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LIGHT-DEPENDENT CHANGES OF THE Mg^{2+} CONCENTRATION IN THE STROMA IN RELATION TO THE Mg^{2+} DEPENDENCY OF CO_2 FIXATION IN INTACT CHLOROPLASTS

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SUMMARY

(1) Light-dependent changes of the Mg^{2+} content of thylakoid membranes were measured at pH 8.0 and compared with earlier measurements at pH 6.6. In a NaCl and KCl medium, the light-dependent decrease in the Mg^{2+} content of the thylakoid membranes at pH 8.0 is found to be 23 nmol Mg^{2+} per mg chlorophyll, whereas in a sorbitol medium it is 83 nmol Mg^{2+} per mg chlorophyll.

(2) A light dependent increase in the Mg^{2+} content of the stroma was detected when chloroplasts were subjected to osmotic shock, amounting to 26 nmol/mg chlorophyll. Furthermore, a rapid and reversible light-dependent efflux of Mg^{2+} has been observed in intact chloroplasts when the divalent cation ionophore A 23 187 was added, indicating a light-dependent transfer of about 60 nmol of Mg^{2+} per mg chlorophyll from the thylakoid membranes to the stroma.

(3) CO_2 fixation, but not phosphoglycerate reduction, could be completely inhibited when A 23 187 was added to intact chloroplasts in the absence of external Mg^{2+}. If Mg^{2+} was then added to the medium, CO_2 fixation was restored. Half of the maximal restoration was achieved with about 0.2 mM Mg^{2+}, which is calculated to reflect a Mg^{2+} concentration in the stroma of 1.2 mM. The further addition of Ca^{2+} strongly inhibits CO_2 fixation.

(4) The results suggest that illumination of intact chloroplasts causes an increase in the Mg^{2+} concentration of 1–3 mM in the stroma. Compared to the total Mg^{2+} content of chloroplasts, this increase is very low, but it appears to be high enough to have a possible function in the light regulation of CO_2 fixation.

INTRODUCTION

There is ample evidence that CO_2 fixation in chloroplasts is controlled by light (cf. see refs. 1, 2). Such a light control may be important in order to avoid CO_2 fixation in the dark at the expense of ATP and also at the expense of reducing power generated in the cytosol by oxidative metabolism [3]. The influence of illumination on the enzymatic activity of the CO_2 fixation cycle seems to be due to different mechanisms working parallel to one another. It has been shown recently by our laboratory that illumination causes a considerable alkalization in the stroma and that CO_2 fixation has a strong pH dependency. The pH changes occurring in the stroma after illumination are almost sufficient to switch CO_2 fixation from zero to maximal activity [3]. Furthermore, evidence has been presented that light regulation of CO_2 fixation may involve the activation of enzymes by SH groups, which are reduced by photosynthetic electron transport (cf. refs. 1, 2, 4).

Finally it has been speculated that CO_2 fixation may be also influenced by light-dependent changes of the Mg^{2+} concentration [5]. Studies of isolated enzymes have revealed a stimulation by Mg^{2+} of the activity of hexosediphosphatase (EC 3.1.3.11), ribulosebisphosphate carboxylase (EC 4.1.1.39) and sedoheptulose bisphosphatase (EC 3.1.3.11) (cf. refs. 1, 2), and it was shown that light-dependent CO_2 fixation in a reconstituted system is stimulated by increasing the Mg^{2+} concentration [6].

Light-dependent movements of Mg^{2+} have also been observed. The Mg^{2+} content of intact pea chloroplasts isolated from illuminated leaves was reported to be higher than was the case when the leaves were kept in the dark [7]. Such changes in the total Mg^{2+} content of the chloroplasts would involve a transfer of Mg^{2+} across the envelope. However, it has been convincingly demonstrated that the envelope of spinach chloroplasts is relatively impermeable to Mg^{2+} [8, 9]. Thus, at least in spinach chloroplasts, light-dependent Mg^{2+} movements across the envelope would not appear to be sufficiently rapid to play a major role in the light-control of CO_2 fixation. On the other hand, a light-dependent release of Mg^{2+} from isolated thylakoid membranes has been observed with spinach [10, 11]. Coupled with the impermeability of the envelope to Mg^{2+}, such a release would result in an increase in the Mg^{2+} concentration in the stroma space. Other indications of a light-dependent release of Mg^{2+} from the thylakoid membranes have been obtained from comparative measurements of chlorophyll fluorescence in broken and intact chloroplasts [12, 13]. These observations have led to the suggestion that Mg^{2+} is one of the major counter-ions of the light-dependent proton transport across thylakoid membranes and that the resulting light-induced Mg^{2+} concentration increase in the stroma may be as high as 10–15 mM [7, 11, 14].

