PHOSPHATE REQUIREMENT FOR THE LIGHT ACTIVATION OF RIBULOSE- 1,5-BIPHOSPHATE CARBOXYLASE IN INTACT SPINACH CHLOROPLASTS

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1. Introduction

Although the fixation of CO_2 during photosynthesis is a dark reaction, it is generally accepted that some of the enzymes of the reductive CO_2 fixation cycle are indirectly regulated by light. Fructose and sedoheptulose bisphosphatase and ribulose bisphosphate carboxylase have been identified as major regulatory sites in the reductive CO_2 fixation cycle [1–3].

Ribulosebisphosphate carboxylase (EC 4.1.1.39) converted from an inactive into an active form by reaction with CO_2 and Mg^{2+}, this activation being enhanced by a pH-shift from pH 7.0–8.5 [4–6]. The activation is a relatively slow process with a half-time in the range of 1–3 min. The activated enzyme is stable enough to be assayed for 90 s without change of activity [4]. A number of effectors such as 3-phosphoglycerate, 6-phosphogluconate, fructose-1,6-bisphosphate and NADPH have been found to increase the activation of the enzyme especially at pH values below 8.0 [7–9].

Using a rapid procedure for the lysis of chloroplasts and assay of the carboxylase activity, the factors controlling the activation state of the enzyme in situ within the intact chloroplast have been investigated [10]. The enzyme was activated in situ by incubation of the chloroplasts with CO_2, an additional activation being observed upon illumination. The light-dependent activation was decreased upon adding acetate to the chloroplast suspension. Since acetate causes acidification of the stroma by facilitating proton transport across the envelope it was concluded that light activation was dependent upon alkalization of the stroma. Light-dependent changes of the stromal Mg^{2+} concentration were also presumed to be involved in the light activation of the carboxylase.

In this report we demonstrate that the in situ light activation of RuBP carboxylase within intact isolated spinach chloroplasts does indeed require Mg^{2+} and further that the presence of inorganic phosphate is a pre-requisite for this light activation.

Abbreviations: RuBP, ribulose-1,5-biphosphate; PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; P_i, inorganic phosphate; chl., chlorophyll

2. Methods

Spinach (*Spinacia oleracea*, Hybrid 124, Ferry Morse, Mountain View, Calif.) was grown in water culture according to [11]. For the preparation of chloroplasts see [12,13]. If not stated otherwise, the chloroplasts (0.05 mg chlorophyll/ml) were incubated at 20°C in a medium containing 0.33 M sorbitol,

2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM $NaHCO_3$, 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (Hepes), pH 7.6 and 2000 units/ml catalase from beef liver (Boehringer, Mannheim). Other additions are indicated in the figure legends. Illumination of the samples was carried out with a tungsten halogen lamp provided with a RG 630 cut off filter (Schott, Mainz). The light intensity was 100 W/m^2. Before the beginning of the experiments shown in fig.1–5 the chloroplasts were preincubated in the dark for 4 min. For the assay of RuBP carboxylase activity 50 μl chloroplast suspension was rapidly transferred to 400 μl assay medium containing 0.1 M Tris-(hydroxymethyl)aminomethane HCl (Tris) pH 8.12, 0.04% Triton X-100, 0.5 mM RuBP, 5 mM $MgCl_2$ and $H^{14}CO_3^-$ (final conc. 1.4 mM). Temperature 20°C. The reaction was terminated after 30 s by the addition of 0.5 ml 4 M formic acid and the incorporation of the radioactive label into non-volatile material was measured.

In order to obtain full activation of the enzyme in lysed chloroplasts, the chloroplasts were first incubated in a medium containing 0.1 M Tris–HCl, pH 8.12, 0.04% Triton X-100, 20 mM $MgCl_2$ and 5 mM $NaHCO_3$ for 6 min at 20°C. Then 50 μl lysed chloroplasts were transferred to the assay medium and RuBP carboxylase measured as described above.

