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CONTROL OF CO_2 FIXATION

CHANGES IN THE ACTIVITY OF RIBULOSEPHOSPHATE KINASE AND FRUCTOSE- AND SEDOHEPTULOSE-BISPHOSPHATASE IN CHLOROPLASTS *

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The activation state of the chloroplast stromal enzymes fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-5-phosphate kinase was assayed by injecting the chloroplasts into an assay medium where the chloroplasts are immediately lysed and the enzymic assay is terminated 15–30 s later. (1) After the onset of illumination the activities of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase rise 20- and 30-fold, respectively, and rapidly decrease again when the light is turned off. The light activation of both enzymes shows a substrate requirement. The catalytic activity of the activated enzyme is dependent on the pH and the Mg^{2+} concentration in the assay medium, with the fructose-1,6-bisphosphatase more markedly affected. (2) The light activation of ribulose-5-phosphate kinase occurs even more rapidly than that of the other two enzymes and leads to about 10-fold activation. Moreover, the inactivated form of the enzyme, as extracted from chloroplasts in the dark, is strongly inhibited when ADP is added to the assay medium. (3) These data clearly indicate that chloroplasts have the ability to inactivate the three mentioned Calvin cycle enzymes virtually completely in the dark phase by a combined effect of changes of the reductive state of the electron-transport carrier on all three enzymes, changes of stromal Mg^{2+} and pH on fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase and changes of stromal ATP/ADP ratios on ribulose-5-phosphate kinase.

Introduction

The reductive pentose phosphate pathway appears to be regulated by light. As possible sites for such a regulation, a number of enzymes of the Calvin cycle have been discussed, including fructose- and sedoheptulose-bisphosphatase, ribulose-5-phosphate kinase and ribulose-bisphosphate carboxylase (for a review see Ref. 2). The effect of light on the activity of these enzymes is mediated by several parameters.

Some enzymes have been shown in vitro to be activated by a transfer of reducing equivalents, e.g., from ferredoxin as mediated by thioredoxin [3]. In order to identify the stromal enzymes which are most sensitive to this redox-mediated regulation in vivo, H_2O_2 has been used as a tool to prevent enzyme activation by reducing equivalents in illuminated chloroplasts. The inhibition of CO_2 fixation by H_2O_2 was found to be accompanied by a dramatic increase of stromal levels of fructose and sedoheptulose bisphosphate. Furthermore, there was a large decrease of the ribulose bisphosphate level observed, whereas the level of pentose monophosphates was unaltered and

* Some of these results have been included in a preliminary report [1].

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

the level of ATP even increased [4,5]. These findings suggest that the activities of fructose- and sedoheptulose-bisphosphatase and also of ribulose-5-phosphate kinase are actually modulated in intact chloroplasts by redox processes.

Another regulatory parameter is the stromal pH. With isolated chloroplasts, a change of this pH from 7.2 in the dark to 7.8–8.0 in the light is observed [6]. CO_2 fixation by chloroplasts is strongly dependent on the stromal pH. Those pH changes observed when the light is turned off were found to be sufficient to switch CO_2 fixation from almost zero to maximal activity [6]. Metabolite analysis in chloroplasts, in which CO_2 fixation was inhibited by artificially lowering the stromal pH, indicated that fructose-1,6-bisphosphatase and sedophetulose-1,7-bisphosphatase were inhibited under these conditions [7–9].

Finally, there are various observations suggesting that CO_2 fixation may be controlled by the Mg^{2+} concentration in the stroma. Studies with isolated thylakoid membranes suggested that Mg^{2+} is one of the major counterions of light-induced proton transport across the thylakoid membranes [10–13]. It could be demonstrated with isolated chloroplasts that illumination causes an increase of the stromal Mg^{2+} concentration of 1–3 mM [14,15]. The stromal concentrations of free Mg^{2+} in the dark are not accurately known yet. In one report it was estimated at 1–4 mM [16], another investigator gave an estimate of 1–3 mM [17]. CO_2 fixation by intact chloroplasts was found to be very sensitive to stromal Mg^{2+} levels. Estimates of the Mg^{2+} concentrations necessary for maximal rates of CO_2 fixation indicated that the changes of the stromal Mg^{2+} level observed on illumination are high enough to have a possible function in the light regulation of CO_2 fixation [14]. Subsequent measurement of metabolite levels in chloroplasts, where CO_2 fixation was inhibited by artificial lowering of the stromal Mg^{2+} level, indicated that fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase are the Mg^{2+}-sensitive steps in the overall reaction of CO_2 fixation [18].

These findings about the regulatory role of fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-5-phosphate kinase have been derived from the study of stromal metabolite levels. A full understanding of enzyme regulation also requires a study of enzyme activities in the stroma. Several investigators have incubated chloroplasts under various conditions, subjected them to osmotic shock and then assayed the released enzymes under standard conditions. In this way, the activities of fructose-1,6-bisphosphatase [19–25], sedoheptulose-1,7-bisphosphatase [19], and ribulose-5-phosphate kinase [19,21–23,26–30] extracted from illuminated chloroplasts were found to be higher than those from chloroplasts kept in the dark. These findings showed that the activated state of these enzymes had a certain stability, and that the activation was a time-requiring process. In these studies the extent of activation varied greatly. The assays, mostly carried out by spectrophotometric recording, took at least several minutes. Thus, there was a danger that the activation state of enzymes was altered during the assay. The effect of stromal conditions, e.g., pH and Mg^{2+} concentration, on the catalytic activity of the enzymes extracted from illuminated chloroplasts was not studied. Furthermore, in all these assays (except that of Ref. 24) the chloroplasts had been ruptured by injecting them into a medium of comparatively low osmotic strength. Under such conditions, the rupture of the chloroplasts may require up to 30 s which also may significantly alter the activation state of enzymes. These technical difficulties might be responsible for the large variability of results obtained so far. The data published so far give no conclusive evidence as to whether these enzymes can be inactivated in the dark completely. This especially applies to ribulose-5-phosphate kinase, where relative high activities have been also reported in the dark.

