Ethylene-Induced Increase in Fructose-2,6-Bisphosphate in Plant Storage Tissues

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Received for publication July 19, 1985 and in revised form September 23, 1985

ABSTRACT

Treatment of carrot roots with ethylene led to: (a) a doubling of the fructose-2,6-bisphosphate content; (b) a general increase in the concentration of glycolytic intermediates; and (c) an increase in the extractable activity of fructose-6-phosphate, 2-kinase, the enzyme synthesizing fructose-2,6-bisphosphate from fructose-6-phosphate and adenosine triphosphate.

Fructose-2,6-bisphosphate is a regulatory metabolite that makes an important contribution to the regulation of carbohydrate metabolism in different types of eukaryotic organisms. In mammalian and yeast cells, Fru-2,6-P₂-promotes glycolysis and restricts gluconeogenesis by respectively activating phosphofructokinase and inhibiting fructose-1,6-bisphosphatase (5, 15). Through a similar inhibition of fructose-1,6-bisphosphatase, Fru-2,6-P₂ functions in higher plants in regulating sucrose synthesis. A process which is analogous to gluconeogenesis, in leaves (3, 13), and in germinating castor bean seedlings (6). The contribution of Fru-2,6-P₂ to regulation of respiratory metabolism in plants is, however, not clear. To gain information on this point, we have exposed plant storage tissues to ethylene—a hormone known to increase respiration (9). We report that Fru-2,6-P₂ increases when such tissues are treated with ethylene and that this is accompanied by an increase in the activity of the enzyme synthesizing Fru-2,6-P₂—Fru-6-P₂₂K.

MATERIALS AND METHODS

Plants. Freshly topped carrots (10–13 cm long, 1.5–2.5 cm maximum diameter) or potatoes (4–6 cm cross-section) purchased from a market, were pretreated in water-saturated air for at least 30 h before introducing 50 µL/L ethylene for 19 to 22 h. Each sample was made from a single carrot or potato. Control experiments were with matched carrots and potatoes incubated for 50 h in the absence of added ethylene. The flow rate was 0.5 L min⁻¹ per carrots, and 1.0 L min⁻¹ per four potatoes. The air was water-saturated.

Extraction for Fru-2,6-P₂ and Metabolites. Within 5 sec of cutting the storage tissue, a cross-sectional sample (0.6–0.9 g fresh weight) was frozen in liquid N₂. Samples were cut so that the width was 2 mm or less. The samples were deproteinized using a chloroform-methanol mixture at sub-zero temperatures as described in Stitt et al. (10) for carrot, and as modified in Stitt and Heldt (12) for potato. The extracts were stored at −85°C. Fru-2,6-P₂ was measured by the PFP bioassay as in Stitt and Heldt (12) and Glu-6-P, Fru-6-P, DHAP, and PGA were assayed spectrophotometrically in a dual wavelength spectrophotometer as in Stitt et al. (10). In experiments where Fru-6-P₂₂K was assayed (see below), the activation of PFP by the zero-time sample (compared to PFP activity in the absence of sample) was also used to provide an estimate of the Fru-2,6-P₂ present in the same sample of carrot tissue. There was no significant hydrolysis of Fru-2,6-P₂ during the time required to prepare the samples under these conditions.

Extraction of Fru-6-P₂₂K. Within 60 s of slicing, a cross-sectional sample (0.3–0.5 g fresh weight) was homogenized with glass powder (200–400 µm mesh) at 4°C in a pestle and mortar with 1500 µL of a solution containing 50 mm Tricine-KOH (pH 7.7), 5 mm MgCl₂, 2 mm EDTA, 14 mm 2-mercaptoethanol, 20 mm diethyldithiocarbamate, 2 mm benzamide HCl, 2 mm e-amino-n-caproic acid, 0.5 mm phenylmethylsulfonyl fluoride, 1.5% (v/v) insoluble PVP, 10% (v/v) glycerol. The homogenate was centrifuged, 2 min, and aliquots of the supernatant fraction were assayed immediately for Fru-6-P₂₂K. Protein was measured as in Cséke and Buchanan (2).

Assay of Fru-6-P₂₂K. Fru-6-P₂₂K was assayed as in Stitt et al. (14), using 20 µL aliquots of extract in a final volume of 100 µl containing 100 mm Tris-HCl (pH 7.3), 5 mm MgCl₂, 1 mm EDTA, 2 mm ATP, 5 mm Fru-6-P, 5 mm Pi, and with no or 1 mm PGA. At 0 (two samples), 400, and 800 sec, 20 µL aliquots were transferred into 30 µL 0.25 M KOH and stored on ice (5–15 min) before assaying for Fru-6-P₂₂K using the PFP bioassay. One of the two samples taken at zero-time received 1 pmol Fru-2,6-P₂, and acted as an internal standard. For practical details see Stitt et al. (14).