3. Results and discussion

3.1. *The inhibition of CO_2 fixation by phosphate deficiency*

The main product of CO_2 fixation released from the chloroplast to the cytosol is DHAP and to a lesser extent PGA. For this reason the export of fixed carbon from the chloroplasts requires the uptake of P_i. CO_2 fixation by intact chloroplasts is inhibited when there is a deficiency of P_i in the medium. One might expect that lack of P_i would lead to a decreased rate of photophosphorylation, in turn to a decline in the concentration of RuBP and hence to a decrease in the rate of CO_2 fixation. In table 1 the effects of limiting and non-limiting P_i concentrations upon the rate of CO_2 fixation and upon the stromal concentrations of the major metabolites determined during CO_2 fixation are presented. Although the rate of CO_2 fixation was clearly reduced under conditions of P_i limitation, contrary to expectation, the stromal RuBP concentration was increased. This suggests that RuBP carboxylase may be inhibited or inactivated under conditions of P_i deficiency.

3.2. *Light activation of RuBP carboxylase*

Intact spinach chloroplasts were incubated in the presence of CO_2 and P_i (fig.1). At the times indicated,

Table 1
The effect of $[P_i]$ in the medium on CO_2 fixation and the metabolite levels in the stroma of spinach chloroplasts

	Phosphate in the medium at beginning of experiment (mM)	
	0.50	0.10
CO_2 fixation (μmol (mg chl)$^{-1}$h^{-1})	108	60
Metabolite concentrations in stroma (mM)		
Inorganic phosphate	7.0	2.2
3-Phosphoglycerate	6.0	8.9
Triosephosphates	0.33	0.25
Hexosemonophosphates	3.7	4.9
Fructose-1,6-bisphosphate	0.29	0.31
Sedoheptulose-1,7-bisphosphate	0.29	0.15
Ribulose-1,5-bisphosphate	0.28	0.57

Chloroplast conc.: 0.025 mg chl/ml. The rates of CO_2 fixation were measured between 5–10 min and the samples for metabolite assay were taken 7 min after the start of illumination. For details of metabolite assay see [21] expt. 101

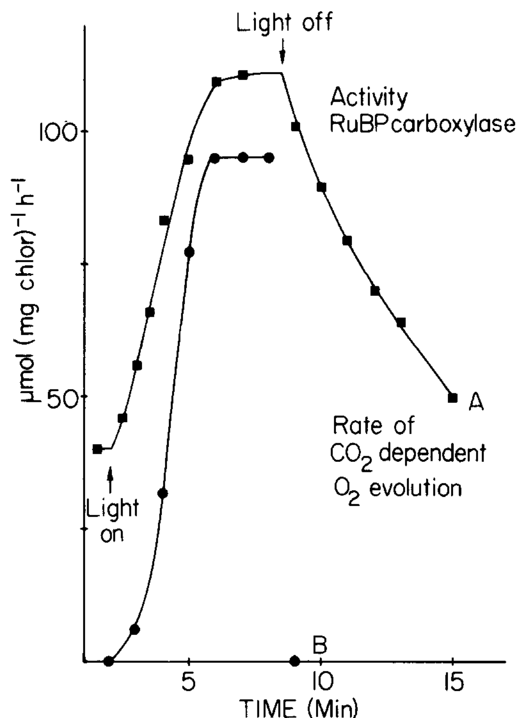

Fig.1. RuBP carboxylase in chloroplasts. Light activation. Simultaneous measurement of O_2 evolution. P_i in medium: 1 mM. expt. 302 B.

samples of the chloroplast suspension were withdrawn and injected into a hypotonic assay medium containing detergent $H^{14}CO_3^-$ and RuBP. In this way the chloroplasts were immediately ruptured and the RuBP carboxylase allowed to react for 30 s. The fixation of $^{14}CO_2$ during the enzyme test was linear for at least 60 s regardless of whether the enzyme was derived from darkened or illuminated chloroplasts. This indicates that the activation state of the enzyme did not undergo appreciable change during the assay itself and that the observed activities accurately reflected the activation state of the enzyme immediately before the rupture of the chloroplasts.

