Plant Physiol. (1983) 72, 0259–0261
0032-0889/83/72/0259/03/$00.50/0

Short Communication

Leaf Cytosolic Fructose-1,6-bisphosphatase[1]

A POTENTIAL TARGET SITE IN LOW TEMPERATURE STRESS

Received for publication December 28, 1982 and in revised form March 23, 1983

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ABSTRACT

Leaf cytosolic fructose-1,6-bisphosphatase (FBPase), partially purified from both spinach (*Spinacia oleracea*, var Hipack) and peas (*Pisum sativum*, var Progress No. 9), is reversibly inactivated by exposure to low temperature. Thus, even though assays were conducted at 22°C, samples incubated at 0 to 12°C had greatly reduced activity relative to controls maintained at 22°C. Following incubation at 22°C prior to assay, the inactivated samples regained their initial activity. Chloroplast FBPase, by contrast, was unaffected by low temperature treatment. This feature as well as lack of a response of cytosolic FBPase to thioredoxins *f* or c_f and to chloroplast FBPase antibody indicate that the FBPase isozymes of leaves are different proteins.

Cytoslic FBPase[3] (EC 3.1.3.11) of leaves is a key enzyme in the synthesis of sucrose, an important sugar of most plants (7, 17). In contrast to chloroplast FBPase (1), the cytosolic isozyme has been shown to be inhibited by AMP and high substrate concentrations (17). Results from our laboratory have demonstrated that cytosolic FBPase, also unlike the chloroplast isozyme, is inhibited by F-2,6-P_2, a regulatory metabolite recently identified in plant tissues, including leaves, where it occurs partly if not totally in the cytosol (5, 14). The two inhibitors, AMP and F-2,6-P_2 act synergistically, suggesting that the activity of the leaf cytosolic FBPase is controlled by the prevailing levels of these metabolites (5).

In an extension of our earlier study, we have found that temperature is also a factor affecting the activity of cytosolic FBPase. When stored briefly at 0 to 12°C, cytosolic FBPase purified from spinach leaves showed diminished activity when assayed at 22°C. In other words, cytosolic FBPase activity was decreased by low temperature in a manner such that it remained low during subsequent assay. The changes effected by low temperature exposure were reversed by incubation at 22°C. These results, which are summarized below in relation to other properties of the cytosolic FBPase, may be relevant to our understanding of low temperature stress in plants.

[1] Supported in part by a grant from the National Science Foundation and by a gift from Mr. Frank Weeden.

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[3] Abbreviations: FBPase, fructose-1,6-bisphosphatase; F-2,6-P_2, fructose-2,6-bisphosphate; F-1,6-P_2, fructose-1,6-bisphosphate; ELISA, enzyme-linked immunosorption assay(s).

MATERIALS AND METHODS

Plant Material. Spinach plants (*Spinacia oleracea*, var Hipack; Asgrow Seed Co., Tracy, CA) were grown in a nutrient solution in a greenhouse (8). Green peas (*Pisum sativum*, var Progress No. 9; Ferry Morse Co., Mountain View, CA) were grown outdoors in vermiculite.

Reagents. Except for fructose-6-P, which was from Boehringer Mannheim, biochemicals and the 'coupling enzymes' for FBPase assays (yeast phosphoglucose isomerase, baker's yeast glucose-6-P dehydrogenase) were obtained from Sigma Chemical Co.

Preparation of Thioredoxins and Chloroplast FBPase. Chloroplast thioredoxin *f* and cytosolic thioredoxin c_f were purified from spinach leaves (4, 16). An earlier procedure was also used for purification of spinach chloroplast FBPase (3).