In order to clarify these uncertainties and to be able to study the kinetics and extent of the activation and the inactivation of these enzymes systematically, we developed improved techniques for the measurement of the activation state of stromal enzymes. The chloroplasts are immediately ruptured by the aid of the detergent Triton X-100 [31], enabling an instantaneous start of enzyme activity measurement, which is terminated only 15–30 s later. In this way the time of the assay is kept short in relation to the kinetics of activation and inactivation. These assays have been used to investigate to what extent the activity of Calvin cycle enzymes is controlled by light, either by alterations of the activation state per se of these enzymes, or by producing changes in the catalytic

activity of the activated or inactivated enzymes due to alterations of stromal ion and metabolite levels.

Materials and Methods

Spinach. Spinach (*Spinacea oleracea*, U.S. Hybrid 424, Ferry-Morse Co., Mountain View, CA, U.S.A.) was grown in hydroponic culture [32]. For the preparation of chloroplasts see Ref. 6.

Chloroplasts. The chloroplasts (40–50 μg Chl/ml) were suspended in a reaction medium containing 0.33 M sorbitol, 50 mM Hepes adjusted to pH 7.6 with KOH, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM K_2HPO_4, 5 mM $NaHCO_3$ and 0.04 mg/ml catalase from bovine liver (Boehringer, Mannheim), if not stated otherwise. The temperature was 20°C. The light intensity (white light) was about 100 W/m^2.

Preparation of ribulose-1,5-bisphosphate carboxylase. The enzyme was purified from spinach leaves according to the method of Paulsen and Lane [33] until after the purification step employing DEAE-cellulose.

Preparation of fructose 1,6-[1-^{32}P]bisphosphate. First, [^{32}P]ATP was prepared from 4 mCi of carrier-free phosphate (Amersham, U.K.) according to the method of Post and Sen [34]. The reaction was allowed to proceed until 90% of the ^{32}P label had been incorporated into ATP, and was stopped by boiling for 3 min. To this, 5 mM fructose 6-phosphate and 6 U fructose-6-phosphate kinase from rabbit muscle (Boehringer, Mannheim) were added and the mixture was incubated for 1 h at room temperature. It was then added directly onto a Dowex 1 X 8-400 mesh Cl column (15 X 1 cm) at 4°C. The column was first eluted with 30 ml H_2O and then with a linear gradient from 0.04 to 0.08 M HCl (300 ml). Approx. 90% of the ^{32}P label was in the fructose 1,6-bisphosphate peak which eluted at approx. 0.07 M HCl. The peak fraction was collected, evaporated by freeze drying, redissolved in water, distributed in different vials, evaporated by freeze drying again and stored at –85°C.

Preparation of sedoheptulose 1,7-[1-^{32}P]bisphosphate. Fructose 1,6-[1-^{32}P]bisphosphate obtained as described above was dissolved in 2.85 ml of a reaction mixture containing 7 mM erythrose 4-phosphate (Sigma, St. Louis), 4.5 U aldolase, 500 U triose isomerase (both from rabbit, Boehringer, Mannheim), 50 mM Tris-HCl, pH 7.6, and 5 mM $MgCl_2$, incubated at room temperature for 1 h and then applied to an ion-exchange column and eluted as described above. The eluate showed only one peak appearing prior to where fructose 1,6-bisphosphate was to be expected. This chromatographic system was previously shown to separate sedoheptulose 1,7-bisphosphate from fructose 1,6-bisphosphate. The peak fraction was collected, evaporated twice by freeze drying and stored at –85°C.

The purity of ^{32}P-labelled fructose and sedoheptulose bisphosphate was verified by the following criteria: (i) Ion-exchange chromatography according to a method of Heldt et al. [35] showed only single peaks appearing in the region of fructose and sedoheptulose bisphosphate. Addition of fructose 1,6-[^{32}P]bisphosphate to a sedoheptulose 1,7-[^{32}P]bisphosphate sample and chromatography of the mixture showed that less than 5% contamination of the sedoheptulose 1,7-bisphosphate by the fructose 1,6-bisphosphate could be detected. (ii) Enzymatic spectrophotometric analysis of fructose and sedoheptulose [^{32}P]bisphosphate according to the method of Stitt et al. [36] showed that there was no detectable (less than 0.2%) triose phosphate present in the fructose and sedoheptulose bisphosphate. Analysis for fructose bisphosphate in the sedoheptulose bisphosphate was performed by sequentially adding glucose-6-phosphate dehydrogenase, phosphohexose isomerase and fructosebisphosphatase (all from Boehringer, Mannheim) to the ^{32}P-labelled substrate in 50 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$ and 0.2 mM NADP. This assay showed that there was less than 0.1% fructose 1,6-bisphosphate (limit of detection) in the prepared sedoheptulose 1,7-[^{32}P]bisphosphate but up to 2% fructose 1,6-bisphosphate in the commercially available sedoheptulose 1,7-bisphosphate (Sigma, St. Louis). The amounts of glucose 6-phosphate and fructose 6-phosphate in the ^{32}P-labelled bisphosphates was negligible.

Assay of ribulose-5-phosphate kinase.

Ribulose 5-phosphate + ATP

→ ribulose 1,5-bisphosphate + ADP

Ribulose 1,5-bisphosphate + $^{14}CO_2$

→ 3-phospho[2-^{14}C]glycerate

50 µl of chloroplast suspension (40–50 µg Chl/ml) were injected into 200 µl reaction mixture containing 0.2% Triton X-100, 0.5 mM ribulose 5-phosphate, 2 mM ATP, 10 mM $NaH^{14}CO_3$ (spec. act. 0.5–1 Ci/Mol), 100 mM Tris-HCl, pH 7.8, and 10 mM $MgCl_2$ (temperature 20°C). After 30 s the reaction was stopped by adding 20 µl of 2 M HCl and the sample was frozen at –20°C. It was then neutralized with 20 µl of 2 M NaOH containing 8 mM dichlorophenolindophenol (oxidized form). The addition of dichlorophenolindophenol inactivates any traces of ribulose-5-phosphate kinase in ribulose-1,5-bisphosphate carboxylase preparation.

