LIGHT INDUCED ACTIVATION OF FRUCTOSE-1,6-BISPHOSPHATASE IN ISOLATED INTACT CHLOROPLASTS

G.J. Kelly, G. Zimmermann and E. Latzko

Abteilung Chemische Pflanzenphysiologie
Technische Universität München
8050 Weihenstephan, Germany (BRD)

Received March 10,1976

SUMMARY: Chloroplasts isolated from spinach leaves previously held in darkness contained no fructosebisphosphatase activity measured at pH 7.5 or 7.9, although high activity at pH 8.8 was observed. Following illumination of these chloroplasts for 7 min, enzyme activity at pH 7.9 was clearly detected, and after 24 min illumination was equal to 36% of the pH 8.8 activity; at this time activity at pH 7.5 also became apparent. The activity at pH 8.8 was not affected by illumination. Similar activation of fructosebisphosphatase in isolated pea chloroplasts was recorded.

INTRODUCTION: The hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate is thought to be an important regulatory step in the Calvin cycle of photosynthetic CO_2 fixation (1-3). The enzyme catalysing this reaction, chloroplast fructosebisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), has been purified and shown to possess properties which could ensure that the enzyme is active only during illumination (4-7). One property of interest is activation by the sulphydryl reducing agent dithiothreitol (5-7) since this effect could be analagous to an in vivo activation mediated by a photosynthetically produced reductant. Unfortunately, investigations of fructosebisphosphatase in intact chloroplasts have failed to provide evidence for any in vivo activation following illumination. Studies in this laboratory (7) have recently shown, however, that activation of the purified enzyme by dithio-

threitol is more pronounced at pH 7.9 which approximates the pH of the stroma in illuminated chloroplasts (3) but which is considerably lower than pH 8.8, the optimum for enzyme activity. Measurements of fructosebisphosphatase activity in intact chloroplasts have now revealed that, at this lower pH, the enzyme is inactive in the dark but remarkably activated following several minutes illumination. Results of these experiments are presented in this communication.

MATERIALS AND METHODS: Spinach (Spinacea oleracea L.) and peas (Pisum sativum L.) were grown under glasshouse conditions. The plants were left overnight in darkness prior to experiments. Chloroplasts were isolated by homogenising 8 g leaves for 5 sec in 40 ml of the isolation medium described by Lilley et al. (8). The homogenate was filtered through Miracloth, centrifuged at 2500 x g for 80 sec and the supernatant discarded. Two ml resuspending medium containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM HEPES and adjusted to pH 7.6 (8) was added without disturbing the chloroplast pellet and the tube gently agitated to suspend the upper layer of chloroplasts which were discarded. The remaining chloroplasts were suspended in 2 ml of resuspending medium. All operations were at 4°C and in dim light.

For illumination, one volume of chloroplast suspension was added to four volumes of resuspending medium in a glass tube (10 cm x 1.3 cm^2) held in a water bath at 22°C. Light was supplied from a Paximat-S-500 projector by an Osram 300 W lamp. At the times indicated (see Results) chloroplasts were removed in 0.2 ml samples and broken by direct addition to reaction mixtures containing 100 µmoles buffer (Tris-HCl, pH 8.8 or 7.9, or imidazole-HCl, pH 7.5), 10 µmoles $MgCl_2$, 0.6 µmole fructose-1,6-bisphosphate, 1 µmole EDTA, 0.3 µmole NADP, 2 units phosphoglucose isomerase and 0.6 unit glucose-6-phosphate dehydrogenase. The final volume was 1 ml. Fructosebisphosphatase activity was calculated from the change in absorbance at 340 nm measured with a UNICAM SP1800 spectrophotometer connected to an automatic sample changer and recorder; with this system three reactions could be followed simultaneously (Figure 1). No activity at pH 7.9 was detected with illuminated chloroplasts when fructose-1,6-bisphosphate was omitted or replaced with glycerate-3-phosphate.

RESULTS: Light activation of spinach chloroplast fructosebisphosphatase is shown in Figure 1. Fructosebisphosphatase released from chloroplasts which were isolated from leaves left overnight in darkness was initially inactive at either pH 7.5

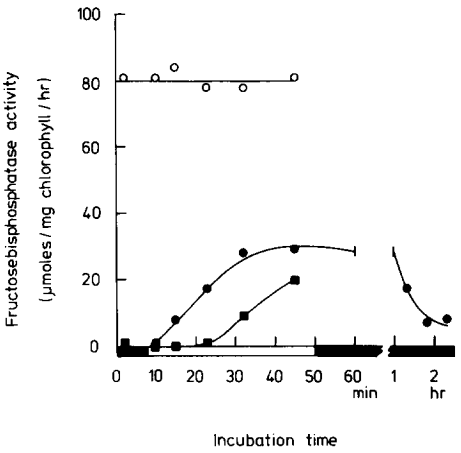

FIGURE 1: Activation of fructosebisphosphatase following illumination of isolated intact spinach chloroplasts. Incubation conditions and determination of enzyme activity are described under methods. The light intensity was 30 000 lux and the chlorophyll concentration 100 μg per ml. Illumination was begun after 8 min incubation and was removed after 50 min. At the times indicated, enzyme activity was measured at pH 7.5 (■—■), 7.9 (●—●) and 8.8 (○—○).

