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FRUCTOSE- AND SEDOHEPTULOSEBISPHOSPHATASE

THE SITES OF A POSSIBLE CONTROL OF CO_2 FIXATION BY LIGHT-DEPENDENT CHANGES OF THE STROMAL Mg^{2+} CONCENTRATION

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SUMMARY

1. The enzymatic steps of the CO_2 fixation cycle responsible for the overall inhibition of CO_2 fixation caused by the lowering of the Mg^{2+} concentration in the stroma were investigated. For this the Mg^{2+} concentration in the stroma was decreased by addition of the ionophore A 23187, and the levels of the intermediates of the CO_2 fixation cycle in the stroma of intact chloroplasts were assayed by ion exchange chromatography.

2. The addition of the ionophore caused an increase of NADPH, ATP, fructose- and sedoheptulosebisphosphate and a dramatic decrease of phosphoglycerate in the stroma. These changes were reversed by the addition of Mg^{2+} and again affected by a subsequent addition of Ca^{2+}. Ribulosebisphosphate and pentosemonophosphate levels in the stroma were only a little affected under these different conditions.

3. The increase of the NADPH and ATP reflects the decreased utilization of these compounds due to the overall inhibition of CO_2 fixation. As phosphoglycerate and triosephosphate appear to be in near equilibrium with NADPH and ATP, the decrease of phosphoglycerate seems to be a consequence of the changes in the nucleotide levels.

4. The rapid increase of fructose- and sedoheptulosebisphosphate after the addition of the ionophore A 23187 clearly demonstrates that the overall inhibition of CO_2 fixation caused by lowering the stromal Mg^{2+} is due to the inhibition of the hydrolysis of these sugar bisphosphates. It is concluded that the activities of fructose- and sedoheptulosebisphosphatase can be controlled by light dependent changes of the stromal Mg^{2+} concentration.

INTRODUCTION

CO_2 fixation in chloroplasts is controlled by light. As possible sites of control,

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Abbreviations: HMP, mixture of hexose and heptose monophosphates; triose-*P*, trisosephosphate = dihydroxyacetonephosphate + D-glyceraldehyde-3-phosphate

several enzymatic steps of the CO_2 fixation cycle have been discussed (for refs. see 1 and 2) and it appears that there is more than one mechanism involved in the influence of illumination on the enzymatic activity of the CO_2 fixation cycle. It was shown from our laboratory that CO_2 fixation has a very strong pH dependency and that the change of the pH in the stroma caused by illumination is sufficient to switch CO_2 fixation from almost zero to maximal activity [3]. Furthermore, evidence has been obtained that some of the enzymes involved in CO_2 fixation may be activated in the light following the reductive formation of sulfhydryl groups by photosynthetic electron transport (for refs. see 1 and 2). Finally, there are various observations suggesting that CO_2 fixation may be controlled by the Mg^{2+} concentration in the stroma. Previous studies of the various enzymes involved in CO_2 fixation have shown that Mg^{2+} stimulated the activities of hexosebisphosphatase and ribulosebisphosphate carboxylase (for refs. see 1, 2). It was also demonstrated that light dependent CO_2 fixation in a reconstituted system is stimulated by increasing the Mg^{2+} concentration [4]. Studies with isolated thylakoid membranes suggested that Mg^{2+} is one of the major counter ions of light induced proton transport across the thylakoid membranes [5–8], and it was shown recently by Portis and Heldt [9] and Krause [10] that illumination of intact chloroplasts causes an increase in the stromal Mg^{2+} concentration of 1–3 mM.

The influence of such changes of the stromal Mg^{2+} on the CO_2 fixation of intact chloroplasts was studied with the aid of the divalent cation ionophore A 23187, which is known to catalyze a H^+/divalent cation exchange across membranes [11]. By increasing the permeability of the envelope for Mg^{2+} this ionophore allowed us to artificially vary the Mg^{2+} concentration in the stroma. Estimates of the Mg^{2+} concentration necessary for maximal rates of CO_2 fixation indicated that the changes of the stromal Mg^{2+} observed on illumination are high enough to have a possible function in the light regulation of CO_2 fixation [9]. It is the aim of the present paper to identify the enzymatic step(s) of the CO_2 fixation cycle being controlled by such changes of the stromal Mg^{2+} A tool for these investigations is the measurement of the steady state levels of the intermediates of the CO_2 fixation cycle in the stroma.

