Effect of Benzyladenine on Some Enzymes of Mitochondria and Microbodies in Excised Sunflower Cotyledons

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ABSTRACT

Benzyladenine (BA) increases the rate of expansion of dark-grown sunflower (Helianthus annuus L.) cotyledons. The hormone slightly enhances the development of the two glyoxysomal enzymes, isocitrate lyase and malate synthetase, during the first 3 days of germination and greatly accelerates their decay in the 2 following days. The levels of the peroxisomal enzymes, glycolate oxidase and glyoxylate reductase, are enhanced by BA more than those of the two glyoxysomal enzymes. These effects of BA on microbody enzymes are very similar to those of white light. Mitochondrial enzyme activities are increased to a varying extent by BA: the increase is minimal for fumarase, and maximal for cytochrome oxidase. The level of cytochrome oxidase is enhanced 346% at the 5th day of germination. Also, the rate of O₂ consumption is increased by BA, but the time course of this increased O₂ consumption does not match with that of cytochrome oxidase. Fusicoccin, a fungal toxin, mimics the effect of BA on cotyledon expansion, but fails to duplicate its action on microbody enzymes. This suggests that the effect of BA on microbody enzymes is not closely linked with the mechanism of growth promotion.

Cytokinins promote the expansion of cotyledons in several plant species (7, 17, 19). The effect on growth is associated, at least in some cases, with an accelerated mobilization of starch reserves (1, 9, 10). We have recently observed that BA also accelerates the disappearance of reserve fat in excised sunflower cotyledons (O. Servettaz and C. P. Longo, unpublished data). Since mitochondria and glyoxysomes collaborate in the utilization of fat reserves during germination, we have investigated whether the treatment with BA raises the level of some representative glyoxysomal and mitochondrial enzymes. Our results show that BA stimulates the development of several enzymes of mitochondria and microbodies and that its effect on microbody enzymes is very similar to that of white light.

MATERIALS AND METHODS

Sunflower (Helianthus annuus L.) seeds of the oil-rich Rumanian strain H.S. 52 were selected for uniform size. After removal of the seed coat, the embryos were soaked for 24 hr between two layers of moist filter paper at 27 °C in the dark. The cotyledons were then excised from the embryonic axis and cultured in darkness at 27 °C in Petri dishes on two discs of filter paper saturated with distilled H₂O or with a 0.1 mM BA solution. The time of excision of the cotyledons was taken as day zero. The cotyledons increased in fresh weight for at least 6 days after excision. No signs of contamination with bacteria or fungi were detected during this time span.

Crude extracts for determination of total enzyme activities were prepared as follows. Thirty cotyledons were thoroughly ground in a mortar with 4 volumes of medium (50 mM K phosphate, pH 7.4; 2 mM Na-EDTA; 10 mM mercaptoethanol). The brei was squeezed through four layers of cheesecloth and centrifuged at 27,000g for 15 min. An aliquot of the supernatant was carefully removed with a Pasteur pipette without disturbing the fat layer that floated on the top of the centrifuge tube. The precipitate was resuspended in 2 ml of the grinding medium. Enzyme activities were determined in the precipitate and in the supernatant. The sum of the total activities in the precipitate and in the supernatant was used to estimate total activity per cotyledon. We found that this method was the best one for obtaining reliable values of enzyme activities. The centrifugation step was necessary for removing the fat that severely interfered with the absorbance readings. Mixing the precipitate and the supernatant after removal of fat did not yield as reproducible results as the separate assay of the two fractions. It was not possible to discard the 27,000g pellet since several reextractions with the grinding medium failed to solubilize a conspicuous fraction of some enzyme activities. The use of detergents resulted in an effective solubilization of enzymes from the pellet, but the variability among replicate samples was larger than with our method.