In the experiment of fig.1 illumination of the chloroplasts caused a 2.8-fold activation of the carboxylase. In similar experiments (fig.4, table 2) the activation was 2.1-fold and 4-fold. No change occurred in the activity of the caboxylase when the chloroplasts were kept in the dark (data not shown). It is feasible that the relatively high activity found in the dark may represent an artefact due to the isolation of chloroplasts, and that in vivo the enzyme may become fully inactivated in the dark. Measurements of RuBP carboxylase activity in intact plant cells may clarify this matter.

Table 2
RuBP carboxylase activity in chloroplasts depending on the preincubation conditions

	Medium		
	P_i	PGA	DHAP
	RuBP carboxylase (µmol/mg chl.h)		
Dark			
Control	28	38	25
$NaNO_2$ (6 mM)	27	36	19
A 23187 (2 µM)	24	27	22
Light			
Control	119	36	29
$NaNO_2$ (6 mM)	62	31	26
A 23187 (2 µM)	18	25	23

The medium contained either 1 mM P_i, PGA or DHAP. $MgCl_2$ and $MnCl_2$ were omitted and the EDTA conc. was only 0.1 mM. After 2 min preincubation of the chloroplasts in the dark the inhibitors were added if indicated. After 4 min subsequent illumination or dark incubation samples were taken for RuBP carboxylase assay. The activity of the fully activated enzyme was 102 µmol (mg chl)$^{-1}$h^{-1}. expt. 305

The RuBP carboxylase activity measured with illuminated chloroplasts (table 2) was marginally higher than the activity measured after pretreatment of the lysed chloroplasts with 20 mM Mg^{2+} and 5 mM HCO_3^-, a procedure known to cause maximal activation of the purified enzyme [4]. These data clearly show that in illuminated chloroplasts the RuBP carboxylase was 100% catalytically active. It may be noted that our assay of RuBP carboxylase activity was carried out with sub-saturating CO_2 concentrations (14 μM) and in the presence of air levels of O_2. To obtain maximal activities (V_{max}) the activity values shown here should be multiplied by a factor of about 5. The half-time of the activation (~ 100 s) was found to be similar to the half-time for the induction period of CO_2-dependent O_2 evolution (fig.1). Evidence has been presented that the lag phase of CO_2 fixation after the onset of illumination is mainly due to the accumulation of metabolites in the stroma brought about by the autocatalytic action of the reductive CO_2 fixation cycle [14]. The results of fig.1 indicate that the light activation of RuBP carboxylase may also be responsible, at least in part, for this lag period. When the illumination was discontinued there was an immediate decrease of the RuBP carboxylase activity (fig.1). This shows that the light activation of the enzyme is a reversible process.

3.3. *P_i requirement of the light activation*

In the experiment of fig.2A the chloroplasts were incubated with a low P_i concentration (0.05 mM). Four minutes after the start of illumination when most of the added P_i had been consumed by CO_2 fixation, a decline of RuBP carboxylase activity was found. The subsequent addition of 1 mM P_i resulted in a full activation of the enzyme. No marked light stimulation of the enzyme was observed when PGA or DHAP, which are also transported by the phosphate translocator across the envelope [15], were added to the chloroplasts instead of P_i (fig.3). The subsequent addition of P_i resulted again in a full activation of the enzyme. In agreement with these findings, the light activation of the enzyme by incubation of the chloroplasts with P_i was found to be diminished when an excess of PGA was added (fig.2B). As shown in table 2, the RuBP carboxylase activity measured in chloroplasts kept in the dark with either P_i, PGA or