In order to elucidate the role of Mg^{2+} in the regulation of CO_2 fixation, we attempted to measure light-dependent changes of the Mg^{2+} content of the stroma in intact chloroplasts and investigated the dependency of CO_2 fixation on the Mg^{2+} concentration in the stroma.

METHODS

(a) Spinach (*Spinacia oleracea* L. cv. True Hybrid 102, Arthur Yates and Co., N.S.W., Australia, was grown in water culture according to Lilley and Walker [15].

(b) Chloroplasts with intact envelopes were prepared from fully grown leaves according to the method of Cockburn et al. [16] modified by Heldt and Sauer [17]. In those experiments in which Mg^{2+} was to be assayed, EDTA, $MnCl_2$ and $MgCl_2$ were omitted from medium B.

(c) Thylakoid membranes were prepared by a 10-fold dilution of an intact chloroplast suspension with distilled water. The medium was then made up to 110 mM sorbitol, 20 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid (HEPES) pH 7.6 and 2 mM $MgCl_2$. The thylakoid membranes were collected by centrifugation and washed once more in this medium.

(d) The incubation of intact chloroplasts was normally carried out in a medium containing 0.33 M sorbitol and 50 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid (HEPES) pH 7.6. Other additions are indicated in the legends. If not stated otherwise, the temperature was 20 °C.

(e) The measurements of oxygen evolution were carried out with an oxygen electrode as described by Delieu and Walker [18]. Illumination of the samples contained in the centrifuge tubes or in the vessel with the oxygen electrode was carried out with a tungsten halogen light source provided with an RG 630 cutoff filter (Schott, Mainz, Germany) and a Calflex heat filter (Balzers, Lichtenstein). The light intensity was 80 000 $ergs \cdot s^{-1} \cdot cm^{-2}$. Illumination was continued during centrifugation.

(f) Chlorophyll was assayed according to the method of Whatley and Arnon [19]. The method of Heldt et al. [20] was used to determine the pH in the stroma (for details of silicon layer filtering centrifugation see ref. 21).

(g) The Mg^{2+} content of thylakoids was determined by centrifugation of the membranes through a layer of silicone oil (AR 50/AR 100, 2 : 1, Wacker Chemie, München, Germany) into a lower layer of 12 % sorbitol. The lower layer was then added to 500 μl of 1 mM EDTA and the thylakoid membranes resuspended and again centrifuged. The Mg^{2+} content of the supernatant was assayed by atomic absorption measurement (Perkin Elmar 290). This procedure avoided the measurement of the high chlorophyll-Mg^{2+} content of the thylakoid membranes, but may not measure tightly bound thylakoid-protein Mg^{2+} [22].

(h) The determination of the Mg^{2+} in the stroma after the osmotic shock of intact chloroplasts, as shown in Table II, was carried out as follows:

In order to label the pool of phosphate and phosphorylated intermediates the chloroplasts (0.15 mg chlorophyll/ml) were first preincubated at 4 °C for 5 min with 0.125 mM radioactive inorganic phosphate (spec. act., 16 Ci/mol), and washed afterwards. For determination of the Mg^{2+} in the stroma, 100 μl of chloroplasts (0.2 mg chlorophyll/ml, containing 2 mM oxaloacetic acid) were layered over 100 μl of silicone oil (AR 100/AR 50, 4 : 1) supported by a lower layer of 20 % (w/w) Ficoll 400 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and 2 mM *N*-Tris(hydroxymethyl)-methylglycine (TRICINE) NaOH, pH 8.0, in a 400 μl centrifugation tube (Sarstedt, Nümbrecht-Rommelsdorf, Germany, Nr. 46/6). After 3 min of illumination or darkness at 4 °C the samples were centrifuged for 15 s at a speed sufficient to pull the chloroplasts into the Ficoll layer, followed by centrifugation at maximal speed (10 000 × *g*) for 45 s and were then frozen. The chloroplast material at both the upper and lower portion of the Ficoll layer was cut off. From the remaining solution 50 μl were taken for Mg^{2+} assay, and 20 μl for the counting of ^{32}P radioactivity. The Mg^{2+} measured was normalized to 100 % breakage of the chloroplasts by the corresponding ^{32}P radioactivity released. Assay of the ^{32}P distribution in separate samples indicated that 60 % of the stroma material could be recovered in the Ficoll layer after removal of the chloroplast material.

(i) The ionophore A 23 187 (Eli Lilly and Company, Indianapolis, U.S.A., Lot 361-D66-282) was dissolved in ethanol. Usually, 1–2 μl of the stock solution were added to 1 ml of chloroplast suspension.

