Short Communication

A Rapid Increase in Spinach Leaf Fructose 2,6-Bisphosphate Occurs during a Light to Dark Transition

Received for publication June 21, 1985 and in revised form August 2, 1985

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ABSTRACT

Spinach leaf fructose 2,6-bisphosphate levels increase rapidly during the first 15 minutes of a normal dark period followed by a gradual decline during the next 5 hours. The regulatory mechanism responsible for the dark-induced rise in fructose 2,6-bisphosphate levels can be counteracted by a brief exposure to light intensities greater than 1 microeinstein per square meter per second.

The regulatory metabolite F2,6-P2 is a potent inhibitor of cytoplasmic FBPase (4, 7, 16), a key enzyme in the sucrose biosynthetic pathway. As such, an increase in the level of F2,6-P2 should tend to decrease the flow of carbon through FBPase. Carbon flow through FBPase decreases rapidly during the initial part of the dark period (18) and, at this time, F2,6-P2 levels increase in the leaves of pea (3) and soybean (9). In spinach leaves, however, F2,6-P2 levels have been reported to decrease (13), rather than increase, at the beginning of the dark period. To resolve this difference, we have re-examined the effect of darkness on spinach leaf F2,6-P2 levels.

MATERIALS AND METHODS

Plant Material. Spinach (Spinacia oleracea L. cv America) plants were grown as previously described (10). The photoperiod in these experiments was 12 h. Dark samples were taken at a light intensity of 0.1 µE m⁻² s⁻¹.

Manipulation for Light Intensity Experiments. In experiments where the light intensity for sampling was varied, groups of four plants each were removed from an illuminated chamber at the end of a normal light period. Each group was removed at 5-min intervals and placed in a darkened chamber (0.1 µEm⁻²s⁻¹) for 20 min. The plants were then exposed to varying intensities of light (0.1, 1.0, 10, or 500 µE m⁻²s⁻¹) for 7 min at which point samples were taken.

Extraction. Leaf discs (1 cm²) were punched from fully expanded secondary leaves. The discs were rapidly frozen in liquid N₂ and then extracted by a modification of the method of Stitt et al. (13). Three discs from each leaf (100–200 mg fresh weight) were ground in a glass/glass homogenizer with 0.75 ml extraction solution (50 mM Tris-HCl [pH 8.2], 5 mM EDTA, 65% [v/v] methanol, and 25% [v/v] chloroform). The extract was centrifuged and the pellet re-extracted twice with 0.2 ml extraction solution. Supernatants were combined, the phases separated with 0.3 ml H₂O, and the aqeous phase vaporated almost to dryness under N₂. The samples were brought to 100 µl with 50 mM Tris-HCl (pH 8.0) and then stored at −20°C.

Fructose 2,6-Bisphosphate Assay. The assay for F2,6-P2 was essentially that of Van Schaftingen et al. (19). The reaction mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.15 mM NADH, 3 mM EDTA, 2 mM DTT, 0.5 mM Ppi, 1 mM F6P, 0.25 unit of aldolase, 5 units of triosephosphate isomerase, 0.5 unit of a-glycerophosphate dehydrogenase, and 0.02 unit of pyrophosphate/F6P phosphotransferase. Appropriate aliquots (0.5–3 pmol F2,6-P2) of the sample were added and the rate of reaction compared to a standard curve prepared with known quantities of F2,6-P2. The standard curve was prepared using an acidified (0.1 N HCl, 15 min) and then neutralized (0.1 N NaOH) aliquot of the sample. An equivalent amount of NaCl was also added to the unknown. All reactions were run at 25°C. Using this extraction and assay procedure, the percentage recovery of F2,6-P2 added to the sample during grinding was 68 ± 8 (mean ± SE of four experiments).

Other Methods. Chl was estimated according to the method of Arnon (1). Glucose 6-phosphate, sucrose, and starch were determined as in the procedures of Robinson (10).

RESULTS AND DISCUSSION

Fructose 2,6-Bisphosphate during a Light to Dark Transition. Spinach leaf F2,6-P2 levels show a rapid initial increase (1.0–1.4 nmol/mg Chl) during the first 15 min in the dark followed by a gradual decrease (to 0.9 nmol/mg Chl) during the next 5 h (Fig. 1). The gradual decrease in F2,6-P2 coincides with a rapid depletion of the leaf sucrose pool and a more gradual decline in starch levels (Fig. 1). Similar light/dark F2,6-P2 profiles have also been observed in the leaves of barley (R. Sicher, personal communication), pea (3), and soybean (9).

The dark-induced rise in F2,6-P2 reported here is in contrast to the results of an earlier study in which spinach leaf F2,6-P2 levels were found to decrease by 50% upon darkening (13). It seems unlikely that this difference is the result of varying growth conditions since these are similar in many respects. Our chambers produce plants with high photosynthetic rates (2); the plants of Stitt et al. (13) had similarly high rates. In our study, the day length was 12 h as opposed to their 9 h, however plants given only 7 h of light also show the dark induced rise in F2,6-P2 (2). Similarly, analytical discrepancies are unlikely since we find the same trends in F2,6-P2 levels during the rest of the diurnal period.
FIG. 1. F2,6-P2 (●), G6P (○), sucrose (□), and starch (■) content of spinach leaves during a light to dark transition. The first sample was taken after 11 h in the light and the lights were turned off at 12 h. Plants were 26 d old at time of sampling. Dark samples were taken at a light intensity of 0.1 μE m⁻² s⁻¹. Values are the mean ± se of four plants per time point. For clarity, only one side of the error bars are shown.