METHODS

a. Spinach (*Spinacia oleracea* L cv. True Hybrid 102, Arthur Yates and Co , NSW, Australia) was grown in water culture according to Lilley and Walker [12]

b Chloroplasts with intact envelopes were prepared from fully grown leaves according to the method of Cockburn et al. [13] modified by Heldt and Sauer [14]

c. The incubation of the intact chloroplasts was normally carried out at 20 °C in a medium containing 0 33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid (HEPES) pH 8 0 and 0.04 mg/ml catalase from beef liver (Boehringer, Mannheim) Other additions are indicated in the legends.

d. Measurements of oxygen evolution were carried out in parallel samples with an oxygen electrode as described by Delieu and Walker [15]. Illumination of the samples in the centrifuge or in the vessel with the oxygen electrode was carried out with a tungsten halogen light source provided with an RG 630 cut off filter (Schott, Mainz, Germany) and a Caltex heat filter (Balzers, Lichtenstein). The light intensity was about 40 000 ergs $\cdot$ s^{-1} $\cdot$ cm^{-2}. Illumination was continued during centrifugation.

e. Chlorophyll was assayed according to the method of Whatley and Arnon [16]. The method of Heldt et al. [17] was used to determine the pH in the stroma and the thylakoid space. For details of silicone layer filtering centrifugation see [18].

f. Phosphate containing metabolites were labelled by including $^{32}P_i$ in the reaction mixtures, which were preincubated with the chloroplasts for 5 min in the dark before illumination. The reaction was terminated by centrifugation of the chloroplasts through a silicon fluid layer into 20 μl of 10 % $HClO_4$. Following centrifugation the upper 200 μl supernatant solution was also acidified with 5 μl of 60 % $HClO_4$. Extracts were prepared by centrifugation of the precipitated protein, and neutralization of the supernatant with KOH or K_2CO_3.

g. Ion exchange chromatography on Bio-Rad AG1-X8 was performed as described elsewhere [19, 20]. The values for the metabolites in the stroma were corrected for the metabolites in the medium, which had been carried through the silicone layer with the chloroplasts.

In the experiment of Table II the effluent from the ion exchange column was also monitored at 254 nm with an ISCO UA-5 Absorbance Monitor with a Type 6 Optical Unit and SemiMicro Flow Cells of 5 mm pathlength (Instrumentation Specialities Corporation, Lincoln, Nebr. U.S.A.)

RESULTS AND DISCUSSION

The effect of A 23 187, Mg^{2+} and Ca^{2+} on CO_2 fixation and the metabolite levels in the stroma

a. CO_2 fixation We previously reported that the addition of A 23 187 to chloroplasts undergoing active CO_2 fixation in a Mg^{2+} free medium containing EDTA causes a complete inhibition of CO_2 dependent O_2 evolution [9] This is shown in the experiment of Table I. The subsequent addition of 5 mM Mg^{2+} restores the O_2 evolution almost to the initial rate, indicating that the effect of the ionophore is due to a loss of the stromal Mg^{2+}. A further addition of 5 mM Ca^{2+} again fully inhibits CO_2 fixation. It was found in a parallel experiment that phosphoglycerate reduction, which is a partial step of CO_2 fixation, was only slightly affected by the addition of the ionophore [9]. This observation indicated that the decrease of the stromal Mg^{2+} concentration by the ionophore has only a minor effect on the photosynthetic generation of ATP and NADPH.

