin.

Capacity and Contribution of the AP. Figure 3 shows changes in the capacity for the AP, V̇O₂, of washed mitochondria. This value increased markedly during the first day, and subsequently declined. It was shown by determining ρ values that the AP was engaged in the state 4 respiration of the mitochondria from 1-d-old cotyledons (ρ = 0.65), but not in the state 3 (ρ = 0). In the mitochondria from 3-d-old cotyledons, no contribution of the AP was observed (ρ = 0 in both state 3 and 4), although a capacity for the AP still remained (Fig. 3).

When the washed mitochondria isolated from 6-h-, 1-d-, and 3-d-old cotyledons were treated with BHAM, RC values were altered; the mitochondria from 3-d-old cotyledons showed a notable increase in the value, while those from the other two cotyledons showed a little decrease (Table II).

Effects of CH and CP. The seeds were imbibed in CH (0.01 and 0.3 mm) or CP (3 mm) solution for 6 h, then transferred to filter paper moistened with the same CH or CP solution, and incubated for 1 or 2 d. In Table III are shown the activities of mitochondria from the antibiotic-treated cotyledons. CH treatment brought about a stimulation of mitochondrial respiration. At present, we have no explanation for this stimulating effect of CH. CP showed little effects on respiration rates. Treatments with 10 µM CH or 3 mM CP resulted in a reduction of RC ratio, while treatment with 0.3 mM CH caused an increase. ADP/O ratios were little altered by these treatments (data not shown). Changes in CN sensitivity of respiration are shown in Figure 4. The increase in CN resistance during the 1st d was almost completely inhibited by CH (0.3 mM) treatment. Inhibition of the development of the AP by CH has also been reported in yeast (5, 10), potato (4), and chick pea (2). On the other hand, CP accelerated the development of CN resistance during the 1st d. The presence of CH (0.3 mM) during incubation with CP completely canceled this accelerating effect of CP. The restoration of CN sensitivity during the 2nd d was blocked by CH and CP treatment.

The membrane integrity of mitochondria from antibiotic-treated cotyledons was determined by measuring the activity of Cyt oxidase (12). The intactness was not impaired by these.
antibiotic treatments (data not shown). Furthermore, CH and CP at the concentrations used in the present study had little influence on the activities of mitochondria when added after isolation (data not shown).

Effects of Anaerobiosis and of Treatments with Some Respiratory Inhibitors. In Table IV are shown the activities and the CN sensitivity of mitochondria from 12-h-old cotyledons treated with N₂, KCN, or CCCP during the period between 6 and 12 h after the start of imbibition. The respiratory rates of mitochondria were reduced by these treatments. The ADP/O ratios decreased in the case of KCN treatment. On the other hand, these treatments brought about an increase in the RC ratios. The development of CN resistance during the first 12 h was inhibited by these treatments.

DISCUSSION

It has been reported in potato slices (9), cultured sycamore cells (19), and germinating chick peas (2) that molecular O₂ is involved in the appearance of the AP. This is also confirmed in the present study by the fact that incubation of cotyledons under anaerobic conditions after the transfer prevented the development of the AP (Table IV). It is as yet not clear whether O₂ acts as a trigger of the induction of the AP or whether its absence has an indirect effect, i.e. the prevention of respiration producing a lack of ATP for protein synthesis (10). In black gram cotyledons, inhibition of the Cyt-mediated respiration by KCN or of ATP formation by an uncoupler (CCCP) resulted in disturbance of the development of the AP (Table IV). In addition, the appearance of the AP was dependent on cytoplasmic protein synthesis (Fig. 4). Therefore, it is probable that, in black grams, the inhibition of the appearance of the AP under anaerobic conditions is brought about through the prevention of Cyt-mediated respiration. A similar result has been reported in chick peas (2).

Taking this O₂ effect into consideration, there is a possibility that the lack of the activity of the AP in mitochondria from cotyledons in the early stage of imbibition (during the first 6 h) was due to a limitation of O₂ supply during this period. However, the respiration of the mitochondria from 6- to 12-h-old cotyledons which were imbibed in aerated water was also completely inhibited by CN. Therefore, the absence of the activity of the AP during the first 6 h of imbibition is apparently not due to the inhibition of the development of the pathway resulting from the lack of O₂, although it is possible that the aeration by bubbling was not adequate.