FIG. 2. F2,6-P2 levels in spinach leaves given 20 min of darkness followed by a 7-min exposure to light of varying intensities. The experiment was conducted at the beginning of the normal dark period. Plants were 58 d old at time of sampling. Values are the mean ± se of four plants per time point. The last light value (15 min before the dark period) was 0.7 nmol F2,6-P2/mg Chl ± 0.1. (see “Materials and Methods” for protocol).

(2), as do Stitt et al. (13). In four separate experiments, we consistently observed a dark-induced rise in F2,6-P2 levels. However, the magnitude of the increase did vary. Consequently, it may be that in certain physiological states, F2,6-P2 levels would decrease rather than increase in the dark.

Spinach leaf F2,6-P2 levels in this study are about 3 times higher on a Chl basis (0.4–1.4 nmol/mg Chl) than previously reported values (0.1 to 0.5 nmol/Chl) (13–15). At least a part of this increase can be attributed to the relatively low Chl content in the fully expanded leaves of young 'America' spinach, 0.5 to 0.7 mg Chl/g fresh weight at 26 DAP as compared with 0.9 to 1.2 mg Chl/g fresh weight at 58 DAP.

Regulation of Sucrose Synthesis. The rate of sucrose synthesis has been shown to drop within the first few minutes of darkness (18). At this time, preformed (vacuolar) sucrose rather than newly synthesized sucrose is being utilized for export (5, 6, 11). As the vacuolar sucrose pool is depleted, an increase in the rate of sucrose synthesis (from starch) is observed (5, 6). Thus, in the darkened leaf, sucrose synthesis rates may be inversely related to F2,6-P2 levels, being low at the beginning of the dark period when F2,6-P2 levels are elevated (Fig. 1; 18) and increasing later on as F2,6-P2 levels decline (Fig. 1; 5, 6).

Although a role for F2,6-P2 in controlling sucrose synthesis in the dark is attractive, several uncertainties remain. For example, is the level of fructose 1,6-bisP ever high enough at night to permit carbon flow through FBPase at any level of F2,6-P2 (7)? Also, in the present study, spinach leaf starch levels decline as soon as the lights are turned off (Fig. 1), suggesting that sucrose synthesis may start immediately in this species. If so, does sucrose synthesis increase once the vacuolar sucrose pool is depleted, as the decline in F2,6-P2 levels would suggest? Answers to these questions will require the simultaneous measurement of F2,6-P2 levels and carbon flow through FBPase and the in vitro demonstration that FBPase activity can be significantly altered by the observed changes in F2,6-P2 levels at in vivo concentrations of enzyme substrates and effectors.

Influence of Light Intensities on Fructose 2,6-Bisphosphate Levels. In some initial experiments, the response of F2,6-P2 levels to darkening was quite variable. For example, after 45 min of darkness, F2,6-P2 levels in one group of plants ranged from 0.5 to 1.5 nmol/mg Chl. The samples for these experiments were taken at variable, but low, light levels (1 to 10 μE), suggesting that leaf F2,6-P2 levels might be responsive to low photon flux densities. To test this, plants were kept in darkness (0.1 μE m⁻² s⁻¹) for 20 min after a normal light cycle then transferred to 0.1, 1, 10, or 500 μE m⁻² s⁻¹ for 7 min at which time the samples were taken. The results (Fig. 2) show that at light intensities of 0.1 and 1.0 μE m⁻² s⁻¹, F2,6-P2 levels increase. However, exposure to light intensities greater than 1.0 μE m⁻² s⁻¹ results in a significant and immediate reduction in F2,6-P2 levels. Therefore, an accurate determination of dark F2,6-P2 values requires that samples be taken at a light intensity equal to or less than 1.0 μE m⁻² s⁻¹.

Concluding Remarks. Although F2,6-P2 is probably involved in the regulation of sucrose synthesis (8, 13–15), the mechanisms responsible for increasing the level of F2,6-P2 during a light to dark transition are not immediately obvious. A decrease rather than an increase in the level of the precursor to F2,6-P2, F6P, occurs in the dark (17, 18). A similar decrease was also observed in the amount of G6P, a metabolite in equilibrium with F6P, in this study (Fig. 1) and in the work of Stitt et al. (13, 17, 18). In addition, the level of cytoplasmic ATP (the phosphorlyl donor in F2,6-P2 synthesis) does not increase during a light to dark transition (18). Thus, precursor levels are not enhanced in the dark and cannot, therefore, be responsible for the dark-induced increase in F2,6-P2. It has been suggested that the level of DHAP, an inhibitor of the enzyme synthesizing F2,6-P2 (F6P,2K), could
be important in controlling F2,6-P2 levels (12). In this context, the rapid drop in cytoplasmic DHAP that occurs in the dark (7, 18) could play a crucial role in increasing F2,6-P2 levels. Control may also be exercised via in vivo phosphorylation of F6P,2K as the spinach leaf enzyme can be phosphorylated in vitro with a resulting change in enzyme activity (C. Baysdorfer, J. M. Robinson, unpublished data).

Acknowledgment—We wish to thank Dr. Rich Sicher for many stimulating conversations during the course of this work.

LITERATURE CITED

4. CSEKE C, NF WEEDEN, BB BUCHANAN, K UEEDA 1982 A special fructose bisphosphate functions as a cytoplasmic regulatory metabolite in green leaves. Proc Natl Acad Sci USA 79: 4322–4326
15. STITT M, B KURZEL, HW HELDT 1984 Control of photosynthetic sucrose synthesis by fructose 2,6-bisphosphate. II. Partitioning between sucrose and starch. Plant Physiol 75: 554–560