or 7.9, although the enzyme showed substantial activity at pH 3.8. However, following 7 min illumination, enzyme activity at pH 7.9 was clearly detected and increased in response to further illumination until after 24 min it was more than one-third of the activity measured at pH 8.8. The enzyme remained inactive at pH 7.5 during 15 min illumination, but thereafter the activity at this pH responded almost as rapidly to light as that at pH 7.9. When the light was turned off the activity at pH 7.9 returned to a low value. Illumination did not alter the pH 8.8 activity. Control experiments showed that both light and chloroplast intactness were required for activation: no fructosebisphosphatase activity at pH 7.9 developed in chloroplasts incubated for 70 min in darkness, or after 45 min illumination of chloroplasts broken at the start of incubation by addition to resuspending medium containing no sorbitol, although in both

experiments high activity at pH 8.8 was recorded. However, fructosebisphosphatase active at both pH 7.5 and 7.9 was observed in a suspension of broken chloroplasts incubated in the dark in the presence of 10 mM dithiothreitol; the activity at pH 7.9 was about 40% of that at pH 8.8 and 1.5-times that at pH 7.5, results almost identical to those obtained after illumination of intact chloroplasts for 37 min. Thus, from the viewpoint of response to pH, light activation in vivo and dithiothreitol activation in vitro were very similar.

Activation of spinach chloroplast fructosebisphosphatase by light of various intensities is shown in Figure 2. Enzyme activity at pH 7.9 increased more sharply at light intensities below 5000 lux, but the light activation process did not appear to be saturated even with the relatively high light intensity of 45 000 lux.

Pea chloroplast fructosebisphosphatase was also activated by light. Illumination of these chloroplasts increased the enzyme activity at pH 7.9 from no activity in darkness to 60% of the pH 8.8 activity after 45 min of light.

It is unlikely that the observations described here were an artifact of chloroplast isolation; in preliminary investigations evidence for light mediated activation of chloroplast fructosebisphosphatase in intact pea leaves has also been obtained. Enzyme activities at pH 7.5 and 7.9 in extracts from leaves previously held in darkness were respectively 30% and 29% of the activity at pH 8.8 and were most probably due entirely to the leaf cytoplasmic fructosebisphosphatase (9). After illumination of leaves for 22 min under prevailing conditions (sunshine, 7°C) the activity at pH 7.9 increased to 47% of that measured at pH 8.8 whereas no change in the activities at pH 7.5 or 8.8 took place.

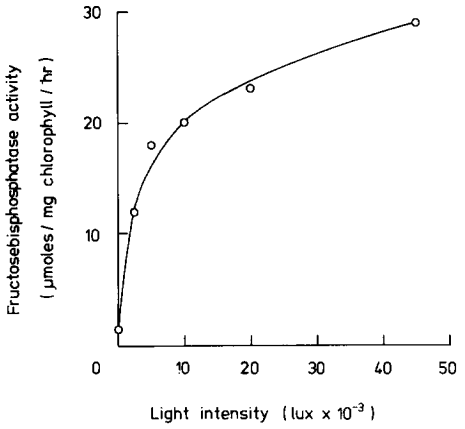

FIGURE 2: Influence of light intensity on the activation of fructosebisphosphatase in isolated intact spinach chloroplasts. Incubation conditions and determination of enzyme activity are described under methods. The chlorophyll concentration was 80 µg per ml and illumination was for 20 min. The pH of reaction mixtures was 7.9.

DISCUSSION: These experiments have clearly demonstrated a light activation of chloroplast fructosebisphosphatase which can be detected only when the enzyme is assayed at a pH near to that of the stroma of illuminated chloroplasts. Reductive formation of sulphydryl groups is the activation mechanism most logically proposed from evidence presently available; the responses to pH of light activated enzyme, on the one hand, and dithiothreitol activated enzyme, on the other, were very similar, and in other experiments from this laboratory (Zimmermann et al., manuscript in preparation) the number of sulphydryl groups in purified chloroplast fructosebisphosphatase more than doubled when the enzyme was incubated with dithiothreitol and this was associated with the appearance of activity at pH 7.5. If light activation does involve a reductive process, the nature of the in vivo reductant becomes an intriguing question. Reduced ferredoxin, which Buchanan et al. (5) have reported to activate chloroplast fructosebisphosphatase, may be one contender for

this role, while natural dithiols offer an attractive alternative. Vallejos and Andreo (10) recently obtained evidence that greater exposure of natural vicinyl dithiols follows light-induced conformational changes of chloroplast membranes and it is not inconceivable that similar dithiols were closely involved with the light activation of fructosebisphosphatase reported here.

Photosynthetic CO_2 fixation at a rate of 100 µmoles per mg chlorophyll hr^{-1} would require chloroplast fructosebisphosphatase activity of at least 50 µmoles per mg chlorophyll hr^{-1} (for starch synthesis in chloroplasts) or 33 µmoles per mg chlorophyll hr^{-1} (for sucrose synthesis in the cytoplasm). The enzyme activity measured at pH 7.9 in illuminated spinach chloroplasts (Figure 1) was therefore close to this minimum requirement. Somewhat higher activities will quite probably be detected in the future, but fructosebisphosphatase is likely to remain one Calvin cycle enzyme with activity not greatly in excess of the rate of CO_2 fixation, thus increasing the likelihood that the sigmoid response of the enzyme activity to concentrations of substrate and cofactor (4,7) is utilised to regulate CO_2 fixation. It will be interesting to examine the interaction between these regulatory properties and the light mediated activation since both are particularly sensitive to pH (7, Figure 1) over the physiological range of 7.2 to 8.0 (3).

ACKNOWLEDGEMENTS: We thank E. Wirth and the Lehrstuhl für Gemüsebau for growing the spinach, and the Deutsche Forschungsgemeinschaft for generous support.

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