The actions of CH and CP show that the changes in mitochondrial properties during and following imbibition, i.e. appearance and disappearance of the AP, are dependent on protein-synthesizing systems; the appearance seems to depend on cytoplasmic protein synthesis but not on mitochondrial protein synthesis, and the disappearance may depend on both cytoplasmic and mitochondrial protein synthesis. The specificity of these inhibitors, however, can be questioned. There are some reports of inhibitory actions of CH or CP on electron flow and the energy coupling in mitochondria (6, 18). In our case, CH and CP showed little effect on activities of isolated mitochondria. Thus, the changes in mitochondrial properties induced by CH or CP treatment are apparently due to a direct effect of the antibiotics on protein-synthesizing systems. As to stimulating effect of CP on the development of the AP, the problem seems to be complex. Edwards et al. (5) reported that treatment of Neurospora cells with CP induces the appearance of the AP and that CH treatment cancels the effect of CP. Some similarity of effects of the antibiotics seemingly exists between Neurospora and black grams. Edwards et al. (5) proposed a model of a mitochondrialy synthesized repressor protein action on nuclear genes coding for the alternative oxidase. It is not known whether this is also the case with black grams.

It is generally believed that oxidation of succinate (substrate used here) through the AP is not linked to phosphorylation (3), although there is some objection to this (20). Therefore, if the AP contributes to state 3 respiration, ADP/O ratio will be diminished. However, no depression was observed even in mitochondria from 1-d-old cotyledons which had a high AP capacity (Fig. 1), indicating no contribution of the AP to state 3. In fact, ρ values show that on day 1 the AP contributes to state 4, but not to state 3. The decreased RC ratio of the mitochondria from 1-d-old cotyledons (Fig. 1) can be explained in terms of participation of the AP only in state 4 respiration. This is substantiated from the fact that the decreased RC ratio was reversed by the addition of BHAM (Table II). In this connection, it can be reasonably thought that the increase in RC ratios observed in mitochondria from cotyledons treated with CH (0.3 mM), N₂, KCN, or CCCP was caused by the inhibition of the development of the AP (Tables III and IV). Decreased RC ratio in mitochondria from CP-treated cotyledons (Table III) can also be explained in terms of acceleration of the development of the AP by the CP treatment.

Respiratory transitions similar to that in black grams have been reported in chick peas and peas by Burguillo and Nicolas (2), and James and Spencer (13), respectively; that is, CN-sensitive O₂ uptake in the early stages of germination and subsequent shift to the alternative respiration. However, the time in which the capacity of the AP reaches a maximum is quite different among them; 3 to 4 d after the start of imbibition in chick pea, 6 to 7 d in pea, and 12 to 16 h in black gram. James and Spencer (13) mentioned that mitochondria in pea cotyledons are fully developed by day 4 to 5 of germination and that, after this time, both structure and enzyme activities deteriorate. Unfortunately, in chick peas no data are presented concerning mitochondrial development (2). As to peas, therefore, it is indicated that the AP reaches a maximum capacity in the stage of deterioration of mitochondria. In black grams, on the contrary, the AP develops early in the imbibitional stages, hence long before the stages of mitochondrial deterioration. In this sense, the developmental pattern of the AP is quite different between peas and black grams. This inconsistency might be due to a difference in plant material used. But we have no definite explanation for this.

Yentur and Leopold (21) and Esashi et al. (7) presumed that the early stages of germination of soybean and cocklebur seeds, respectively, are dependent on the alternative respiration. The results presented here also suggest a possibility that the AP, whose appearance and disappearance are dependent on protein synthesis, plays some role in the early stages of germination of black gram seeds. However, in order to confirm and define the nature of this role, further investigations are necessary.

LITERATURE CITED

8. GOLDSTEIN AH, JO ANDERSON, RG MCDANIEL 1981 Cyanide-insensitive and
12. JACKSON C, AL MOORE 1979 Isolation of intact higher plant mitochondria. In E Reid, ed, Plant Organelles. Ellis Harwood Ltd., Chichester, pp 1–12