RESULTS AND DISCUSSION

Light dependent changes in the Mg^{2+} content of thylakoid membranes

Previous studies of Mg^{2+} efflux from thylakoid membranes have been done with a pH of 6.6–7.0 in the medium, where the light-induced proton uptake is maximal [10, 11]. The conditions of these measurements appear to be unphysiological when considering that the optimum of CO_2 fixation is about pH 8.0 [3]. Therefore, in order to evaluate the extent of the Mg^{2+} movement across the thylakoid membrane during CO_2 fixation, we compared the efflux of Mg^{2+} from thylakoid membranes at pH 8.0 with the earlier experiments at lower pH (Table I). The illumination of thylakoid membranes at pH 6.6 caused a considerable decrease in the Mg^{2+} content of the membranes. The amount of Mg^{2+} being released concurs with the earlier data obtained by Hind et al. [11] at this pH, using ion-specific electrodes. The presence of KCl and NaCl in the medium lowered the Mg^{2+} content of the membranes in the dark and there was less Mg^{2+} released on illumination. A similar decrease of light-induced Mg^{2+} release by KCl has been also found by Hind et al. [11].

TABLE I

Mg^{2+} CONTENT OF THYLAKOID MEMBRANES

The incubation mixtures contained either 100 mM sorbitol or 75 mM of both KCl and NaCl, either 50 mM *N*-tris(hydroxymethyl)methylglycine (TRICINE) NaOH (pH 8.0) or 50 mM piperazine-*N*,*N'*-bis-2ethane sulphonic acid (Pipes) NaOH (pH 6.6), 50 μM phenazine methosulfate, 0.5 mM K_2HPO_4, thylakoids equivalent to 200 μg chlorophyll/ml. The Mg^{2+} concentration in the incubation mixtures due to the addition of the thylakoids was determined to be 0.25 mM. Aliquots (100 μl) were centrifuged after 3 min in the light or 4 min in darkness. The Mg^{2+} contents have been corrected for the small amount of incubation medium carried along with the membranes. This correction amounted to between 10–25 nmol/mg chlorophyll depending on the incubation conditions as estimated from separate samples that contained ^{32}P-inorganic phosphate (2.5 μCi/ml). The mean and standard deviation is based on 5–6 samples.

Conditions			Mg^{2+} content (nmol/mg chlorophyll)		
pH 6.6, Sorbitol,		Light	70 ± 6		
		Dark	164 ± 7	Δ(Light − dark)	−94
	NaCl + KCl,	Light	62 ± 6		
		Dark	124 ± 2	Δ(Light − dark)	−62
pH 8.0, Sorbitol,		Light	182 ± 6		
		Dark	265 ± 14	Δ(Light − dark)	−83
	NaCl + KCl,	Light	63 ± 7		
		Dark	86 ± 6	Δ(Light − dark)	−23

At the more physiological pH of 8.0, the Mg^{2+} content of the thylakoid membranes and the extent of light-induced Mg^{2+} efflux were strongly depressed when KCl and NaCl were present in the medium. The amount of Mg^{2+} released under these conditions was less than one third of the amount released in a sorbitol medium.

Disregarding the open question whether KCl and NaCl or sorbitol resemble more the milieu of the stroma, these data indicate that the extent of Mg^{2+} efflux from the thylakoid membranes of intact chloroplasts at pH 8.0 may be between 20–80 nmol/mg chlorophyll.

Light dependent changes of the Mg^{2+} content of the stroma in intact chloroplasts as measured by osmotic shock

It may be a matter of debate to what extent the data obtained with isolated thylakoid membranes can be applied to the metabolic situation of the intact chloroplast. In order to ascertain the importance of the Mg^{2+} concentration in the stroma for the regulation of CO_2 fixation, we attempted to measure these changes in the intact chloroplast. In one approach to this problem, we devised a technique by which the incubation of illuminated or darkened chloroplasts is terminated by rapid centrifugation of the chloroplasts through a layer of silicone oil into a hypotonic medium. Immediately on arrival in the hypotonic medium, the majority of the chloroplasts are disrupted by osmotic shock, and the thylakoid membranes move rapidly to the interface between the hypotonic phase and the silicone oil. In this way, the Mg^{2+} of the stroma should be released to the hypotonic medium between the pellet of still unbroken chloroplasts and the upper layer of thylakoid membranes. Therefore a sample of this fraction was withdrawn for Mg^{2+} assay.