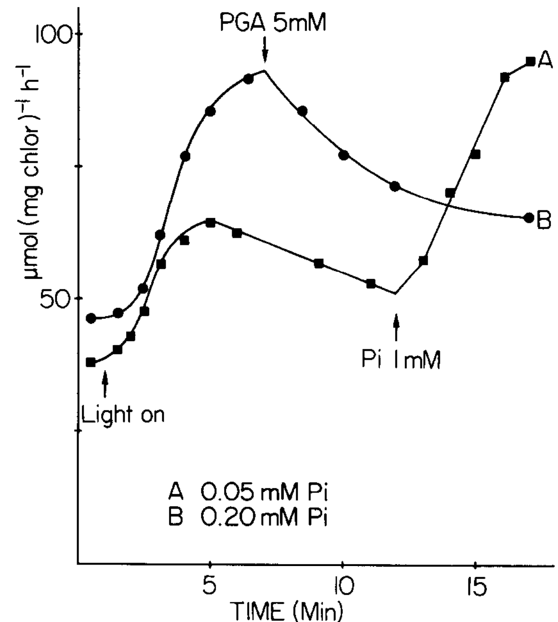

Fig.2. RuBP carboxylase in chloroplasts. P_i dependency of the light activation. Expt. A: 0.05 mg chl/ml; Expt. B: 0.1 mg chl/ml; P_i in medium and further additions are indicated in the figure. expt. 303 and 308.

DHAP was about the same and light activation was only observed in the presence of P_i. It appears from these findings that P_i is required for the light activation of the enzyme, and a depletion of the stromal P_i either by consumption or by exchange with either DHAP or PGA prevents or reverses this activation.

3.4. *CO_2 and Mg^{2+} requirement and pH dependency of the light activation*

The P_i dependent light activation of RuBP carboxylase requires CO_2, as shown in fig.4. The very low rate of enzyme activation in the absence of added CO_2 may be due to traces of CO_2 present.

In the experiment of fig.5 the enzyme activation by stromal Mg^{2+} was investigated. It has been previously shown that illumination of intact chloroplasts causes an increase of the stromal Mg^{2+} concentration by about 3 mM [16,17] due to Mg^{2+} transport across the thylakoid membrane. It was also shown that this increase can be rapidly reversed by the addition of the ionophore A 23187 rendering the envelope

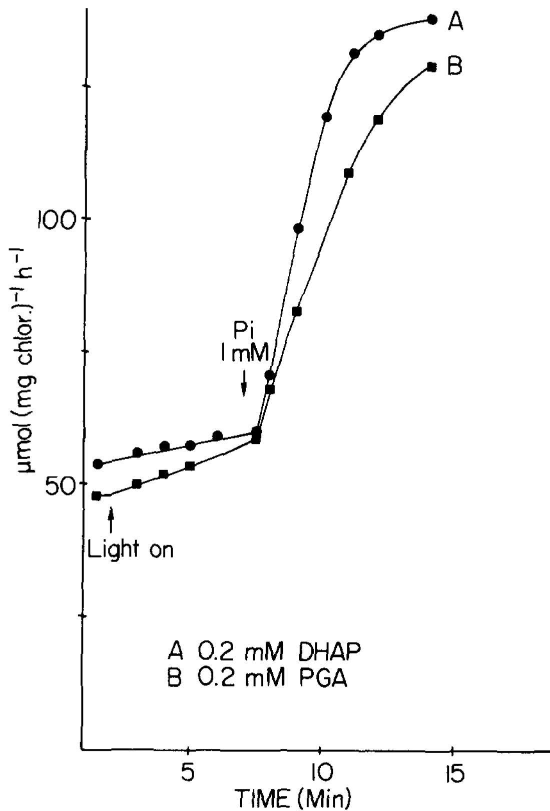

Fig.3. RuBP carboxylase in chloroplasts. P_i dependency of the light activation. The medium contained in expt. (A) 0.2 mM DHAP and in expt. (B) 0.2 mM PGA. expt. 303.