The addition of the ionophore leads to an acidification of the stroma, probably caused by a Mg^{2+}/H^+ counter exchange [11]. We have shown recently that the rate of CO_2 fixation is strongly dependent on the pH in the stroma [3]. In chloroplasts not treated with the ionophore, a shift of the pH in the stroma from 7 88 to 7.55 would inhibit CO_2 fixation by about 50 % Therefore the 100 % inhibition observed on adding the ionophore cannot be entirely due to that pH shift, but has to be attributed to the observed loss of Mg^{2+}. On the other hand, the addition of 5 mM Mg^{2+} causes only a slight alkalinization of the stroma, whereas the rate of CO_2 fixation is restored to over 90 % Apparently, the inhibitory effect of the acidification in the stroma on CO_2 fixation may be partially counterbalanced by an excess of Mg^{2+}.

In order to identify the Mg^{2+}-sensitive steps of the CO_2 fixation cycle the metabolite levels in the chloroplasts were measured in parallel experiments (Table I). Since all the metabolites of the CO_2 fixation cycle contain phosphate, these can be

TABLE I

THE EFFECT OF DIVALENT CATIONS ON CO_2 FIXATION AND METABOLITE LEVELS IN INTACT CHLOROPLASTS

The chloroplasts (0 1 mg chlorophyll/ml) were incubated in the presence of 5 mM $NaHCO_3$ 0 5 mM EDTA and 0 5 mM [^{32}P]K_2HPO_4 (specific activity 80 Ci/mol). The incubation of the different samples was started by illumination. At the times indicated additions were made and the incubation terminated by silicone layer filtering centrifugation. CO_2 fixation and the pH in the stroma and the thylakoid space were assayed in parallel samples. For chromatographic assay of ^{32}P-labelled compounds extracts from the sedimented chloroplasts and the remaining supernatant, each equivalent to about 10 μg chlorophyll, were applied to the columns. The sorbitol impermeable ^3H_2O spaces were. 39 7 (1), 37.8 (2), 32 9 (3) and 29.9 (4) μl/mg chlorophyll

Sample No.	1	2	3	4
Time schedule of the experiment (s after start of illumination)				
Addition of 2 μM A 23187	–	180	180	180
Addition of 5 mM Mg^{2+}	–	–	270	270
Addition of 5 mM Ca^{2+}	–	–	–	360
Taking of sample	180	270	360	450
pH in stroma	7 88	7 55	7 60	7 71
Δ pH stroma/thylakoid space	2 26	1 92	1 97	1 60
CO_2 fixation (μmol O_2/mg chlorophyll per h)	92	0	85	0
Metabolites in stroma (nmol/mg chlorophyll)				
ATP	4 7	16 7	7.9	14 7
ADP	20 5	9 0	13 8	10.4
Triosephosphate	6 6	11 5	6 2	9 3
Phosphoglycerate	85 5	1 0	62 8	1 9
P_i	125	172	108	102
Fructosebisphosphate	4 2	22 0	6 8	35 9
Sedoheptulosebisphosphate	8 1	23 3	6 6	23 4
Hexose- and heptosemonophosphate	53 2	11 4	38 8	13 2
Pentosemonophosphate	3 3	1 2	2 6	1 5
Ribulosebisphosphate	17 8	18 4	30 7	8 8
Metabolite ratios in stroma				
$\frac{ATP}{ADP}$	0 23	1 86	0 58	1 42
$\frac{Triosephosphate}{Phosphoglycerate}$	0 08	11	0 10	4 9
$\frac{Fructosebisphosphate}{Hexose\text{- and heptosemonophosphate}}$	0 08	1 9	0 17	2 7
$\frac{Pentosemonophosphate}{Ribulosebisphosphate}$	0 19	0.07	0 08	0 18

conveniently labelled when CO_2 fixation is carried out in the presence of [^{32}P]phosphate. A very rapid separation of the chloroplasts from the surrounding medium by silicone layer filtering centrifugation, followed by a quantitative determination of the labelled substances using ion exchange chromatography permits a sensitive and accurate measurement of the metabolites in the stroma in the steady state of CO_2 fixation [20].