Unfortunately these measurements are complicated by the fact that breakage of the chloroplasts is not complete. In order to determine the degree of chloroplast disruption, the chloroplasts were preincubated before the experiment with radioactive inorganic phosphate and washed carefully afterwards. In this way the pool of inorganic phosphate and phosphorylated intermediates was radioactively labelled. Since there was no phosphate present in the medium during the experiment and phosphate transport is known to occur by a strict counter-exchange [23], the ^{32}P radioactivity is confined to the chloroplasts. As the thylakoid membrane has been found to be virtually impermeable to inorganic phosphate and phosphorylated compounds (Chon, C., Portis, A. R. and Heldt, H. W., unpublished), all of the radioactively labelled phosphate can be attributed to the stroma. Thus the degree of chloroplast disruption can be determined from the relative amount of ^{32}P radioactivity released into the hypotonic medium. Under the reasonable assumption that the Mg^{2+} in the stroma is as equally well released on osmotic shock as inorganic phosphate and phosphorylated compounds, the total amount of extractable "stroma" Mg^{2+} can be calculated from the Mg^{2+} assay of the hypotonic fraction. Table II presents the results of such measurements. A large quantity of Mg^{2+} is released from chloroplasts kept in the dark and illumination increases this value by only about 7 %. Because of this slight difference, a large number of parallel experiments were carried out in order to verify the significance of the light-dark difference by statistical means (Students *t* test). Taken at face value, these data indicate that the increase of the stroma Mg^{2+} in the light is about 30 nmol which seems to concur with the results obtained in Table I with thylakoid membranes.

It is a prerequisite for this method that during the osmotic shock and the following separation of the thylakoid membranes, the pH gradient of the illuminated thylakoid membranes does not collapse. In order to check this, [^{14}C]methylamine was added to the chloroplasts. Illumination caused a large uptake of the radioac-

TABLE II

RELEASE OF Mg^{2+} FROM INTACT CHLOROPLASTS ON OSMOTIC SHOCK

For details, see Methods. The total amount of the Mg^{2+} releasable by osmotic shock in the presence of 1 mM EDTA was 500 nmol/mg chlorophyll. The difference between the light and dark values is significant by the Student t test (95 % reliability). Results are given ± standard deviation.

Conditions	Mg^{2+} released (nmol/mg chlorophyll)	
Light	405 ± 37	$n = 14$
Dark	379 ± 30	$n = 14$
Δ(Light−dark)	26	

tive material into the chloroplasts due to its accumulation in the acidic thylakoid space. From this, a pH of 5.6 was calculated for the thylakoid space. When the chloroplasts were shocked according to the method described above, this ^{14}C radioactivity was still found in the thylakoid fraction, indicating that there was no major flux of protons from the thylakoid space into the medium during the shock procedure. However, it cannot be definitely excluded that there may be some portion of the Mg^{2+} which is normally bound to the thylakoids in the dark, released during the separation and which thereby reduces the observed light-dependent Mg^{2+} increase. Because of these possible objections, we attempted to verify the results of Table I by an alternative experimental approach.

Light dependent changes of the Mg^{2+} content of the stroma as measured by ionophore facilitated Mg^{2+} release

The ionophore A 23 187 has been shown to catalyze a H^+/divalent cation exchange across membranes [24]. It would be expected that this ionophore also renders the chloroplast envelope permeable to divalent cations. We tried therefore to detect light-induced changes in the Mg^{2+} concentration in the stroma through changes in the Mg^{2+} release to the medium by applying the ionophore, as shown in Fig. 1. In this experiment, the chloroplasts were preincubated in the light and in the dark and the time course of Mg^{2+} release was measured upon the addition of A 23 187. 30 s after the addition of the ionophore, 70 nmol Mg^{2+} were released from the chloroplasts kept in the dark, while about twice as much Mg^{2+} was released from the illuminated chloroplasts. This light-dependent Mg^{2+} release was somewhat increased by longer incubation times, but it was again decreased when the illumination was discontinued. The experiment of Fig. 1 was carried out in a Mg^{2+}-free medium. In the presence of EDTA (1 mM), almost all the Mg^{2+} associated with the chloroplasts could be removed within 5 min in the light and also in the dark (experiment not shown here). In the experiment of Fig. 1, the EDTA-releaseable Mg^{2+} amounted to 430 nmol/mg chlorophyll. In agreement with the results in Table II, these data show that illumination causes a rise of the Mg^{2+} concentration in the stroma and that there is reversible binding of this Mg^{2+} in the dark. According to the data of Table I, this light-induced Mg^{2+} release appears to be due to ion transport by the thylakoid membranes.