RESULTS

Change in Fru-2,6-P₂. Whole carrots were treated for 20 h either in air or in air supplemented with 50 µL/L ethylene, in seven separate experiments carried out during a period of 6 weeks, using immature carrots (4 per treatment). The Fru-2,6-P₂ content (mean ± se of 28 carrots) increased from 140 ± 12 pmol·g⁻¹ fresh weight in the absence of ethylene to 250 ± 21 pmol·g⁻¹ fresh weight after exposure to ethylene. A similar increase was observed with mature carrots, from 80 ± 29 to 170
Changes in Glycolytic Metabolites. To relate the ethylene-induced changes in Fru-2,6-P2 to other metabolites, we determined the effect of ethylene on intermediates of glycolysis which are known to regulate Fru-6-P,2K (2, 6, 8, 11, 14). In the experiments summarized in Table I, Fru-2,6-P2, and glycolytic intermediates were measured in the same samples of carrot roots or potato tubers. Consistent with an enhanced mobilization of carbohydrates, we observed in both cases a small but general increase in metabolite levels after exposure to ethylene. The percentage increases produced by ethylene, expressed respectively for carrots and potatoes, were: Fru-2,6-P2, 108 and 100%; Glu-6-P, 34 and 19%; Fru-6-P, 7 and 16%; DHAP, 124 and 200%; and PGA, 32 and 13%.

Change in Fru-6-P,2K. To determine whether there was a correlation between Fru-2,6-P2 levels and extractable Fru-6-P,2K activity, we tested the activity of the enzyme in extracts rapidly prepared from control and ethylene-treated carrot roots (Table II). In these experiments, less than 5 min elapsed between the time at which a sample was removed from the exposure chamber and the time at which the Fru-6-P,2K assay was initiated. By minimizing preparation time, we considered that any alterations in enzyme activity following exposure to ethylene would be best preserved. Fru-6-P,2K was assayed in both the absence (control) and presence of PGA, which is an inhibitor of Fru-6-P,2K from carrot and other plant tissues (2, 6, 8, 11, 14). For comparative purposes, the Fru-6-P,2K content was estimated in the sample used to measure Fru-6-P,2K activity.

Considering first the measurements in the absence of PGA (in which assay conditions were arranged to give about 80% of the \( V_{max} \) activity), the results demonstrated that there was a small increase in Fru-6-P,2K activity in carrot tissues that had been exposed to ethylene (Table II). This increase was somewhat larger when Fru-6-P,2K activity was measured in the presence of 1 mM PGA. With PGA, the activity of Fru-6-P,2K was, in three separate experiments, 94, 51, and 40% higher in carrots that had been exposed to ethylene (versus respective values of 29, 15, and 32% for assays carried out in the absence of PGA). Parallel to these increases in Fru-6-P,2K activity, were increases in Fru-6-P2 content of 130, 63, and 110% (Table II). These results are related to protein content, but similar increases were found when activity was related to fresh weight.

DISCUSSION

In this communication, we present evidence that ethylene, a known stimulator of respiration in plants, brings about a significant increase in Fru-2,6-P2 content of nonphotosynthetic storage tissues (carrot roots, potato tubers). This increase was found whether Fru-2,6-P2 content was related to fresh weight or to protein content. Although higher in ethylene-treated tissues, the Fru-2,6-P2 content of individual carrots was found to vary, even within single lots. It seems likely that this variation may reflect the prehistory of each individual carrot and result from isolated treatments, such as wounding during handling and storage, which could result in up to a 10-fold increase in Fru-2,6-P2 (A Balogh, JH Wong, BB Buchanan, unpublished data; M Stitt, unpublished data). A similar increase in Fru-2,6-P2 has also been found in response to ethylene production in naturally ripening avocado (AB Benent, unpublished data).

The increase of Fru-2,6-P2 in response to ethylene treatment provides new evidence that this regulatory metabolite is involved in promoting carbohydrate mobilization and breakdown in plants. A similar correlation between increased Fru-2,6-P2 and carbohydrate breakdown is found (a) during wound-induced respiration in Jerusalem artichoke (16) and carrot or potato storage tissues (A Balogh, JH Wong, and BB Buchanan, unpublished data; M Stitt, unpublished data); (b) after making tissues anaerobic (7); (c) after supplying endogenous sugars to leaves (10) or roots (M. Stitt, unpublished data); and (d) during malate accumulation as guard cell protoplasts swell (4). It remains to be seen how Fru-2,6-P2 brings about this stimulation. As Fru-2,6-P2 does not increase the linear rate of phosphofructokinase activity in plants (1), one site of action could lie in its activation of PFP, either the forward or reverse reaction. Depending on the direction in which PFP operates in vivo, increasing Fru-2,6-P2 could enhance the glycolytic direction, thereby increasing the use of PPI for phosphorylation of Fru-6-P, or, alternatively, Fru-2,6-P2 could stimulate the reverse reaction, thereby increasing the pool of PPI needed for sucrose mobilization via sucrose synthase and UDP-glucose phosphorylase.