To the neutralized sample at 20°C, 100 µl of a reaction mixture were added containing 20 mM $NaH^{14}CO_3$ of exactly the same specific activity as above, ribulose-1,5-bisphosphate carboxylase (1 U/ml), 0.5 mM dichlorophenolindophenol, 100 mM Tris-HCl, pH 8.2, and 20 mM $MgCl_2$. After 1 h, 300-µl aliquots were removed into a scintillation vial containing 200 µl of 1 M formic acid, and the sample was dried at 60°C. The sample was then dissolved in 200 µl of 1 M HCl and 7 ml of scintillation fluid (toluene/methylglycol, 6 : 4 (v/v) with 6 g/l 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole), and counted in a scintillation counter for ^{14}C radioactivity.

Assay of fructose- and sedoheptulose bisphosphatases.

Fructose 1,6-[1-^{32}P]bisphosphate

→ fructose 6-bisphosphate + $^{32}P_i$

Sedoheptulose 1,7-[1-^{32}P]bisphosphate

→ sedoheptulose 7-phosphate + $^{32}P_i$

50 µl of chloroplast suspension (40–50 µg Chl/ml) were injected into 200 µl of reaction medium containing 0.2 mM fructose 1,6-[1-^{32}P]bisphosphate or sedoheptulose 1,7-[1-^{32}P]bisphosphate (spec. act. 5–20 Ci/mol), 10 mM $MgCl_2$, 0.2% Triton X-100 and 100 mM Tris-HCl, pH 7.8 (temperature 20°C). After 30 s the reaction was stopped by adding 0.2 ml of 4.5% (w/v) $(NH_4)_4Mo_7O_{24} \cdot 4\,H_2O$ in 3.5 M HCl, and the sample was frozen at –85°C overnight. At 20°C, 10 µl of 0.1 M KH_2PO_4 were added, and the sample was then mixed with 0.9 ml isobutanol/benzene (1 : 1, v/v). After thorough shaking, the two phases were separated by centrifugation. 0.6 ml of the upper phase was added to 7 ml scintillation fluid (see above) and counted for ^{32}P radioactivity.

Results and Discusssion

Measurement of the activation state of stromal enzymes

A spectrophotometric recording of enzyme activity, as usually carried out, is not suitable for immediate measurements of the activation state of stromal enzymes, since minutes of recording are required to achieve accurate evaluation. This is in part due to the dramatic absorbance changes occurring during the disintegration of the chloroplast structure. The relatively long time required for a single activity assay makes it also impossible to establish the time course of light activation and dark inactivation with a single chloroplast incubation by withdrawing samples in short time intervals. For these reasons, our measurements were carried out by fixed-time incubation assay. For assaying stromal enzyme activities immediately after chloroplast rupture, the medium contains the substrates of the enzymic reactions to be assayed. The enzymic reaction, as initiated by the disruption of the chloroplasts, is terminated 15–30 s later by the addition of acid. In this way, several activation and inactivation kinetics can be carried out within a short time, with the sample being frozen and analyzed later. For the assay of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, the release of [^{32}P]phosphate from fructose 1,6-[^{32}P]-bisphosphate or sedoheptulose 1,7-[^{32}P]bisphosphate, respectively, is assayed. For the assay of ribulose-5-phosphate kinase, ribulose 5-phosphate is allowed to react with ATP to form ribulose 1,5-bisphosphate. The addition of ^{14}C-labelled bicarbonate and the presence of ribulose-1,5-bisphosphate carboxylase in the lysed chloroplasts converts about 20% of the newly formed ribulose 1,5-bisphosphate to ^{14}C-labelled 3-phosphoglycerate. After quenching the reaction with acid, the bulk of the newly formed ribulose 1,5-bisphosphate is then also converted to 3-phospho[^{14}C]glycerate by incubation with more [^{14}C]bicarbonate and ribulose-1,5-bisphosphate carboxylase.

Fig. 1 shows time courses for the assay of fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-5-phosphate kinase. At various times after the injection of chloroplasts into the reaction medium, samples were withdrawn and analyzed for P_i released or ^{14}C incorporation. The assay is linear from the very beginning, demonstrating an immediate lysis of the chloroplasts. Linear time courses were also observed for the low enzyme activities extracted from dark-pretreated chloroplasts, indicating that the dark-inactivated enzymes were not reactivated during the assay.

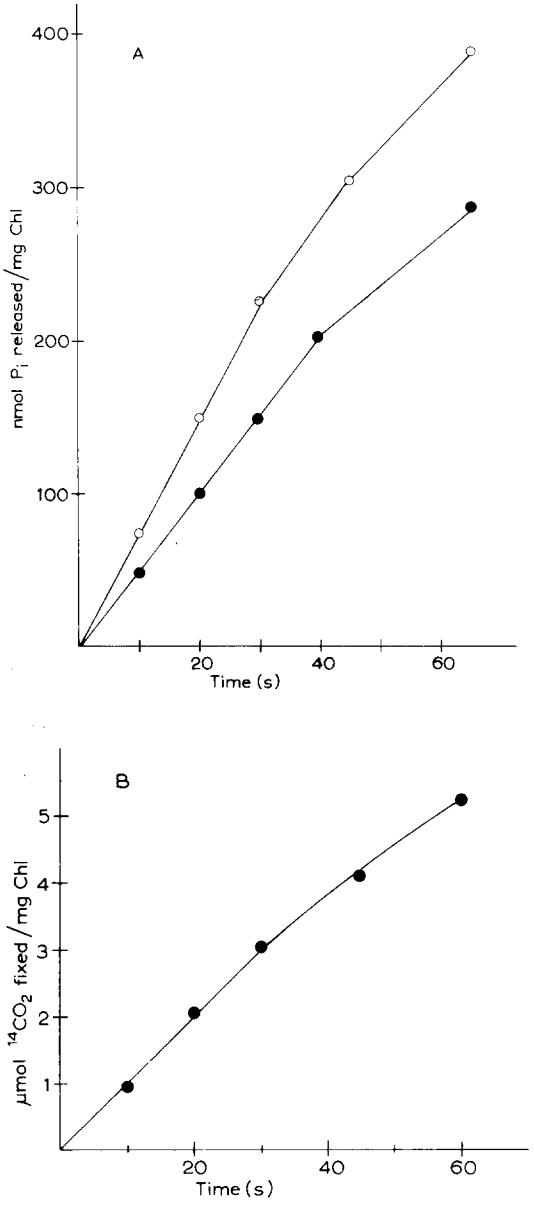

Fig. 1. Time course for the assay of enzyme activities extracted from illuminated chloroplasts. (A) Fructose-1,6-bisphosphatase (○———○), sedoheptulose-1,7-bisphosphatase (●———●). (B) Ribulose-5-phosphate kinase.