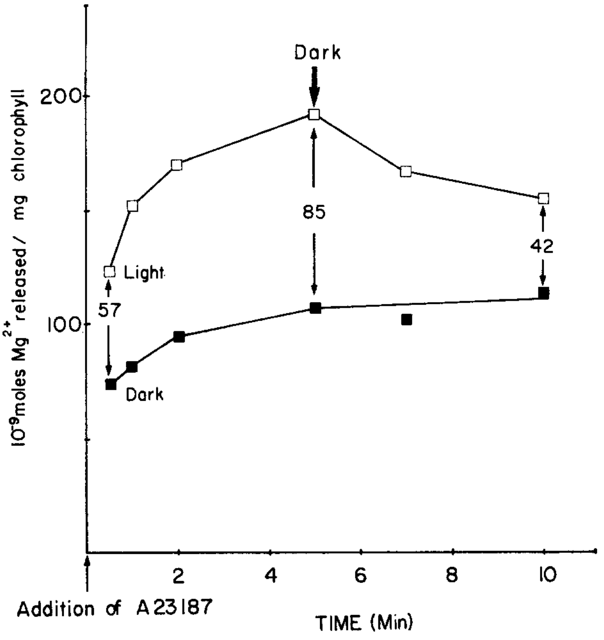

Fig. 1. Effect of illumination on the release of Mg^{2+} from intact chloroplasts as facilitated by the ionophore A 23 187. The incubation mixture contained in addition 10 mM $NaHCO_3$, 0.5 mM K_2HPO_4 and chloroplasts equivalent to 200 μg chlorophyll/ml. A 23 187 (4 μM) was added after 5 min in the light or in the dark and aliquots (100 μl) were centrifuged at the indicated times after the addition of the ionophore. The illuminated samples were returned to darkness 5 min after addition of the ionophore. An EDTA releasable amount of 430 nmol/mg chlorophyll was obtained by the addition of 1 mM EDTA. An initial value of 200 nmol Mg^{2+}/mg chlorophyll equivalent to an external Mg^{2+} concentration of 40 μM has been subtracted from all values, as this represents Mg^{2+} introduced into the medium by the addition of the chloroplasts. In the absence of the ionophore, a Mg^{2+} release of 25 nmol/mg chlorophyll after 5 min light followed by 5 min darkness was observed.

The effect of A 23 187 on CO_2 fixation

Since such large amounts of Mg^{2+} can be released when A 23 187 is added to intact chloroplasts, it would be expected that CO_2 fixation could be inhibited under these conditions, as the result of the loss of Mg^{2+} from the stroma. This possibility was tested in the experiment of Fig. 2A. The addition of 2 μM A 23 187 to chloroplasts (0.1 mg chlorophyll/ml) undergoing active CO_2 fixation in the presence of 0.1 mM EDTA and in a Mg^{2+}-free medium, caused a complete inhibition of O_2 evolution within 30 s. Parallel experiments, not shown here, confirmed that CO_2 fixation was also completely inhibited. If EDTA was omitted, CO_2 fixation could still be inhibited if the final Mg^{2+} concentration in the medium was kept very low. Lower concentrations of the ionophore were also sufficient, but required longer times for full inhibition to be attained. The inhibition of CO_2-dependent O_2 evolution by A 23 187 is reversed by the addition of 5 mM Mg^{2+}, indicating that the inhibition was indeed due to the loss of Mg^{2+}. If 1 mM Ca^{2+} was then added, O_2 evolution was again inhibited rapidly. The strong inhibition of CO_2 fixation by Ca^{2+} was only observed in the presence of the ionophore. This concurs with the observation by Gimmler (personal

