Relationship between Fructose 2,6-Bisphosphate and Carbohydrate Metabolism in Darkened Barley Primary Leaves

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ABSTRACT

Initial dark fructose 2,6-bisphosphate levels in 10-day-old barley (Hordeum vulgare L.) leaves increased when the photosynthetic period was lengthened, when the temperature during the prior photosynthetic period was reduced, and following leaf excision. These treatments also increased the leaf sucrose concentration. Conversely, a decrease in dark fructose 2,6-bisphosphate occurred during extended darkness, with increasing leaf age and when photosynthetic in the leaf was reduced by earlier low light treatments. These variations in fructose 2,6-bisphosphate content correlate with known changes in dark respiration. These findings suggest, but do not conclusively prove, a causal relationship between dark fructose 2,6-bisphosphate levels and dark respiration rates.

The concentration of F2,6-P2,1 in barley leaves increases about 10-fold at the onset of darkness and then decreases slowly over the next several hours (17). The metabolic significance of this changing F2,6-P2 profile is poorly understood. A decrease in F2,6-P2 in the light and a subsequent increase in effector level in the dark has been interpreted as evidence for feedforward regulation of sucrose synthesis in source leaves (17). Based on in vitro enzyme analysis, an increase in F2,6-P2 concentration could simultaneously activate PPI-PFK (7, 8, 12) and inhibit cytosolic FBPase (10, 21, 22). Accordingly, in darkened photosynthetic tissue, as in nonphotosynthetic tissues (7, 14, 20), high F2,6-P2 levels might stimulate glycolysis as well as inhibit sucrose synthesis.

In the present study we have examined the role of F2,6-P2 in the regulation of glycolysis in darkened barley leaves. Leaf metabolite levels were measured under conditions known to alter the rate of dark respiration, a major consumer of glycolytically directed carbon in mature leaves. The results show a positive correlation between dark F2,6-P2 levels and dark respiration.

MATERIALS AND METHODS

Plant Materials. Barley seedlings (Hordeum vulgare L. cv Brant) were grown in controlled environment chambers (model M-3, Environmental Growth Chambers, Chagrin Falls, OH)2

1 Abbreviations: F2,6-P2, fructose 2,6-bisphosphate; PPI-PFK, pyrophosphatase:fructose 6 phosphate 1-transferase; FBPase, fructose 1,6-bisphosphatase; G6P, glucose 6-phosphate; PPFD, photosynthetic photon flux density.
2 Names of products are included for the benefit of the reader and do not imply an endorsement or preferential treatment by the United States Department of Agriculture.

from single seeds planted in 25 ml conical, plastic tubes filled with vermiculite. Growth conditions were 510 to 550 μmol m−2 s−1 PPFD, 12-h photoperiod, 20 ± 2°C and 60 to 65% RH. Plants were watered daily with a complete mineral nutrient solution. Unless otherwise noted, experiments were conducted on the primary leaf of 10-d-old seedlings. For extended dark treatments, plants were placed in a humidified incubator at 25°C and were watered, as needed, with one-sixth strength nutrient solution. Watering and harvesting were performed under green safelights. Samples of four individual leaves were harvested at indicated times and were quick-frozen in liquid N2 to stop metabolic activity. Leaves were extracted immediately or, if necessary, were stored overnight at −80°C.

Leaf Extraction and Metabolite Analysis. Barley primary leaves were extracted using buffered methanol-chloroform-water as described previously (17). Leaf starch, Chl, sucrose, glucose, fructose, G6P, and F2,6-P2 were measured as previously described (6, 16). Unless otherwise noted, values represent means ± se of four plants per measurement.

Leaf Gas Exchange Measurements. Rates of net carbon exchange by single, intact barley primary leaves were measured with a differential IR gas analyzer (model 865, Beckman Instr.) equipped with water vapor filters. Temperature, light, CO2, and humidity regimes were as used for plant growth. Individual leaves were placed in an acrylic plastic chamber that was connected to an open gas exchange system and measurements were made continuously over the dark period.