A crude particulate fraction was prepared from batches of 150 cotyledons. The cotyledons were chopped in a Petri dish for 10 min with 4 volumes of grinding medium (20 mM HEPES, pH 7.5; 10 mM KCl; 0.1 mM MgCl₂; 1 mM Na-EDTA; 10 mM mercaptoethanol; 0.1% BSA). The brei was filtered through four layers of cheesecloth and centrifuged at 480g for 10 min. The supernatant was recentrifuged at 27,000g for 15 min. The resulting pellet was suspended in 8 ml of the grinding medium (without mercaptoethanol).

Enzyme assays were those described in the literature as follows: isocitrate lyase (6), malate synthetase (14), glyoxylate reductase (27), glycolate oxidase, oxygen electrode method (3), Cyt oxidase (24), succinate dehydrogenase (16), malate dehydrogenase (20), fumarase (21). Protein was determined according to Breidenbach et al. (2).

Oxygen consumption was determined on samples of 4 to 10 cotyledons in Warburg vessels at 27°C. Respiration rates were linear for at least 6 hr.

RESULTS

Effect of BA on Growth. Preliminary trials indicated that the optimal concentration of BA for promoting cotyledon expansion (measured as increase in fresh weight) is 0.1 mM. This concentration was maintained throughout in the following experiments. Figure 1 shows the effect of BA on the growth pattern of excised sunflower cotyledons. The cotyledons (both control and treated) did not show significant changes in dry weight during the observed growth period.

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Effect on Glyoxysomal Enzymes. The development of isocitrate lyase and malate synthetase, two typical glyoxysomal enzymes, follows the well known pattern that has been described in a large number of fat-storing seeds (Fig. 2). Enzyme activities are very low in the dry cotyledon (not shown); they increase quickly during the hydration phase, and peak on day 1 (isocitrate lyase) or on day 3 (malate synthetase). In the interval from day 3 to day 5, the levels of both enzymes sharply decrease. BA increases the activity of both enzymes in the first 2 days and promotes their decline in the following 3 days. The effect is more conspicuous for malate synthetase than for isocitrate lyase and the decline of enzyme activities seems to be affected to a higher degree then the rise. At day 5, malate synthetase has practically disappeared in the BA-treated cotyledons, whereas in the water control, its activity is still as high as 40 to 50% of the peak value. Thus, the overall effect of BA on the two glyoxysomal enzymes is a steepening of their activity curve on both sides.

The compartmentation of isocitrate lyase and malate synthetase is apparently not affected by BA. The percentage of total activity that is recovered in the crude particulate fraction stays around 50 to 60% over the whole period studied. The fact that this percentage does not decrease during the declining phase of the enzymes suggests that isocitrate lyase and malate synthetase are not released into the cytosol when the glyoxysomes are destroyed. It is more likely that the enzymes are inactivated as soon as the organelles are broken down.

Effect on Peroxisomal Enzymes. BA stimulates the peroxisomal enzymes, glycolate oxidase and glyoxylate reductase. In dark-grown cotyledons, these enzymes do not exhibit the rise and fall pattern characteristic of the glyoxylate cycle enzymes, but they remain at a low and constant level from day 1 until day 5. BA not only enhances enzyme activities, but also changes somewhat this developmental pattern (Fig. 3). In the treated cotyledons, NADH-dependent glyoxylate reductase reaches a peak on day 2 and declines in the following 3 days, whereas glycolate oxidase increases steadily until day 4. Maximal stimulation is 260% for glycolate oxidase and 120% for glyoxylate reductase. The use of hydroxypropionate as substrate in the glyoxylate reductase assay did not result in higher rates, in contrast with the observations of other authors (26). This seems to be a peculiarity of the sunflower cotyledon; in watermelon cotyledons, we measured 3- to 5-fold higher rates with hydroxypropionate. The activity of the watermelon enzyme is increased 5-fold by BA at day 3.