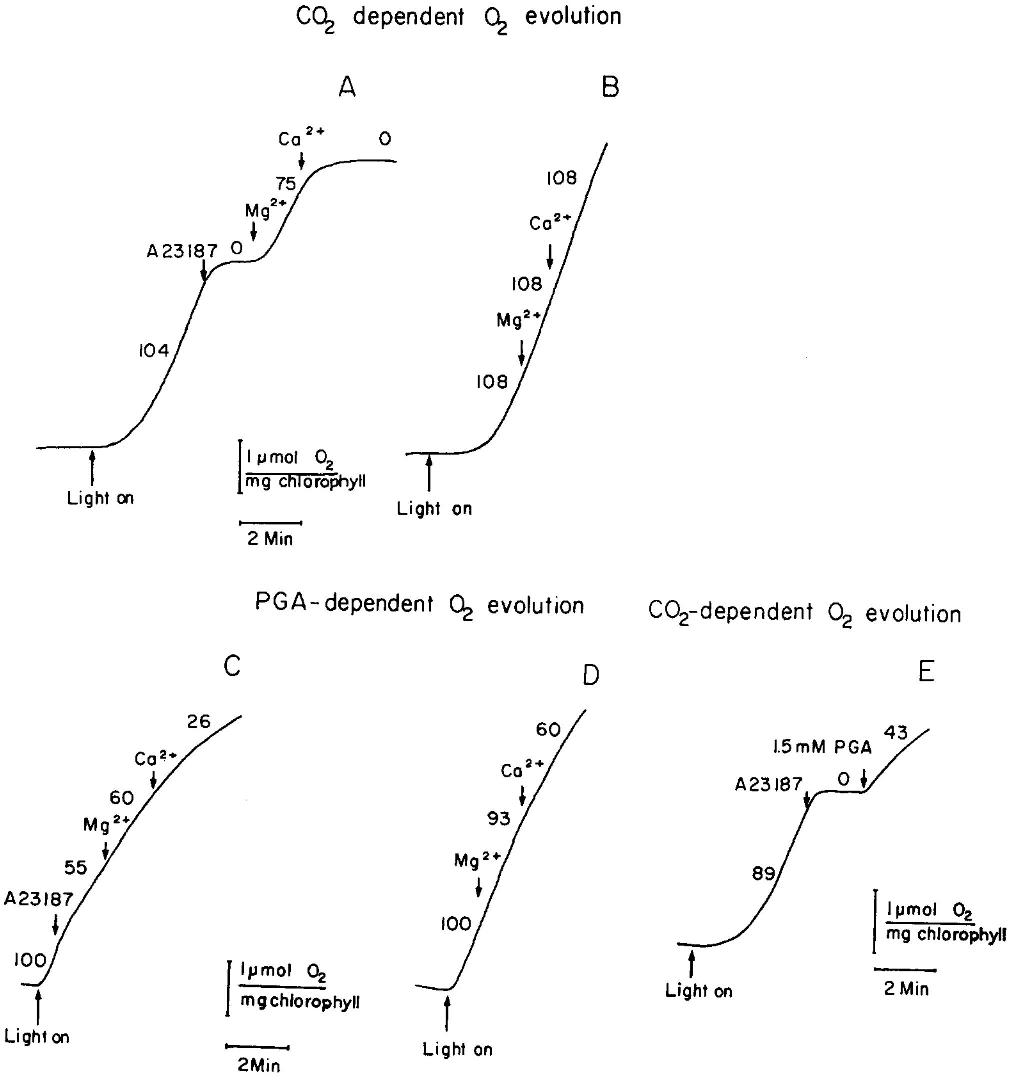

Fig. 2. Recorder traces of CO_2- (Fig. 2A, B, E) and 3-phosphoglycerate- (Fig. 2C, D) dependent O_2 evolution. The chloroplast suspension (0.1 mg chlorophyll/ml) contained 0.1 mM EDTA. In the experiment shown in A, B and E, the medium also contained 10 mM $NaHCO_3$ and 0.5 mM K_2HPO_4; in C and D, 3 mM 3-phosphoglycerate and 2 mM K_2HPO_4. Where indicated, 2 μM A 23 187, 5 mM Mg^{2+} and 1 mM Ca^{2+} were added. The numbers are the rates of oxygen evolution in μmol O_2/mg chlorophyll per h. The experiment shown in E was carried out with a different chloroplast preparation.

communication) that A 23 187 facilitates a fast accumulation of Ca^{2+} within the chloroplasts, whereas the uptake of Ca^{2+} in the absence of the ionophore is low. As shown in Fig. 2B, in the absence of the ionophore, neither Mg^{2+} nor Ca^{2+} had any marked effect on CO_2 fixation. In other experiments, an inhibitory effect of Ca^{2+}

and Mg^{2+} was sometimes observed, which was dependent on the conditions of preincubation. An inhibition of CO_2 fixation and of 3-phosphoglycerate reduction by Mg^{2+} has been reported earlier [25–27].

In contrast to CO_2 fixation, only a partial inhibition of 3-phosphoglycerate reduction was observed when the same concentration of the ionophore was added (Fig. 2C). The subsequent addition of Mg^{2+} (5 mM) caused little or no stimulation while the subsequent addition of Ca^{2+} led to further inhibition. It has been reported by Telfer et al. [29] that A 23 187 strongly inhibited 3-phosphoglycerate reduction. With 2 μM A 23 187 and the same chloroplast concentration as in our experiment, they found an inhibition of about 60 %, which was only 20 % when 3 mM $MgCl_2$ was also present. With higher concentrations of the ionophore, these authors observed total inhibition of 3-phosphoglycerate reduction both in the absence and presence of Mg^{2+}. The differential inhibition was attributed to a loss of Mg^{2+} from the stroma in the absence of external Mg^{2+} and uncoupling by the ionophore in its presence. In contrast, we have been able to obtain a total inhibition of 3-phosphoglycerate reduction only in the presence of both EDTA (1 mM) and relatively high concentrations (approx. 10 μM) of the ionophore (data not shown). These data indicate that uncoupling of photophosphorylation by the ionophore may be a minor cause for the inhibition of 3-phosphoglycerate reduction. On the other hand, the effect of the ionophore on 3-phosphoglycerate reduction cannot be fully explained by an inhibition of 3-phosphoglycerate kinase (EC 2.7.2.3), due to a lack of Mg^{2+} in the stroma, since very little restoration of O_2 evolution was obtained by re-adding Mg^{2+}. This inhibition even in the presence of Mg^{2+} might be due to an inhibition of 3-phosphoglycerate transport [28, 30].