FBPase Assays. Cytosolic FBPase was routinely assayed spectrophotometrically by coupling fructose-6-P formation to the reduction of NADP with P-glucose isomerase/glucose-6-P dehydrogenase and measuring the change in $A_{340\ nm}$ (5). The reaction mixture contained, in a final volume of 0.5 ml, 50 mM imidazole-HCl buffer, pH 7.5; 1 mM $MgSO_4$; 1 mM NADP; and the coupling enzymes as 0.1 unit of glucose-6-P dehydrogenase, and 0.2 unit of P-glucose isomerase. The FBPase sample (20 μl) was added to this mixture and allowed to equilibrate for 30 s before the reaction was initiated by the addition of 0.01 mM F-1,6-P_2. When stored at low temperature, enzyme samples were incubated 60 min at room temperature (22°C) for activation prior to assay.

Starch Gel Electrophoresis. Samples of approximately 50 μl were absorbed onto paper wicks and subjected to horizontal starch gel electrophoresis using a lithium hydroxide/Tris-citrate buffer system (6). Gels were sliced and separate slices stained for 60 min at 37°C in 50 ml of either a FBPase or an acid phosphatase assay. The FBPase assay mixture was modified from (13) and contained 100 mM Tris-HCl buffer, pH 8.0; 2 mM $MgCl_2$; 0.5 mM F-1,6-P_2; 0.2 mM NADP; 0.3 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 0.1 mM phenazine methosulfate; 20 units of glucose-6-P dehydrogenase; and 40 units of P-glucose isomerase. The acid phosphatase mix, also modified from (13), included 100 mM sodium citrate, pH 5.2; 4 mM sodium α-naphthyl acid phosphate; and 20 mg of Fast Black K, salt.

Purification of Cytosolic FBPase. Cytosolic FBPase was partially purified from spinach and pea leaves as described previously (5). Unless a protease inhibitor (phenylmethylsulfonylfluoride, PMSF) and a relatively high sucrose concentration (5–15% w/v) were included in the buffers used during extraction and purification, the cytosolic FBPase preparation from both spinach and peas was insensitive to AMP.

Analytical Methods. ELISA were carried out as described previously (11).