RESULTS AND DISCUSSION

Effects of Environmental Treatments in the Light on Barley Leaf F2,6-P2 Levels in the Dark. Treatments that increase the soluble carbohydrate content of the leaf also result in an increase in the initial rate of respiration in the dark (2, 4, 5, 11, 13). To determine the effect of high soluble carbohydrates on initial dark F2,6-P2 levels, three approaches were used for altering the soluble carbohydrate content of the leaf. The first involved shortening the photosynthetic period. It has previously been reported that decreasing the daily photoperiod from 12 to 4 h decreased the amount of F2,6-P2 present in barley leaves during the first hour of the dark period (17). Barley seedlings exposed to a 12-h photosynthetic period at 500 μmol m−2 s−1 contained 0.27 nmol F2,6-P2/mg Chl in the light, and this value increased 8-fold in the dark (Table 1A). By comparison, when barley seedlings were given a 4-h photosynthetic period at 500 μmol m−2 s−1 followed by 8 h of light at 100 μmol m−2 s−1, there was 0.38 nmol F2,6-
P2/mg Chl at the end of the light period. Only a slight increase in F2,6-P2 was observed in these plants after 30 min of darkness. Total leaf sucrose also increased from 20 to 33 μmol/mg Chl when the photosynthetic period was increased while the photoperiod was held constant. These results suggest that dark levels of F2,6-P2 in barley leaves are affected more by the length of the
prior photosynthetic period (i.e. the extent of soluble carbohydrate accumulation) rather than by the photoperiod per se.

A second method for increasing the carbohydrate content of leaves is by low temperature treatment. The sucrose content of barley leaves was 20 and 44 μmol/mg Chl at 20°C after 4 and 12 h of light, respectively (Table IB). Comparable leaf samples exposed to an air temperature of 10°C contained 36 and 72 μmol sucrose/mg Chl, respectively. For all treatments, F2,6-P2 levels did not vary greatly when the leaves were harvested in the light. However, after 30 min of darkness, F2,6-P2 levels were several-fold higher in leaves exposed to low temperature during the preceding light period. In these experiments all dark samples were harvested at an air temperature of 20°C. Note that 4 h at 10°C resulted in a large increase in dark F2,6-P2 levels similar to that previously observed after 12 h of photosynthesis at 20°C. Taken together, results in Table I show that environmental manipulations that alter the soluble carbohydrate content of barley leaves have a significant effect on F2,6-P2 levels during the early dark period.

Effects of Leaf Excision on Photosynthetic Metabolite Levels in Darkened Barley Primary Leaves. A third approach for altering leaf carbohydrate content is through excision. In the present study, barley primary leaves were excised immediately after a light-to-dark transition and the leaves placed in vials containing 0.5 ml of deionized water to maintain transpiration. Leaves were then maintained in standard conditions in the growth chamber. Sucrose and starch decreased during the dark period in intact leaves, whereas in the excised leaves only small changes in these metabolites were noted (Fig. 1). F2,6-P2 levels were consistently higher in the excised leaves as compared to the intact controls. In spite of the high sucrose content of the excised leaves, at the start of the next light period F2,6-P2 levels decreased to control values. The above results support an earlier suggestion (17) that starch degradation only proceeds in barley leaves after sucrose and F2,6-P2 decrease to low levels in the dark.

Relationship between Leaf Age and F2,6-P2 Levels in the Light or Dark. Dark respiration rates decline with leaf age (3, 9, 11, 18, 24). To determine the effect of leaf age on sucrose and F2,6-P2 levels, these compounds were measured in primary leaves obtained from 9-, 14-, and 21-d-old barley plants (Table II). The developmental stages of these leaf samples correspond to full expansion, maximum photosynthetic rate, and late senescence, respectively. The total amount of sucrose stored in the barley primary leaf during the light period did not vary significantly with age. Light levels of F2,6-P2 were similar for all ages whereas initial dark F2,6-P2 values declined dramatically with leaf age.

Photosynthetic Metabolite Levels in Barley Primary Leaves during Extended Darkness. It has previously been shown that F2,6-P2 levels in barley leaves undergo an initial, rapid increase at the start of the dark period followed by a gradual decline (17). If the function of F2,6-P2 in the dark is primarily to control the rate of glycolysis, a further decrease in F2,6-P2 levels would be expected during an extended period of darkness since respiration rates decline with this treatment (9, 15, 23). Conversely, if F2,6-P2 is primarily involved in the control of sucrose synthesis (starch to sucrose conversion via FBPase) an increase in F2,6-P2 should occur during extended darkness as the rate of sucrose synthesis declines due to the depletion of the starch pools. These alternatives were tested by measuring metabolite levels in barley primary leaves during 48 h of extended darkness (Fig. 2). Following an initial increase during the first 30 min of darkness, whole leaf F2,6-P2 levels decreased throughout the experiment. Starch and sucrose pools were totally depleted after about 18 h of darkness. Glucose, fructose, and G6P also decreased rapidly during the first 18 h of darkness, but thereafter, remained unchanged at about 200, 100, and 50 μmol/mg Chl, respectively. The presence