It can be concluded from our data that the inhibition of CO_2 fixation by A 23 187 is mainly due to the loss of Mg^{2+} from the stroma, which is reversed by the addition of Mg^{2+} to the medium. Similar conclusions have been also drawn by Gimmler (personal communication), who observed in parallel experiments that A 23 187 (approx. 1 μM) inhibited CO_2 fixation without acting as an uncoupler, and also that the inhibition was reversed by Mg^{2+}. The lower sensitivity of 3-phosphoglycerate reduction toward the ionophore indicates that 3-phosphoglycerate kinase, involved in this reaction, is less sensitive to changes of the Mg^{2+} concentration in the stroma than other enzymes of the Calvin cycle. This is confirmed by the results shown in Fig. 2E, where it is shown that CO_2-dependent O_2 evolution, which has been inhibited by the ionophore, can also be partially restored by the addition of 3-phosphoglycerate. This is in agreement with earlier observations that isolated 3-phosphoglycerate kinase had a considerable activity even without added free Mg^{2+} (E. Latzko, personal communication).

Taking these considerations into account, the Mg^{2+}-dependent step should be in the reaction chain of CO_2 fixation between dihydroxyacetonephosphate and 3-phosphoglycerate. In order to identify these steps, we are at present studying the levels of the intermediates of the Calvin cycle in the intact chloroplasts under the conditions of the experiment in Fig. 2, A and B. The data obtained so far indicate that the inhibition of CO_2 fixation by the ionophore A 23 187 is mainly due to the inhibition of the activities of ribulosebisphosphate carboxylase and hexosediphosphatase (Portis, A. R. and Heldt, H. W., unpublished). This concurs with earlier investigations on the Mg^{2+} requirement of the isolated enzymes [31, 32]. The inhibition of CO_2 fixation by Ca^{2+}

in the presence of A 23 187 can be explained by inhibition of hexosediphosphatase, as Ca^{2+} is a very potent inhibitor of this enzyme [33]. At pH 7.9, the K_i of Ca^{2+} was reported to be as low as 30 μM.

Mg^{2+} dependency of CO_2 fixation

The ionophore A 23 187 seems to be a very useful tool for determining the Mg^{2+} concentration dependence of CO_2 fixation in intact chloroplasts. In the experiment shown in Fig. 3, CO_2 fixation was first inhibited by releasing the stroma Mg^{2+} through the action of A 23 187. It was then investigated which Mg^{2+} concentration in the stroma was required to restore CO_2 fixation. Although full restoration could not be achieved, oxygen evolution was half maximal in the presence of 0.2–0.3 mM Mg^{2+} (0.1 mM EDTA was also present) and only 1–2 mM Mg^{2+} were sufficient for maximal restoration at pH 8.0. The lack of complete restoration of CO_2 fixation can be explained by the fact that there is an acidification in the stroma by about 0.3 pH units after the addition of the ionophore, which is probably caused by a Mg^{2+}/H^+ counterexchange catalysed by the ionophore. Since CO_2 fixation has a strong pH dependency [3] this acidification in the stroma would be expected to give rise to an inhibition of CO_2 fixation.

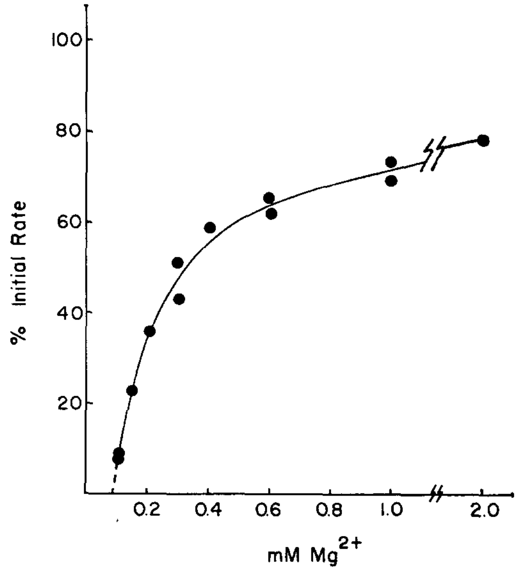

Fig. 3. Dependency of CO_2 fixation on the Mg^{2+} concentration, as measured from CO_2-dependent O_2 evolution after the addition of the ionophore A 23 187. O_2 evolution of illuminated chloroplasts (0.1 mg chlorophyll/ml) containing 0.1 mM EDTA, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$ and 50 mM *N*-tris (hydroxymethyl)methylglycine (TRICINE)-NaOH, pH 8.0, was measured by an oxygen electrode. 2 min after the start of the illumination, when O_2 evolution reached a constant rate (100 % = 83 μmol O_2/mg chlorophyll per h) 2 μM A 23 187 were added. 90 s later, when total inhibition of oxygen evolution had occurred, the indicated concentration of $MgCl_2$ was added. Illumination was continued until a constant rate of O_2 evolution was attained. In a separate experiment, under the same conditions, the pH measured in the stroma was 7.9 before and 7.6 after the addition of A 23 187 and $MgCl_2$.