Effect on Mitochondrial Enzymes and on Respiration Rate. The mitochondrial enzymes studied by succinate dehydrogenase, fumarase, malate dehydrogenase, Cyt oxidase) all show the same developmental pattern in the water control: the activities rise steeply from day 0 to day 1 and remain at a relatively constant level from day 1 until day 5. BA stimulates the development of all of the four enzyme activities: the stimulation is, however, much stronger for Cyt oxidase and succinate dehydrogenase than for fumarase (Fig. 4). Identical results were obtained when the activities were assayed in the crude particulate fraction instead of the total homogenate. Only in the case of malate dehydrogenase, the results did not coincide because of the presence of a very active cytoplasmic isozyme. BA decreases about 20% the level of this cytoplasmic isozyme (asayed in the supernatant of the crude particulate fraction) while the particulate enzyme is stimulated 20 to 40%, depending on the stage of development. This stimulation can be due to an increase of the mitochondrial as well as of the glyoxysomal malate dehydrogenase activity. A run of the crude particulate fraction on a sucrose gradient showed, however, that the increase of particulate malate dehydrogenase induced by BA was due entirely to the mitochondrial component.

Cyt oxidase yields maximal response to BA in comparison to the other enzymes studied by us: the stimulation reaches 346% (mean of five experimental values) at day 5.

The strong increase in succinate dehydrogenase and Cyt oxidase suggested that respiration could be affected by the hormone. We observed, indeed, that BA strongly stimulates O2 uptake. The time course of the increase in respiration does not match, however, that of the two mitochondrial enzymes. Figure 5 shows that the hormone stimulates respiration until day 3,

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**Fig. 1.** Effect of 0.1 mM BA on growth of excised cotyledons in the dark. The time of excision of the cotyledons (after 24 hr of imbibition) was taken as day zero. Closed symbols: BA; open symbols: water control. Vertical segments indicate standard deviations.

**Fig. 2.** Effect of 0.1 mM BA on isocitrate lyase and malate synthetase activities of sunflower cotyledons. Hatched bars: BA; light bars: water control. Each value represents the mean of five independent experiments. Vertical segments indicate standard deviations.

**Fig. 3.** Effect of 0.1 mM BA on glyoxylate reductase and glycolate oxidase activities of sunflower cotyledons. Hatched bars: BA; light bars: water control. Each value represents the mean of five independent experiments. Vertical segments indicate standard deviations.
hormones displays several other effects that are strikingly similar to those of light, e.g. stimulation of chloroplast morphogenesis (13), enhancement of several photosynthetic enzymes (8), stimulation of anthocyanin synthesis (23), prevention of leaf senescence (18, 25). A stimulation of glyoxylate reductase and glycolate oxidase by BA has already been reported for etiolated ryegrass leaves (5), although the effect on the decay of glyoxysomal enzymes had not yet been detected, to our knowledge. Whether the changes in activity of microbody enzymes induced by BA also involve changes in the developmental cycle of the whole organel remains an open question.

By and large, the enzyme data suggest that BA can accelerate the well known transition from glyoxysomes to peroxisomes that occurs when a cotyledon changes its function from storage to photosynthesis. Cytokinins could possibly become a valuable tool for studying the glyoxysome→peroxisome transition.

The effect of BA on the mitochondrial enzymes is apparently not similar to any known effect of light. Kagawa et al. (15) have reported that in watermelon cotyledons, light does not significantly affect the levels of succinate dehydrogenase and fumarase. BA, however, strongly stimulates the development of mitochondria in the same material (C. P. Longo, unpublished results).

The enzymes of mitochondria are not equally stimulated by BA: the effect is much larger for Cyt oxidase and succinate dehydrogenase than for fumarase and malate dehydrogenase. A similar unbalanced increase in mitochondrial enzymes has been described by Sakano and Asahi (22), who observed that in aging sweet potato root slices, Cyt oxidase increases at a faster rate than the other mitochondrial enzymes.