Fructose-1,6-bisphosphatase

The experiment of Fig. 1 reveals dramatic changes between fructose-1,6-bisphosphatase activities released from chloroplasts kept in the dark and in the light. Fig. 2 shows a time course for the light activation and dark inactivation of the enzyme in situ. After turning on the light, the activity of this enzyme rises rapidly to about 20-fold of the dark activity, and it is even more rapidly decreased when the light is switched off again. The mechanism of this activation by light and deactivation in the dark, as studied by various investigators (see Introduction), will not be

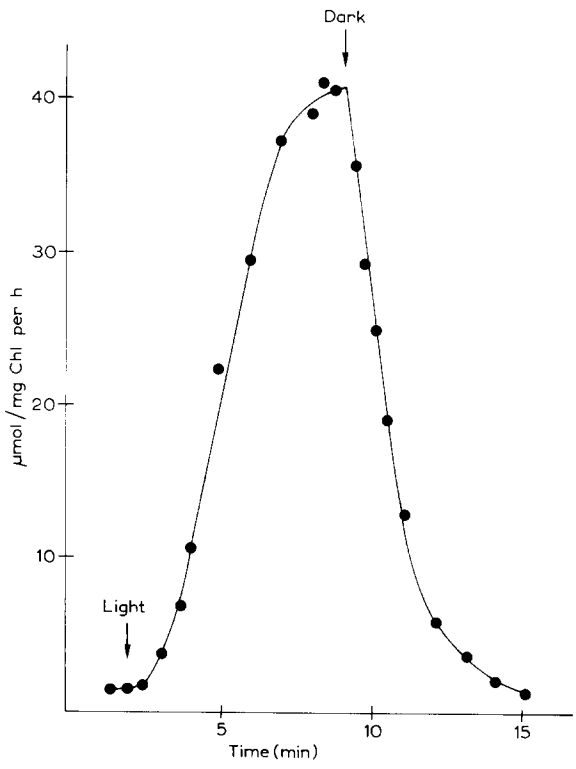

Fig. 2. Light activation of fructose-1,6-bisphosphatase in chloroplasts.

discussed here in detail. Our enzyme activity measurements were carried out at pH 7.8 in the presence of 10 mM $MgCl_2$. Light-dependent changes of the stromal pH and Mg^{2+} concentration may affect the activity of fructose-1,6-bisphosphatase in two different ways. Firstly, the light activation of the enzyme as such seeems to be influenced by the these parameters [22]. Secondly, an effect of stromal pH and Mg^{2+} concentrations on the catalytic activity of the activated (or inactivated) enzyme is to be expected. This is shown in Fig. 3, where illuminated chloroplasts had been injected into assay media of different pH and Mg^{2+} concentrations. At pH 7.8 and high Mg^{2+} concentration, the assay was linear throughout. In a medium of pH 7.0 and low Mg^{2+} concentration, the corresponding activity assays were linear for about 20 s only and then gradually declined. Apparently, in such a milieu resembling the conditions in the stroma in the dark, the activated enzyme can be gradually inactivated. In order to eliminate such time-dependent changes of the activation state of the enzyme and to measure the effect of pH and Mg^{2+} concentration on the catalytic activity of the light-activated enzyme only, the time for the assay of fructose-1,6-bisphosphatase activity was reduced to 15 s. As shown in Fig. 3, the pH and Mg^{2+} dependence of the light-activated enzyme is very strong and resembles the pH and Mg^{2+} dependence of partially purified fructose-1,6-bisphosphatase from spinach which had been activated by dithiothreitol [37–39].

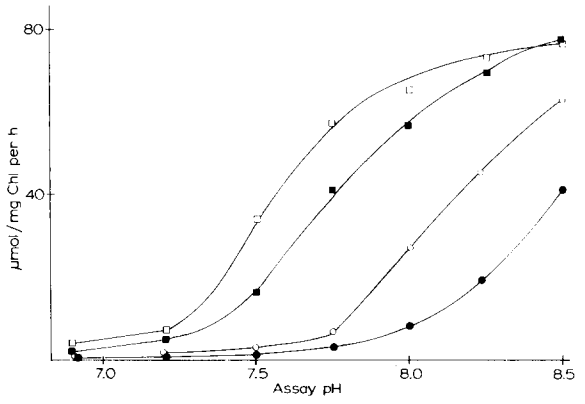

Fig. 3. Fructose-1,6-bisphosphatase from illuminated chloroplasts. Dependence of the catalytic activity on the pH and Mg^{2+} concentration in the assay medium. Mg^{2+} present at 10 mM (□), 5 mM (■), 1 mM (○), 0.5 mM (●).

Thus, a change during a transition from illumination to darkness in the chloroplast stroma from pH 7.8 to 7.0 with 5 mM Mg^{2+} (see Introduction) would decrease the catalytic activity of the light-activated enzyme to about 6%, and any simultaneous decrease of the stromal Mg^{2+} concentration would further decrease this value.