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>F2,6-P2</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>A. Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h at 500 μE plus</td>
<td></td>
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</tr>
<tr>
<td>8 h 100 μE</td>
<td>0.38 ± 0.03</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>8 h 500 μE</td>
<td>0.27 ± 0.03</td>
<td>2.05 ± 0.74</td>
</tr>
<tr>
<td>B. Temperature</td>
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<tr>
<td>4 h 20°C</td>
<td>0.17 ± 0.03</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>4 h 10°C</td>
<td>0.06 ± 0.01</td>
<td>3.19 ± 0.18</td>
</tr>
<tr>
<td>12 h 20°C</td>
<td>0.13 ± 0.01</td>
<td>1.54 ± 0.22</td>
</tr>
<tr>
<td>12 h 10°C</td>
<td>0.08 ± 0.01</td>
<td>6.03 ± 0.30</td>
</tr>
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</table>

Table I. Effects of Temperature and Light Intensity on F2,6-P2 and Sucrose Levels in Barley Primary Leaves

In the temperature study, metabolite levels were measured after 4 and 12 h of light and after an additional 30 min of darkness. Different temperature regimes were imposed during the light period only; all dark samples were taken at 20°C. Light treatments consisted of 4 h at 500 μE followed by 8 additional h at the levels indicated. Samples were taken after 12 h of light and after an additional 30 min of darkness.

Primary leaves.
support for this interpretation is provided by the close correlation between the decline in F2,6-P2 and G6P levels in darkened barley leaves (Fig. 2) and the decline in the rate of respiratory CO2 efflux during the first 12 h of darkness (Fig. 3).

**Fructose 2,6-Bisphosphate and Glycolysis.** As previously noted (16), leaves of species that accumulate substantial amounts of sucrose in the light have greater than 1.0 nmol F2,6-P2/mg Chl during the early dark period. Consequently, a primary function of the high initial dark levels of F2,6-P2 in barley leaves could be to enhance glycolysis using soluble leaf carbohydrate reserves as substrate.

The enzyme PPi-PFK catalyzes the interconversion of F6P and PPi to F1,6BP and Pi. Although this reversible enzyme is stimulated in both directions by F2,6-P2, there is considerable evidence that in most tissues PPI-PFK either has a direct glycolytic function or supplies PPI for use in an earlier step in sucrose degradation (1, 7, 8, 12, 14). Desalted extracts of 10-d-old barley leaves have a PPI-PFK activity of 12 μmol FBP formed/g fresh weight–h (72 μmol C) when assayed at 1 mm F6P and 1 μM F,6-P2, an activity sufficient to account for the maximal rate of CO2 release by darkened barley leaves, 60 μmol CO2/g fresh weight–h (RC Sicher, unpublished observations).

If the major function of F2,6-P2 in the dark is to activate PPI-PFK, one would expect an increase in F2,6-P2 under conditions where respiration is enhanced and, conversely, a decrease under conditions where flow through this pathway is reduced. In the present study this is what was observed. First, treatments that increase carbohydrate levels in photosynthetically active leaves consistently increase initial dark respiration rates (2, 4, 5, 11, 13). Initial dark F2,6-P2 and sucrose levels are considerably higher after longer photosynthetic periods, low temperature treatment, and when loss of carbohydrate is restricted by leaf excision (Table I; Fig. 1). Second, in intact leaves dark respiration (3, 9, 11, 18, 24), PPI-PFK activity (19; RC Sicher, unpublished observations) and initial dark F2,6-P2 levels (Table II) decline with leaf age. Third, respiration rates frequently decline during both normal and extended periods of darkness (9, 15, 23; Fig. 3). Fructose 2,6-P2 and carbohydrate levels also decline during the normal dark period (6, 16, 17) and during extended darkness (Fig. 2).

The data presented above clearly show that a positive correlation exists between dark F2,6-P2 levels and dark respiration. These findings are consistent with, but not sufficient proof for, a role for F2,6-P2 in controlling the rate of dark respiration. It is important to note that F2,6-P2 is also thought to be an important regulator of glycolysis in most nonphotosynthetic tissues (7, 14, 20).

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