Our finding that BA strongly increases O2 consumption was unexpected. Most research workers have found that cytokinins inhibit O2 uptake (25). Some authors have also reported stimulations (11, 12), but these were comparatively small (30-40%),

Fig. 4. Effect of 0.1 mM BA on mitochondrial enzyme activities of sunflower cotyledons. Hatched bars: BA; light bars: water control. Succinate dehydrogenase, fumarase, and Cyt oxidase activities were measured in the whole homogenate; malate dehydrogenase was measured in a crude particulate fraction. Each value represents the mean of five independent experiments. Vertical segments indicate standard deviations.

whereas it has no effect at days 4 and 5, just at the time when Cyt oxidase is maximally stimulated.

Effect of Fuscoxicin. We have compared the effects of BA with those of fuscoxicin, a fungal toxin that has been reported to stimulate cotyledon expansion (23). The results are summarized in Table 1. The effect of FC1 on growth is similar in magnitude to that of BA, although some qualitative differences are observed. (The FC-treated cotyledons are larger in surface area, but thinner and more curled at the edges.) FC enhances Cyt oxidase by 58% and partially prevents the fall of isocitrate lyase and malate synthetase that occurs in the controls between day 2 and day 4. Thus, the effect of the toxin on the decay of the two glyoxysomal enzymes is exactly opposite to that of BA. FC has no significant effect on the two peroxisomal enzymes.

DISCUSSION

The effect of BA on the glyoxylate cycle enzymes of sunflower cotyledons is strikingly similar to that of white light. In both cases, the rise of enzymic activities is slightly stimulated in the first days of germination, whereas their decay is greatly accelerated in the following days (4, 15). The effect of BA on the peroxisomal enzymes, glycolate oxidase and glyoxylate reductase, is also similar to that of light. The light-mimicking action of BA shown by our experiments fits well into the over-all picture of the action of cytokinins. It is known, indeed, that this class of

![Graph showing effect of 0.1 mM BA on mitochondrial enzyme activities of sunflower cotyledons.](https://example.com/fig4)

![Graph showing effect of 0.1 mM BA on respiration rate and Cyt oxidase activity of excised sunflower cotyledons.](https://example.com/fig5)

Fig. 5. Effect of 0.1 mM BA on respiration rate and Cyt oxidase activity of excised sunflower cotyledons. Closed symbols: BA; open symbols: water controls. Each value represents the mean of three independent experiments (respiration) or five independent experiments (Cyt oxidase).

Table 1. Comparison of the Effects of Benzyladenine and Fuscoxicin on Growth and Enzyme Levels of Sunflower Cotyledons

<table>
<thead>
<tr>
<th>Effect</th>
<th>H2O</th>
<th>SA</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>76</td>
<td>150</td>
<td>154</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td>152</td>
<td>195</td>
<td>155</td>
</tr>
<tr>
<td>Malate synthetase</td>
<td>210</td>
<td>312</td>
<td>234</td>
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<tr>
<td>Glyoxylate</td>
<td>72</td>
<td>179</td>
<td>80</td>
</tr>
<tr>
<td>Cyt oxidase</td>
<td>57</td>
<td>200</td>
<td>63</td>
</tr>
</tbody>
</table>

1 Abbreviation: FC: fuscoxicin.

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whereas we have observed stimulations as high as 118% (on a cotyledon basis) or 129% (on a dry weight basis). It is unlikely that BA stimulates respiration by increasing the level of Cyt oxidase since the time courses of the two activities do not coincide. A more likely explanation is that the increase of the over-all metabolic work due to BA induces a higher respiration rate through the mechanism of respiratory control.

The experiments with FC show that this toxin is able to mimic only the effect of BA on cell expansion, but not that on microbody enzymes. We had already obtained similar results with another strain of sunflower that accumulates anthocyanins in the dark in presence of BA (23). FC stimulated growth in this strain as did BA, but failed to duplicate the effect of BA on anthocyanin synthesis. Our data suggest that the effect of BA on microbody enzymes as well as that on anthocyanin synthesis can be considered as side effects, not directly linked with the growth-promoting action of the hormone.

LITERATURE CITED