After the onset of illumination, the activation of fructose-1,6-bisphosphatase occurs with a characteristic lag (Figs. 2 and 4; see also Ref. 20), showing a similarity to the lag during the induction of CO_2 fixation in isolated chloroplasts [40]. This lag can be prolonged, and the velocity of activation decreased when the chloroplasts are incubated in the presence of high external phosphate concentrations (Fig. 4; see also Ref. 21), which are known to deplete the stromal levels of the Calvin cycle intermediates [41]. The

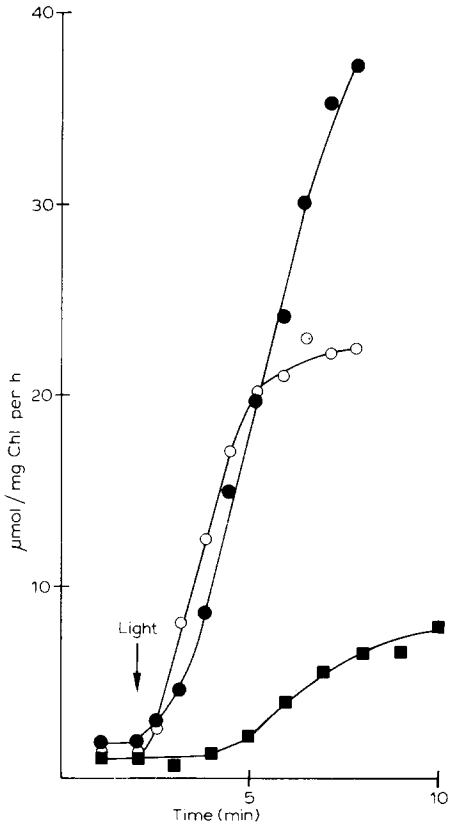

Fig. 4. Light activation of fructose-1,6-bisphosphatase in chloroplasts. Effect of the addition of 3-phosphoglycerate or P_i to the chloroplast incubation medium. (●) Control, (○) 1.2 mM 3-phosphoglycerate, (■) 5 mM P_i.

addition of 3-phosphoglycerate, which is known to shorten the lag in chloroplasts by stimulating the autocatalytic build up of Calvin cycle intermediates [40], decreases the lag of enzyme activation (Fig. 4). These findings indicate that the light activation of this enzyme requires the presence of Calvin cycle intermediates in the stroma. Studies on the activation of chloroplast fructose-1,6-bisphosphatase by reduced thioredoxin in vitro indicated that the activation of the enzyme was stimulated in the presence of fructose 1,6-bisphosphate [42]. It may be noted that with 3-phosphoglycerate the extent of enzyme activation is lowered (Fig. 4). This might be due to a decreased level of reduced thioredoxin present.

Sedoheptulose-1,7-bisphosphatase

Evidence has been presented that fructose- and sedoheptulose-bisphosphatase are separate enzymes [43]. To show that in our measurements sedoheptulose-1,7-bisphosphatase was a separate enzyme from fructose-1,6-bisphosphatase, isotope dilution experiments were performed. Addition of unlabelled sedoheptulose bisphosphate to the ^{32}P-labelled sedoheptulose bisphosphate in the assay decreased the amount of released [^{32}P]phosphate concomitant with the isotope dilution and total sedoheptulose bisphosphate concentration. Addition of unlabelled fructose bisphosphate (0.8 mM) to 0.2 mM sedoheptulose [^{32}P]bisphosphate in the assay inhibited ^{32}P release maximally by 20% (not shown).

As shown in Fig. 5, illumination causes a 30-fold activation, which is reversed after the light has been turned off. The dependence of activity on the pH and Mg^{2+} concentration in the assay medium was investigated in the experiment of Fig. 6. Here the assay time was again reduced to 15 s in order to prevent errors due to time-dependent changes of the extent of activation at low pH and Mg^{2+} concentration which lead to loss of linearity after 15–20 s. The data of Fig. 6 show also for sedoheptulose-1,7-bisphosphatase a marked sensitivity of the catalytic activity of the light-activated enzyme to changes of pH and Mg^{2+} concentration. Thus, at pH 7.8 and 5 mM Mg^{2+}, resembling the stromal conditions in the light (see Introduction), a 6-fold higher activity would be obtained than at pH 7.0 and 1 mM Mg^{2+}.

The light activation of sedoheptulose-1,7-bisphosphatase also requires the presence of substrates in the

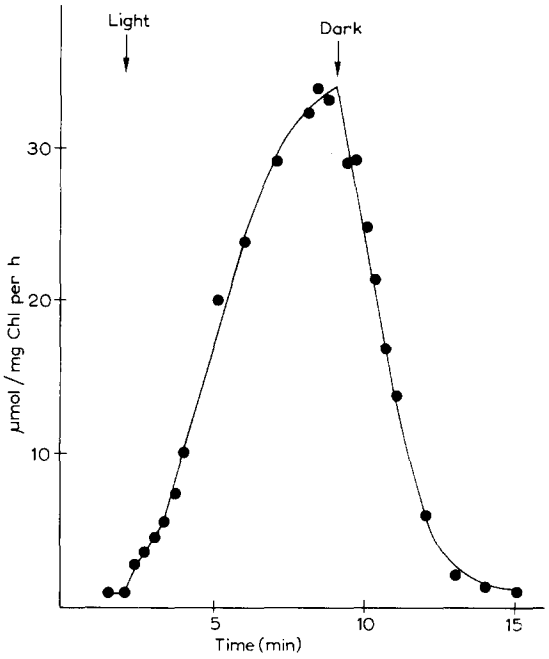

Fig. 5. Light activation of sedoheptulose-1,7-bisphosphatase in chloroplasts.

stroma. In the experiment of Fig. 7, the rate of light activation in the presence of high phosphate levels was almost zero, and after the addition of 3-phosphoglycerate not only the decrease of the lag but also the increase of the rate of activation was considerable. A

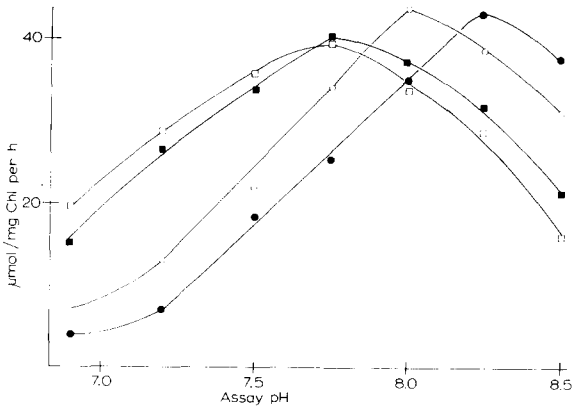

Fig. 6. Sedoheptulose-1,7-bisphosphatase from illuminated chloroplasts. Dependence of the catalytic activity on the pH and Mg^{2+} concentration in the assay medium. Mg^{2+} present at 10 mM (□), 5 mM (■), 1.0 mM (○), 0.5 mM (●).