It should be noted that in the presence of the ionophore, the Mg^{2+} concentration in the stroma may be higher than in the medium. If one assumes the most simple case that the distribution of Mg^{2+} between the medium and the stroma follows the distribution of protons, and that the activity coefficients for Mg^{2+} are the same in both spaces, the Mg^{2+} concentration in the stroma could be calculated in the following way:

$$(\mathrm{Mg^{2+}\ conc.\ in\ stroma}) = (\mathrm{Mg^{2+}\ conc.\ in\ medium}) \cdot 10^{(2 \times \Delta\mathrm{pH})}$$

With a ΔpH of 0.4 between the stroma and the medium (see legend Fig. 3) the Mg^{2+} concentration in the stroma would be about six times higher than in the medium. The data of Fig. 3 therefore indicate that the concentration of free Mg^{2+} in the stroma which is required for half the maximal stimulation of CO_2 fixation is in the range of 0.6–1.2 mM, whereas full restoration may require about 5 mM Mg^{2+}. These data concur with the earlier results of Lilley et al. [6], who showed that in a reconstituted chloroplast system, 2.5 mM Mg^{2+} in the presence of 1 mM EDTA were required to achieve 70–90 % of the full activity of CO_2 fixation.

CONCLUSION

The data presented here clearly demonstrate that CO_2 fixation by intact chloroplasts is sensitive to relatively small changes in the Mg^{2+} concentration in the stroma. Similar changes in the Mg^{2+} concentration in the stroma are found when the chloroplasts are illuminated. Using two different methods, we found a rapid increase of the Mg^{2+} in the stroma of 26–60 nmol/mg chlorophyll. With an average stroma volume of 25 μl/mg chlorophyll, this is equivalent to 1–3 mM. Similar results were also obtained by G. H. Krause (personal communication). From measuring the absorption of Eriochrome-Blue, a weak complexing agent for magnesium ion, he calculated a light-dependent increase of the Mg^{2+} in the stroma of spinach chloroplasts of approximately 2 mM at pH 7.6. These different independent measurements may be regarded as convincing evidence that illumination causes an increase of the Mg^{2+} concentration in the stroma, due to light-dependent proton transport across the thylakoid membrane. Since the total amount of Mg^{2+} in intact chloroplasts was found to be about 400–600 nmol/mg chlorophyll (Table II, see also ref. 9), this implies that only a small portion of the total Mg^{2+} is transported across the thylakoid membrane during illumination.

The results discussed here support the possibility that CO_2 fixation might be regulated by light-dependent changes of the Mg^{2+} concentration in the stroma, but the question still remains whether this feasible regulatory mechanism is of physiological significance. A regulation of CO_2 fixation by Mg^{2+} would imply that, during darkness, the concentration of free Mg^{2+} in the stroma is below 1 mM. This would mean that the relatively large amount of Mg^{2+}, which is found in the stroma also during darkness, has to be in a bound state. Whether this is really the case cannot be decided definitely from the available experimental data. However, the fact that such a binding of larger amounts of divalent cations might be principally possible is illustrated from the effect of Ca^{2+} on chloroplasts. As shown in Fig. 2, low concentrations of Ca^{2+} strongly *inhibited* CO_2 fixation of intact chloroplasts, if these are rendered permeable for Ca^{2+} by the ionophore A 23 187. It is interesting that intact chloroplasts normally contain large amounts of Ca^{2+}. A Ca^{2+} content of 600 nmol/mg chlorophyll of intact chloroplasts has been found by Gimmler (personal communication), and we found 100–200 nmol Ca^{2+} per mg chlorophyll in our chloroplast preparations. All this large amount of Ca^{2+} has to be very effectively bound, otherwise CO_2 fixation would be inhibited all the time, due to the low K_i reported for Ca^{2+} inhibition of hexosediphosphatase [33]. It is quite likely that the Mg^{2+} which is present in the stroma during darkness is also bound. Further investigations will be necessary to elucidate this matter and to prove the physiological importance of the regulation of CO_2 fixation by Mg^{2+}.

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