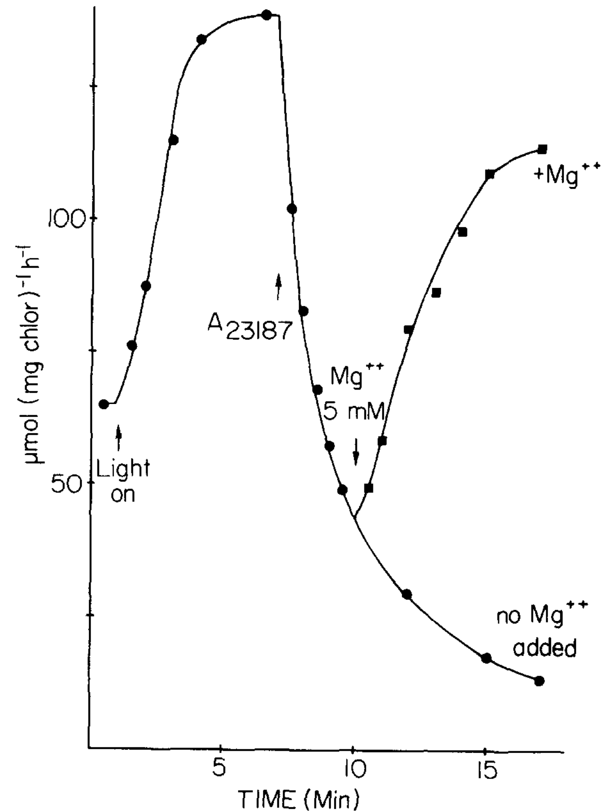

Fig.4. RuBP carboxylase in chloroplasts. CO_2 dependency of the light activation. The medium contained 1 mM P_i, pH 7.2. expt. 310.

permeable to Mg^{2+} [16]. The release of the stromal Mg^{2+} caused by A 23187 results in a rapid inhibition of CO_2 fixation, an inhibition which can be relieved by the subsequent addition of excess Mg^{2+}. As shown in fig.5 the addition of A 23187 very rapidly inactivated the light-activated RuBP carboxylase, whereas the activity of the enzyme in chloroplasts either incubated in the dark or illuminated in the presence of PGA or DHAP was only little affected (table 2). The subsequent addition of excess Mg^{2+} overcame the effect of A 23187 and lead to the reactivation of the carboxylase. These findings clearly demonstrate that the light activation requires stromal Mg^{2+}.

It has been shown earlier that illumination of intact chloroplasts causes an increase of the stromal pH due to light dependent proton transport from the stroma to the thylakoid space [18]. In chloroplasts kept in a pH 7.6 medium, illumination changed the stromal pH from pH 7.0 to pH 7.8–8.0. This light-dependent alkalization of the stroma could be prevented by the addition of nitrite, facilitating an indirect proton transfer via nitrous acid across the envelope [19]. In those experiments the addition of 6 mM nitrite to illuminated chloroplasts decreased the stromal pH from pH 7.8–7.2 and inhibited CO_2 fixation by 96%. The measurement of stromal metabolite levels showed that fructose- and sedoheptulose bisphosphatase and to a lesser extent also RuBP carboxylase were inhibited [18]. In the experiment of table 2, the addition of 6 mM nitrite partially diminished the light activation of RuBP carboxylase, whereas the activity of the enzyme assayed in chloroplasts incubated with either P_i, PGA or DHAP in the dark or with PGA and DHAP in the light appeared to