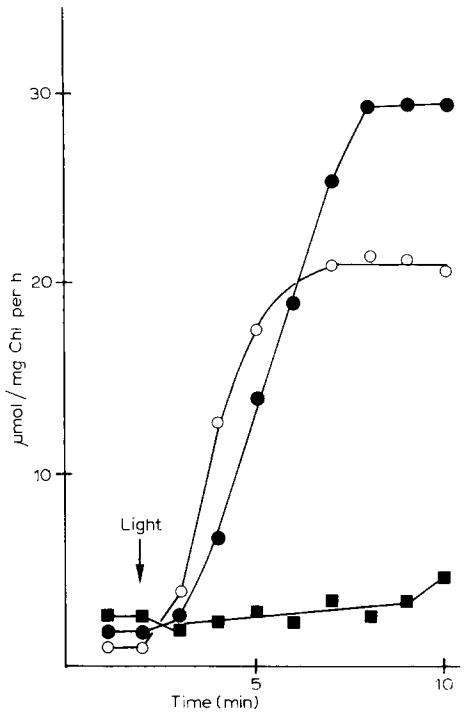

Fig. 7. Light activation of sedoheptulose-1,7-bisphosphatase. Effect of the addition of 3-phosphoglycerate or P_i to the chloroplast incubation medium. (•) Control, (○) 1.1 mM 3-phosphoglycerate, (▪) 5 mM P_i.

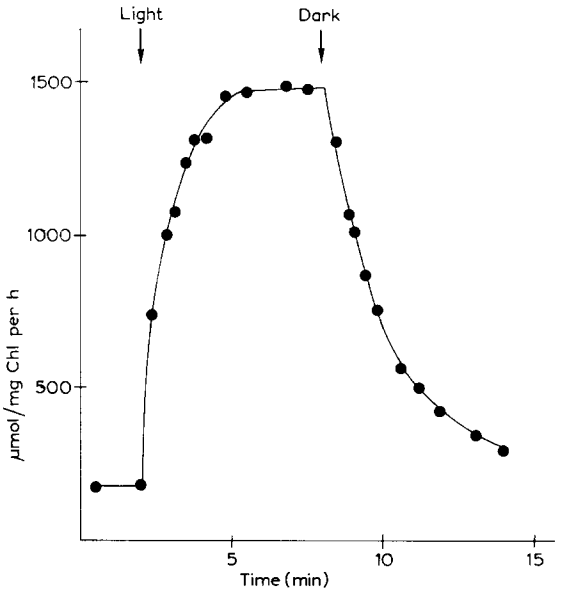

Fig. 8. Light activation of ribulose-5-phosphate kinase in chloroplasts.

stimulation of the rate of activation of this enzyme by the addition of dihydroxyacetone phosphate to chloroplasts has been independently observed by Woodrow and Walker (personal communication). One very interesting difference between sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase concerns the difference in K_m. Whereas the light-activated fructose-1,6-bisphosphatase shows half-saturation with 80 µM fructose 1,6-bisphosphate (for substrate saturation of the purified enzyme see Refs. 38 and 44), 5 µM sedoheptulose 1,7-bisphosphate is sufficient for half-saturation of sedoheptulose-1,7-bisphosphatase. Since the concentration of sedoheptulose 1,7-bisphosphate in the stroma during CO_2 fixation is about 0.25 mM [36], this enzyme seems to be substate saturated in vivo.

Ribulose-5-phosphate kinase

The light activation of ribulose-5-phosphate kinase as shown in Fig. 8 proceeds considerably faster than that of the two bisphosphatases. In the experiment of Fig. 8 the light activation was 9-fold. It may be noted that the light intensities required for a half-maximal activation of ribulose-5-phosphate kinase as well as fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase (3 W/m^2, white light; Laing, unpublished data), is relatively low. It is therefore essential to keep the chloroplasts in complete darkness prior to illumination to observe a large degree of light activation. In contrast to fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, the activation of ribulose-5-phosphate kinase was not affected by the addition of 5 mM phosphate (Fig. 9 and Ref. 21). Neither did the addition of 3-phosphoglycerate increase the rate of activation, although the extent of activation was decreased. This decrease in the extent of activation, as also observed with the two phosphatases, may again be due to a decreased level of reduced thioredoxin under these conditions. It appears from these data that the activation of ribulose-5-phosphate kinase does not require Calvin cycle intermediates. Compared to fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, ribulose-5-phosphate kinase activity is less sensitive to changes of pH and Mg^{2+} concentration (Fig. 10).

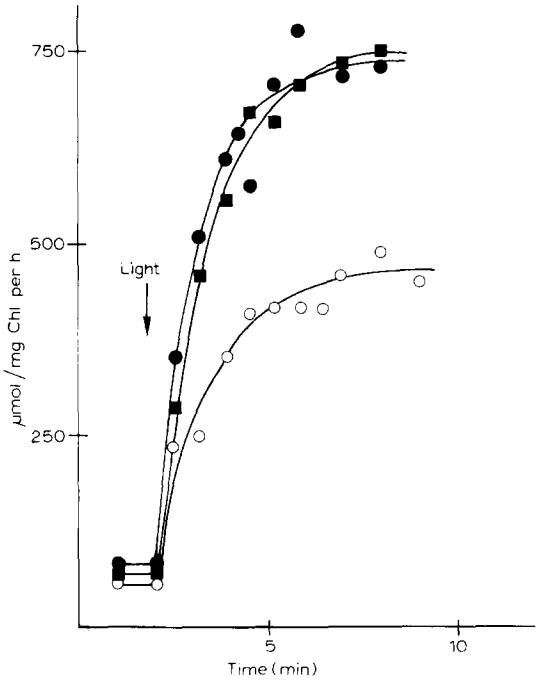

Fig. 9. Light activation of ribulose-5-phosphate kinase. Effect of the addition of 3-phosphoglycerate or P_i to the chloroplast incubation medium. (•) Control, (○) 1.2 mM 3-phosphoglycerate, (▪) 5 mM P_i.