The question arises as to the basis of the ethylene-induced increase in Fru-2,6-P2, i.e. what is responsible for altering the activities of the enzymes catalyzing the synthesis and degradation of Fru-2,6-P2. Current evidence suggests that plants resemble animal systems in having both Fru-6-P,2K and Fru-2,6-Pase activities on a single bifunctional protein (3). Hence the amount of Fru-2,6-P2 depends on the relative extent to which each of these activities functions. In carbohydrate exporting tissues (sources) like leaves (3, 8, 11) and germinating bean endosperm (6), the extent of function seems to be fixed, at least in part, by the level of metabolites. The recent finding that the Fru-6-P,2K preparations from different carbohydrate importing tissues (sinks) (14), including carrot roots, show properties similar to the enzyme from sources opens the possibility that Fru-2,6-P2 content of plant sink tissues could also be controlled by metabolite levels. However, the increase of Fru-2,6-P2 following ethyl-

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**Table 1. Changes of Fru-2,6-P2 and Respiratory Metabolites Following Exposure of Storage Tissues to Ethylene**

Whole carrot roots, or potato tubers, were treated in air, or in air containing 50 μL/L ethylene, and then quenched, extracted and analyzed as in "Materials and Methods." The results are the mean ± SE of three separate experiments with 4 carrots for each treatment (n = 12), and of one experiment with 4 potatoes for each treatment (n = 4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ethylene Added</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μL/L</td>
<td>pmol·g⁻¹ fresh wt</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrot</td>
<td>0</td>
<td>120 ± 9</td>
</tr>
<tr>
<td>Carrot</td>
<td>50</td>
<td>250 ± 13</td>
</tr>
<tr>
<td>Potato</td>
<td>0</td>
<td>30 ± 3</td>
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<tr>
<td>Potato</td>
<td>50</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>

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Table II. Changes of Fru-2,6-P₂ and Fru-6-P,2K Activity Following Exposure of Carrot Storage Tissue to Ethylene

Whole carrots were treated in air, or in air containing 50 μL/L ethylene and extract rapidly prepared and assayed as in "Materials and Methods." The assay in the absence of PGA yielded an activity which was about 80% of the Vₘₐₓ of Fru-6-P,2K. Fru-2,6-P₂ content of the carrots was estimated during the Fru-6-P,2K assay in "Materials and Methods." Three experiments with separate batches of carrots are shown, the number of carrots used per experiment being shown in the footnote, and results are as mean ± SE.

<table>
<thead>
<tr>
<th>Ethylene Added</th>
<th>Fru-2,6-P₂</th>
<th>Fru-6-P,2-Kinase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol·mg⁻¹ prot</td>
<td>nmol·mg⁻¹ prot·h⁻¹</td>
</tr>
<tr>
<td>μL/L</td>
<td>Control</td>
<td>1 mm PGA</td>
</tr>
<tr>
<td>0</td>
<td>2.4 ± 0.4</td>
<td>0.48 ± 0.13</td>
</tr>
<tr>
<td>50</td>
<td>3.1 ± 0.2</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>100 ± 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2.0 ± 0.4</td>
<td>0.70 ± 0.19</td>
</tr>
<tr>
<td>130 ± 18</td>
<td>1.06 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>80 ± 11</td>
<td>0.65 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>100 ± 28</td>
<td>1.9 ± 0.3</td>
<td>0.92 ± 0.09</td>
</tr>
</tbody>
</table>

*The number of carrots used for each of the three experiments were 4, 4, and 5, respectively.

Ethylene treatment could not be accounted for by concordant changes in regulatory metabolites, i.e. a strong activator of Fru-6-P,2K (Fru-6-P) rose, but to a lesser degree than did inhibitors of the enzyme (PGA and DHAP). While uncertainty about the subcellular compartmentation of these metabolites (as well as changes in Pi, another activator) means that a degree of interpretative caution is in order, the results suggest that regulation by metabolites is not primarily responsible for the increased Fru-2,6-P₂ levels following exposure to ethylene but that additional mechanisms may be required.

Acknowledgements—We are grateful to Dr. Mark Tucker and Dr. Joshua Wong for advice on the physiology of ethylene.

LITERATURE CITED

2. Cäre C, BB Buchanan 1983 An enzyme synthesizing fructose-2,6-bisphosphate occurs in leaves and is regulated by metabolite effectors. FEBS Lett 155: 139-142

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