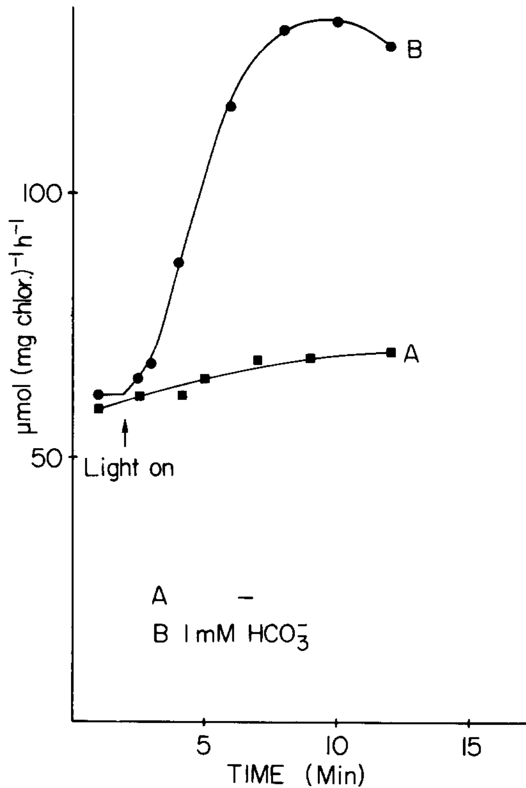

Fig.5. RuBP carboxylase in chloroplasts. Mg^{2+} dependency of the light activation. The medium contained 1 mM P_i, pH 8.0. $MgCl_2$ and $MnCl_2$ were omitted from the medium and the EDTA conc. was only 0.1 mM. At the indicated times 2 μM A 23187 and 5 mM Mg^{2+} were added. expt. 304.

be not markedly altered. We attribute the effect of nitrite upon the light activation of RuBP carboxylase to its ability to reverse in part the alkalization of the stroma which occurs during illumination.

In summary, these results show that the light activation of RuBP carboxylase in intact chloroplasts requires the presence of Mg^{2+} and CO_2 and is furthermore enhanced by an alkalization of the stroma. The influence of light on the enzyme appears to be brought about by light-dependent transport of protons and Mg^{2+} across the thylakoid membrane. Thus, basically the light activation of the enzyme seems to follow the same mechanism as found with the purified enzyme [4]. However, as an additional requirement of the light activation of the enzyme in intact chloroplasts P_i has to be present. This effect of P_i is equally reversed by the addition of DHAP or PGA, both facilitating an exchange with the stromal P_i. Since the addition of DHAP or PGA will have very different effects on stromal metabolites which have been suggested to influence RuBP carboxylase activity [8,9] such as NADPH or fructose bisphosphate, it seems unlikely that the effect of P_i on the light activation is ultimately due to concentration changes of such metabolites.

Further experiments on the activation of the purified enzyme and a systematic comparison of the activation state of the enzyme with the metabolite concentrations in the stroma are required to elucidate the activation mechanism.

3.5. *Phosphate regulating chloroplast metabolism*

In the course of photosynthesis, the soluble products of CO_2 fixation DHAP and PGA are exported from the chloroplast stroma to the cytosol by the phosphate translocator in a strict counter-exchange with P_i [15]. In this way the total pool of P_i and phosphorylated compounds in the stroma is kept constant. The DHAP exported to the cytosol may be converted there to sucrose, and the P_i thus released can re-enter the chloroplast in exchange for more DHAP. If the utilization of triose-phosphate is lower than its production in the chloroplasts, the cellular P_i levels will be decreased. It has been shown earlier that a decrease of the stromal P_i concentration induces an enhanced starch synthesis in the chloroplasts [20]. In this way part of the fixed carbon will be stored in the chloroplasts without the consumption of P_i. The rate of starch synthesis in spinach chloroplasts, however, is normally less than 30% total CO_2-fixing capacity [20]. For this reason, in the case of a limited demand for photosynthesis products by the cell, a lowering of the total rate of CO_2 fixation brought about by a change of the activation state of RuBP carboxylase may be important to avoid the total sequestration of the P_i by CO_2 fixation. It might be the purpose of this regulatory mechanism to maintain minimal cellular P_i levels required for metabolic functions such as photosynthesis and formation of sucrose.

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