LIGHT ACTIVATION OF CALVIN CYCLE ENZYMES AS MEASURED IN PEA LEAVES

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1. Introduction

Several soluble Calvin cycle enzymes, including fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and ribulose 5-phosphate kinase are interconverted from an inactive to an active form by reducing equivalents, which can be generated photochemically via the ferredoxin–thioredoxin system [1]. For FBPase it has been demonstrated that this interconversion involves the reduction of disulfide groups of the enzyme protein [2] and there are indications that also the activation of SBPase and Ru5P kinase proceeds in this mode. In isolated chloroplasts the above mentioned enzymes were shown to be activated in the light and inactivated in the dark, but the rate and extent of activation and deactivation observed by different investigators varied largely [3–9]. To avoid alterations of the activation state of these enzymes after extraction from the chloroplasts, a method has been designed in which the enzyme assays were terminated only 15–30 s after the rupture of the chloroplasts in the assay medium. Upon illumination a 20–30-fold activation of the 2 phosphatases and a 9-fold activation of Ru5P kinase was observed [10]. The physiological function of this enzyme activation is still a matter of debate. Many authors (citations in [1]) infer that the enzyme activation by reducing equivalents plays a major role in the control of CO_2 fixation by light. Other authors [11] have questioned this and regarded it more likely that these enzyme activation systems exist in vivo to prevent inhibition of the reduced forms of enzymes by any light-generated H_2O_2. To decide between these two interpretations, one has to know to what extent in a whole leaf these enzymes are inactivated in the dark and activated in the light. Light activation of enzymes in whole leaves has been investigated, but the times of extraction were often long and relatively small activations were observed [1]. In [12], RuBP carboxylase activity was determined in spinach leaves. In these measurements the grinding procedure for releasing the enzyme from the leaf tissue required ~60 s. Here, we report a method, by which enzymes can be extracted from leaves $\lesssim$5 s. This method has been used to determine the activation state of 6 Calvin cycle enzymes in pea leaves. The results clearly show that in the leaves the activities of FBPase, SBPase and Ru5P kinase undergo very large changes between illumination and darkness.

Abbreviations: FBPase, fructose 1,6-bisphosphatase; SBPase, sedoheptulose 1,7-bisphosphatase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate

2. Materials and methods

The experiments were performed with shoots from 14–16-day-old pea plants (*Pisum sativum*, var. Kleine Rheinländerin), grown in vermiculite. The plants had been kept in complete darkness for 60 min before the experiment. For each measurement, 6 pea leaves were excised and placed into a 5 ml plastic syringe, which contained at the bottom a 200 μM net (Monodur, Verseidag, Krefeld). The outlet of the syringe was provided with a capillary in order to decrease the dead volume. To ensure a constant supply of CO_2 and to prevent the leaves from dessication, a stream of moist air was pumped through the syringe. The syringe was kept in complete darkness. At zero time it was illuminated by white light from a tungsten–halogen light source of ~100 W/m^2. After defined periods of illumination, the leaves were violently squashed against the net at the bottom of the syringe, releasing 20–30 μl cell sap which was collected in 200 μl extraction medium. Within 5–10 s, 20 μl of this extract were transferred into 100 μl assay medium,

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containing the constituents of the assay (see below). The enzyme assays, carried out in principally the same way as described earlier for the assay of enzyme activities in isolated chloroplasts [10], were terminated 30 s later by addition of 250 μl 0.2 M HCl, and the products formed were determined subsequently. All results of enzyme activity measurements were related to chlorophyll. For this, from each extraction an aliquot was taken for the assay of chlorophyll (fig.1).