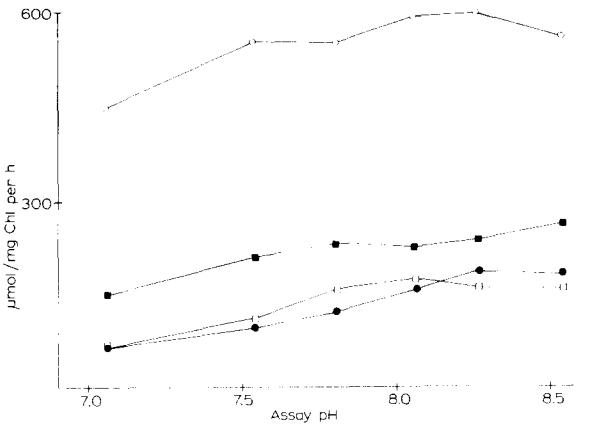

Fig. 10. Ribulose-5-phosphate kinase from illuminated chloroplasts. Dependence of the catalytic activity on the pH and the Mg^{2+} concentration in the assay medium. Mg^{2+} present at 10 mM (○), 5 mM (▪), 1.0 mM (•), 0.5 mM (□).

Nevertheless, in a medium with a pH of 7.8 and 4 mM Mg^{2+}, this enzyme is about 4-times more active than at pH 7.0 and 1 mM Mg^{2+}.

Investigation in our laboratory of stromal metabolite levels during a dark/light transition of intact chloroplasts (Flügge, Stitt and Heldt, unpublished observations) showed that the termination of CO_2 fixation, which was completed within 15 s after turning off the light, was accompanied by a rapid fall of the ribulose 1,5-bisphosphate level and a corresponding rise of the stromal ribulose 5-phosphate. Although the stromal ATP level decreased under these conditions, there was still stromal ATP available in the dark (unpublished data). These findings strongly suggested that cessation of illumination resulted within a few seconds in an almost complete inactivation of ribulose-5-phosphate kinase. As the rate of dark inactivation of this enzyme (Fig. 8) is too low to account for this observation, and a rapid change of stromal pH and Mg^{2+} concentration could at best only lead to 75% inhibition, an additional mechanism regulating this enzyme was to be expected. Earlier studies by Lavergne et al. [27] indicated that the activity of ribulose-5-phosphate kinase is influenced by the 'energy charge' of adenine nucleotides. Anderson [46], on the other hand, reported that the enzyme was not inhibited by ADP or AMP and was unaffected by energy charge levels. In these experiments ribulose-5-phosphate kinase activity was measured by the assay of ADP. Our assay procedure, by directly measuring ribulose 1,5-bisphosphate, enabled us to study the effect of ADP and AMP on the activity of ribulose-5-phosphate kinase systematically.

In Fig. 11 the ATP dependence of light-activated ribulose-5-phosphate kinase is shown. With ATP present alone, a half-saturation by ATP at about 30 µM is found (cf. Refs. 45 and 46). The addition of ADP and AMP to the ATP, with the adenylates in adenylate kinase equilibrium, when the total of AMP + ADP + ATP was 1.28 mM (similar to that found in the chloroplast stroma; unpublished data) changed the ATP concentration required for half-saturation of the enzyme to 220 µM. The experiment of Table I investigates the effect of ADP and AMP on the activity of the enzyme. Whereas ADP causes a strong inhibition, AMP shows only a minor effect. Since the ribulose-5-phosphate kinase-catalyzed reaction is practically irreversible, these data clearly show that ADP has a true inhibitory effect on this enzyme. This inhibitory effect of ADP is dramatically increased when the enzyme is extracted from chloroplasts in

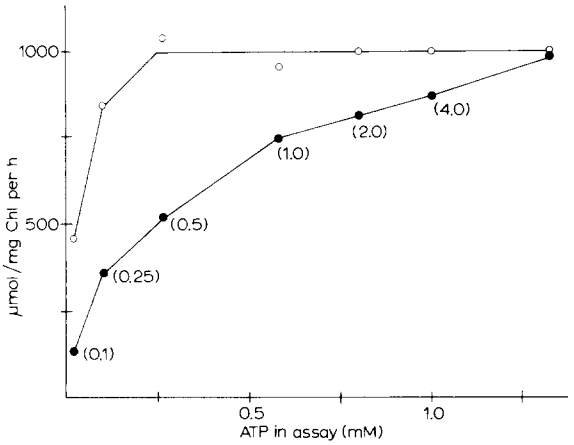

Fig. 11. Ribulose-5-phosphate kinase from illuminated chloroplasts. Dependence of the catalytic activity on the concentration of ATP, ADP and AMP in the assay medium. (A) (○———○) Addition of ATP only. The ATP was maintained in a phosphorylated state through the aid of creatine phosphate (2.5 mM) and creatine kinase (1.3 U/ml). (B) (●———●) Addition of ATP as indicated plus ADP and AMP in equilibrium with the ATP, as catalyzed by adenylate kinase (6 U/ml), yielding a total concentration of AMP + ADP + ATP of 1.28 mM. The figures in parenthesis show the ATP/ADP ratio.

the dark. Thus, a change from illumination to darkness accompanied by a change of the ATP/ADP ratio from 2 to 0.2 reduces the activity of ribulose-5-phosphate kinase to 0.5%.

Enzyme regulation

As has been shown in the preceding text, the activities of ribulose-5-phosphate kinase, sedoheptulose-1,7-bisphosphatase and stromal fructose-1,6-bisphosphatase can be regulated by light through complementary mechanisms. There is an effective but relatively slow activation of these enzymes in the light and deactivation in the dark, occurring in intact chloroplasts only (unpublished data) and being generally understood as chemical modification of enzyme proteins due to redox processes [3]. Additionally, the thus activated or inactivated forms of these enzymes can be effectively and instantaneously modulated by light through the stromal pH, and the concentrations of Mg^{2+} and ADP. These various parameters, e.g., changes of Mg^{2+} levels, do not need to be always effective, but may be a safeguard for a strict control of carbon metabolism in various metabolic situations.