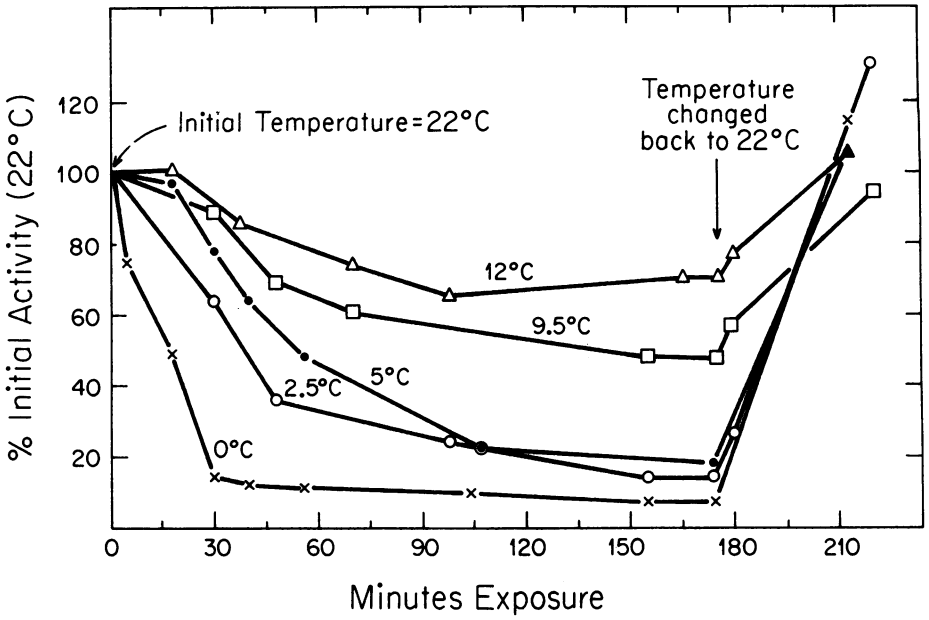

FIG. 1. Reversible inhibition of spinach cytosolic FBPase by exposure to low temperatures. A 0.5-ml aliquot of a concentrated spinach cytosolic FBPase preparation in 50% (w/v) sucrose stored 60 min at 22°C was diluted into 5.5 ml of 0.1 M K-phosphate (pH 7.7) containing 10% (w/v) sucrose. The resulting solution was divided into six 1.0-ml aliquots in 15- × 125-mm glass test tubes, each of which was placed in a separate controlled temperature environment at the indicated temperatures (°C): 0, 2.5, 5, 9.5, 12, and 20. Samples of 20 μl were removed from the test tubes after specified periods of incubation and immediately added to the reaction mixture and assayed at 22°C. The procedure used to determine the low temperature response of chloroplast FBPase was identical except that the buffer concentration was 0.05 M, and 14 mM 2-mercaptoethanol and 5 mM $MgSO_4$ were added to the incubation solution.

RESULTS AND DISCUSSION

In characterizing the partially purified cytosolic FBPase, we observed that the activity associated with the spinach and pea enzyme was dependent on the temperature of storage—*i.e.* the enzyme stored at 0° to 12°C showed a low (nonlinear) activity relative to the enzyme stored at 22°C even though assays were carried out at 22°C. In the case of spinach cytosolic FBPase, a 2-h incubation at 12°C and 0°C resulted in respective activity drops of 30% and 90% relative to the control maintained at 22°C (Fig. 1). Proportional drops in activity were observed at intermediate temperatures. In all cases, the low-temperature induced decrease in activity was reversible—*i.e.* the activity of samples incubated at 12°C or below returned to initial values following incubation for 40 min at 22°C (Fig. 1). Cytosolic FBPase from pea leaves was not subjected to such a thorough investigation; however, it did exhibit a significant and reversible loss of activity when incubated at 5°C. Significantly, the chloroplast FBPase differed and was not inhibited by exposure to low temperatures (data not shown). Sensitivity to low temperature is thus a parameter that distinguishes the chloroplast and cytosolic forms of FBPase. A similar response to low temperature has been reported for a number of enzymes from diverse sources (12), including ribulose 1,5-bisP carboxylase from tobacco (9).

The temperature sensitivity results and the earlier reported differential response to AMP and F-2,6-P_2 (5) suggest that the cytosolic and chloroplast enzymes are different proteins, and in this manner they resemble cytosolic and chloroplast phosphoglucose isomerases (15). Additional evidence for this conclusion comes from the present finding that the cytosolic FBPase differs from chloroplast FBPase in that it (a) was not activated by the reduced forms of thioredoxin (*f* or c_l), (b) was not significantly inhibited by an antibody prepared against spinach chloroplast FBPase, (c) showed no response to this same antibody in ELISA analyses, and (d) showed an electrophoretic mobility relative to the buffer front of 0.2 *versus* 0.5 for chloroplast FBPase (data not shown). In this latter connection, a third (nonspecific) phosphatase also showed a mobility of about 0.2, but this enzyme was easily removed during purification of either of the substrate-specific FBPases. The two FBPases both differ from this nonspecific (acid) phosphatase in their regulatory properties and substrate specificity (1, 2, 17).

A comment on the possible physiological importance of the current findings seems in order. While the physiological consequences of the temperature sensitivity of cytosolic FBPase are not fully apparent, there are earlier reported indications that such a response may be relevant to low temperature stress. Thus, years ago Knott (10) found that spinach varieties which survived well at 40 to 50°F (about 5–10°C) could not grow at these temperatures and remained stunted compared to room temperature controls or to plants grown at 50 to 60°F (about 10–16°C). The inactivation of cytosolic FBPase and the attendant diminution of photosynthate production within this range could provide at least part of the explanation. It will be of future interest to learn whether the cytosolic FBPases from other plants, *viz.*, cold sensitive species such as tomato, show a similar response to low temperature.

Acknowledgment—We thank Prof. D. B. Wake for a sample of Fast Black, K salt.

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