The assay of RuBP carboxylase [13] was done in the presence of 0.5 mM ribulose 1,5-bisphosphate, 10 mM $NaH^{14}CO_3$, 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.1) and 0.04% (v/v) Triton X-100. Extraction medium: 20 mM $MgCl_2$, 100 mM Tris–HCl (pH 8.1), 0.05% (v/v) Triton X-100. Ru5P kinase was assayed as in [10]. The extraction medium was as for RuBP carboxylase assay. The assay of FBPase and SBPase [10] was performed in a medium containing 10 mM $MgCl_2$, 100 mM Tris–HCl (pH 7.8), 0.2% (v/v) Triton X-100 and either 0.2 mM fructose 1,6-[1-^{32}P]bisphosphate or 0.08 mM sedoheptulose 1,7-[1-^{32}P]bisphosphate (spec. act. 0.2–1.0 Ci/mol). The extraction media were the same, but fructose 1,6- or sedoheptulose 1,7-bisphosphate were unlabelled. The activity of NADP-glyceraldehyde 3-phosphate dehydrogenase was assayed from the release of [^{32}P]phosphate in a medium containing 6 mM [γ-^{32}P]ATP (spec. act. 1.5 Ci/mol), 0.3 mM NADPH, 7 mM 3-phosphoglycerate, 15 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 50 mM Hepes (pH 8.0) and 0.2% (v/v) Triton X-100. The extraction medium had the same composition, but ATP and NADH were omitted. For measuring the release of [^{32}P]phosphate see [10]. For measuring the activity of 3-phosphoglycerate kinase the assay medium contained 6 mM 3-phosphoglycerate, 1 mM ATP, 0.2 mM NADH, 0.9 mM EDTA, 2 mM $MgSO_4$, 2.7 U/ml glyceraldehyde 3-phosphate dehydrogenase (Boehringer), 100 mM triethanolamine (pH 7.6) and 0.2% (v/v) Triton X-100. This reaction was stopped with 250 μl 1M $HClO_4$. After neutralization, ADP was determined spectrophotometrically by enzymatic assay. The extraction medium contained 100 mM triethanolamine (pH 7.6) and 0.2% (v/v) Triton X-100.

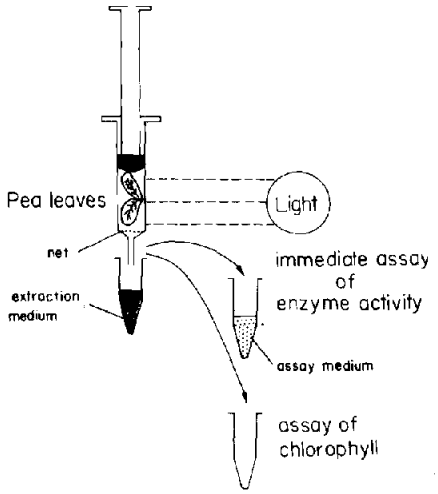

Fig.1. Schematic representation of the method.

3. Results and discussion

Fig.2–5 show the time courses for the assay of enzymes in pea leaves during a dark–light transient. Since each measuring point in the time courses has been obtained with an individual sample of leaves, the observed scatter of the data may reflect the variability to be expected in biological material. Table 1 shows mean values of enzyme activities as measured in several other experiments in the dark and after 15 min illumination.

The changes in the activities of SBPase and FBPase observed upon illumination were similar to those found in isolated spinach chloroplasts [10]. The half-times of activation were about the same, whereas the

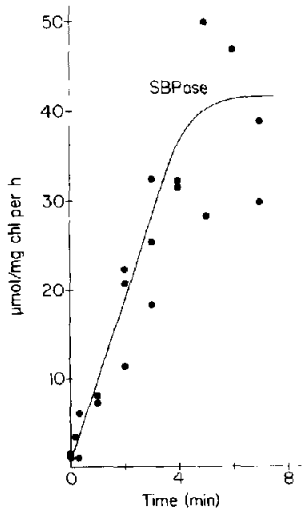

Fig.2. Time course of the light activation of sedoheptulose 1,7-bisphosphatase in pea leaves.

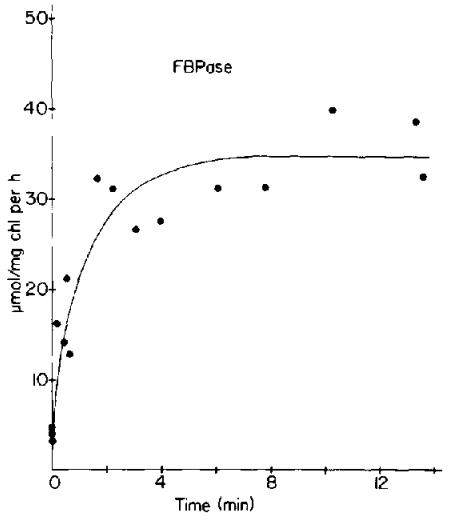

Fig.3. Time course of the light activation of fructose 1,6-bisphosphatase in pea leaves.