TABLE I

RIBULOSE-5-PHOSPHATE KINASE FROM ILLUMINATED AND DARKENED CHLOROPLASTS, AND DEPENDENCE OF THE CATALYTIC ACTIVITY ON THE CONCENTRATION OF ATP, ADP AND AMP IN THE ASSAY MEDIUM

Additions (mM)			Ribulose-5-phosphate kinase (µmol/mg Chl per h)		(% of maximal activity in illuminated chloroplasts)
ATP	ADP	AMP	Light		Dark
1	–	–	437 (≡ 100)		56 (13)
0.1	–	–	369	(84)	43 (10)
1	0.5	–	400	(92)	29 (7)
1	5.0	–	123	(28)	2 (0.5)
1	–	0.5	407	(93)	50 (11)
1	–	20	244	(56)	30 (7)

The measurements of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase shown here were carried out with physiological concentrations of substrates. Thus, in illuminated chloroplasts the measured rates of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase were usually about 40 µmol/mg Chl per h (Figs. 2 and 6), which would be actually required for a usual rate of CO_2 fixation in these chloroplasts of 120 µmol/mg Chl per h. The activity of ribulose-5-phosphate kinase was assayed in our experiments for technical reasons with near-saturating concentrations of ribulose 5-phosphate. With physiological substrate concentrations, for this enzyme the activity in the steady state of CO_2 fixation is similar to the rate of the overall reaction. This also applies to the activity of ribulose-1,5-bisphosphate carboxylase [1]. Thus, for each of these enzymes the in vivo capacity seems not to exceed greatly the overall rate of CO_2 fixation. All these enzymes would be therefore capable of carrying out a fine regulation of the flow of carbon through the Calvin cycle.

One example for such a fine regulation may be the partial inhibition of ribulose-1,5-bisphosphate carboxylase when CO_2 fixation is limited by the supply of phosphate [30]. Fine regulation of ribulose-5-phosphate kinase may also be important for

the following reasons: Of the two phosphorylation steps in the Calvin cycle, the phosphorylation of ribulose 5-phosphate is an irreversible reaction. In the case of ATP shortage in the stroma, this reaction could therefore act as a sink for ATP consumption [47] at the expense of phosphoglycerate reduction. The low K_m of 30 μM for ATP (Fig. 11) would make the ribulose-5-phosphate kinase reaction a particularly strong sink for ATP. As a consequence of this, the chloroplasts would have to export phosphoglycerate instead of triose phosphates as products of CO_2 fixation. It can be visualized that a coordination of the rates of the two phosphorylation reactions, as achieved by the regulation of ribulose-5-phosphate kinase by stromal ATP and ADP levels, may prevent such malfunction of the Calvin cycle.

Beside such fine regulation for optimizing CO_2 fixation in illuminated chloroplasts, it seems equally important that during darkness some enzymes of this reaction are totally inactivated to allow utilization of the starch accumulated in the stroma and to prevent drainage of intermediates out of the cytosol and their conversion to hexose and pentose phosphates in the chloroplast. The phosphorolytic starch degradation, enabling an export of triose phosphate or 3-phosphoglycerate from the chloroplasts in the dark period, requires the phosphorylation of fructose 6-phosphate. With an observed rate of phosphorolytic starch degradation of 6 μmol C/mg Chl per h [48], a phosphofructokinase activity of 1 μmol/mg Chl per h would be required. Phosphofructokinase and fructose-1,6-bisphosphatase being active at the same time would constitute a futile cycle. For a net synthesis of fructose 1,6-bisphosphate from fructose 6-phosphate, this futile cycle should be minimized. The fructose-1,6-bisphosphatase activity in the dark would therefore be reduced to less than 0.5% of its light activity. Moreover, the export of triose phosphates as products of starch degradation in the dark would also require an inhibition of sedoheptulose-1,7-bisphosphatase during this period. As transketolase is functioning in the oxidative pentose phosphate pathway in the dark, the presence of sedoheptulose-1,7-bisphosphatase activity would lead to a conversion of stromal triose phosphate and fructose 6-phosphate to pentose monophosphates. Because of the irreversibility of the sedoheptulose-1,7-bisphosphatase-catalyzed reaction, such a formation of pentose phosphates should act as a sink for the products of starch degradation. An export of the mobilized starch as triose phosphates or 3-phosphoglycerate will therefore depend on an almost complete inhibition of sedoheptulose-1,7-bisphosphatase activity during the dark period. Phosphorolytic starch degradation also provides the substrate for the oxidative pentose phosphate pathway. The pentose monophosphates thus formed are normally recycled within the stroma to triose and hexose monophosphates [48]. As there is ATP present in the stroma also in the dark, as needed during starch degradation for phosphorylation of fructose 6-phosphate, a minimal activity of ribulose-5-phosphate kinase would be sufficient to convert all these pentose monophosphates into ribulose 1,5-bisphosphate. Since ribulose 1,5-bisphosphate is not found to be accumulated in the dark (unpublished observation) and ribulose-1,5-bisphosphate carboxylase does not appear to be completely inactivated in the dark [1], this would imply that there is CO_2 fixation also in the dark. This is not the case. Earlier experiments have clearly shown that chloroplasts kept in the dark in a medium of pH 7.6 containing triose phosphates are practically unable to fix CO_2 (rate 0.005 μmol/mg Chl per h [6]). Therefore, inhibition of ribulose-5-phosphate kinase in the dark is also necessary.

The provision of the cell with fixed carbon, which has been accumulated during the light period, appears to be a major function of the chloroplasts during darkness. It seems a prerequisite for this chloroplast function that certain enzymes of the Calvin cycle are inactivated in the dark, which concurs with the results of enzyme activity measurements shown in this report. The data presented here describe the phenomenon of enzyme activation and deactivation. Further systematic studies are required in order to elucidate fully the mechanism of activation and deactivation processes in the chloroplasts, and to relate these to the properties of enzymes observed in vitro [3].

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