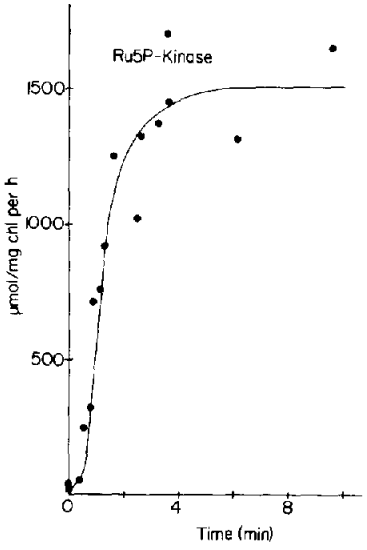

Fig.4. Time course of the light activation of Ru5P kinase in pea leaves.

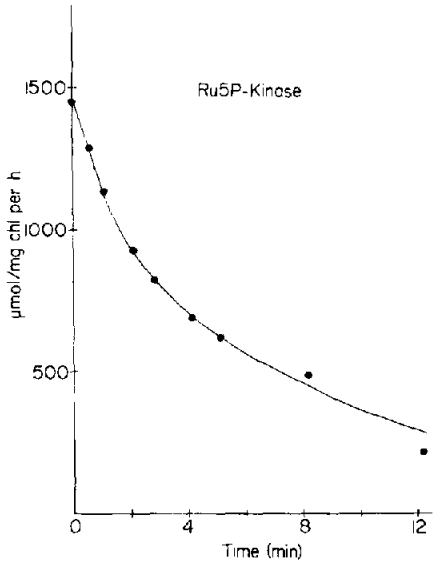

Fig.5. Time course of the dark inactivation of ribulose 5-phosphate kinase in pea leaves.

extent of activation was in the leaves somewhat lower than in spinach chloroplasts. It may be noted, however, that in leaves, part of the FBPase assayed in the dark (and also in the light) reflects cytosolic FBPase [14]. Although the assay conditions had been selected in order to suppress this cytosolic activity, it could not be eliminated completely. Thus in the leaf the dark activity of the stromal FBPase will be lower and hence the light activation higher than shown in our experiments.

For Ru5P kinase, a 40-fold activation is found when the leaves are illuminated (fig.4) which is higher than observed in intact spinach chloroplasts (9-fold [10]). In these chloroplasts, as well as in the leaves the activation of Ru5P kinase was more rapid than that of the two phosphatases. Compared to this, the rate of Ru5P kinase inactivation during a light–dark

Table 1
Light-dependent interconversion of Calvin cycle enzymes

Enzyme	Dark	Light	Activation	No. Exp.
Ribulose 5-phosphate kinase	29	1160	40	3
Ribulose 1,5-bisphosphate carboxylase	363	440	1.2	3
(NADP) Glyceraldehyde 3-phosphate dehydrogenase	2454	3368	1.4	4
3-Phosphoglycerate kinase	4098	8417	2.1	3
Sedoheptulose 1,7-bisphosphatase	2.8	28.5	12	9
Fructose 1,6-bisphosphatase	2.5	37.4	15	4

transient was rather low, which has been also observed with isolated chloroplasts.

For NADP glyceraldehyde 3-phosphate dehydrogenase, the extent of light activation was very low, which concurs with earlier measurements with spinach chloroplasts [3,5,9]. 3-Phosphoglycerate kinase showed only a 2-fold activation. In pea leaves, we did not find any significant light activation of RuBP carboxylase. Earlier investigations had shown that the light activation of this enzyme varied. Studies with intact spinach chloroplasts yielded up to 4-fold light activations [13,15], in wheat protoplasts the activation was <1.4 [16], and in wheat leaves a 3-fold actiation was found [12]. The metabolic regulation of this enzyme is not yet understood.

Our results clearly demonstrate that the interconversion of FBPase, SBPAse and Ru5P kinase from an inactive to an active form occurs when a plant is illuminated. As discussed in [10,17], the resulting activated and inactivated forms of these enzymes can be effectively and instantaneously modulated by light through the stromal pH, the concentrations of Mg^{2+}, ADP, and other metabolites. A combination of this modulation of enzyme activity with the light-mediated interconversion of enzymes will mean that enzymes are virtually switched off in the dark. This can be well illustrated for Ru5P kinase. When this enzyme, extracted from darkened and illuminated spinach chloroplasts, is assayed in the presence of those concentrations of H^+, Mg^{2+}, 3-PGA and ADP found in the stroma of darkened and illuminated chloroplasts, respectively, the activity of Ru5P kinase found under conditions of illumination is found to be 400-times larger than the activity in a simulated dark condition (Gardemann, unpublished). With FBPase and SPBase a similar situation exists. Apparently, light-dependent interconversion of stromal enzymes enables the plant cell to restrict the activity of certain Calvin cycle enzymes in the dark in a very rigid way.

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