b. Adenine- and pyridine nucleotides. In agreement with earlier results [20], the ATP level in chloroplasts performing active CO_2 fixation is found to be rather low Thus, under the conditions of our experiment (saturating bicarbonate concentration and a possible limitation by light), photophosphorylation may be a limiting factor of CO_2 fixation. We have shown earlier that the stromal ATP is largely increased during the lag phase of CO_2 fixation [20]. This is a metabolic situation where CO_2 fixation is partially inhibited due to a lack of CO_2 fixation cycle intermediates. Likewise, the inhibition of CO_2 fixation by the ionophore also causes an almost 4-fold increase of the ATP (Table I). This again clearly indicates that the inhibition of CO_2 fixation by the ionophore is not the result of an uncoupling of photophosphorylation. When CO_2 fixation and the accompanying ATP consumption is restored by the addition of Mg^{2+}, the ATP level decreases, and it increases again when CO_2 fixation is inhibited by Ca^{2+}

In Table I these findings have been expressed also as ATP/ADP ratios. However, a significant amount of the ADP and the ATP in the chloroplasts may be bound to the ATPase [21, 22] and the concentration ratios of the free nucleotides may be actually somewhat different from those evaluated in Table I. It may be noted that the evaluated ATP/ADP ratios (and also the values for log ($\{[ATP]/[ADP]\} \cdot [P_i]$) not shown here) do not correlate well with the obtained values for the pH gradient across the thylakoid membrane, which is widely believed to represent the intermediary driving force of photosynthetic ATP synthesis (for review see ref. 23). The finding of a low ATP/ADP ratio during active CO_2 fixation (Expt 1) together with a high pH gradient might be explained by the high rate of ATP utilization. Further investigations will be required to eludicate this matter.

Table II shows an experiment similar to that of Table I in which the chromatographic assay was carried out by monitoring the ultraviolet absorbancy of the column effluate. This method of detection is less sensitive and therefore requires much larger samples, but it enables the determination of nucleotides which are not detected from ^{32}P incorporation, e g. AMP and pyridine nucleotides. The quantitative determination of pyridine nucleotides is normally rather complicated, since the reduced forms are unstable in acid media, whereas the oxidized forms are decomposed in an alkaline environment. With ion exchange chromatography this complication is overcome because the acid degradation of NADH and NADPH yields defined degradation products which are separated and can be detected by ultraviolet absorbance [24].

In the experiment of Table II the ATP level is again increased by the ionophore The level of NADPH in the stroma closely follows that of ATP. During active CO_2 fixation the NADPH/NADP ratio is relatively low, and there is a strong increase of this ratio observed when the ionophore is added. Obviously, the inhibition of CO_2 fixation results in an accumulation of reducing equivalents in the stroma.

c. Phosphoglycerate and triosephosphate. As the chromatographic assay procedure does not separate dihydroxyacetonephosphate and glyceraldehydephosphate, the term triosephosphate is used for the sum of these substances. If one assumes that

TABLE II

EFFECT OF A 23187 ON CO_2 FIXATION RELATIONSHIP BETWEEN THE LEVELS OF TRIOSEPHOSPHATE, PHOSPHOGLYCERATE, ADENINE- AND PYRIDINENUCLEOTIDES IN THE STROMA

The chloroplasts (0 16 mg chlorophyll/ml) were incubated in the presence of 5 mMNa HCO_3, 0 1 mM EDTA, 0 5 mM [^{32}P]K_2HPO_4 (specific activity 10 Ci/mol), 20 mM HEPES (pH 8 0) The control experiment was terminated 210 s after the start of illumination by silicone layer filtering centrifugation In the other experiment 3 μM A 23187 were added to the chloroplasts 180 s after the start of illumination, and the reaction was then terminated after 120 s CO_2 fixation and the pH in the stroma and the thylakoid space were assayed in parallel samples For the chromatographic assay of the sediment fraction the extracts from 8 parallel samples, each containing 0 032 mg of chlorophyll, were applied to the column The nucleotides were determined by planimetric peak evaluation of the ultra violet (254 nm) recording Rates of CO_2 fixation control, 77, +A 23187, O μmol mg chlorophyll per h

	Metabolite levels 10^{-9}mol/mg chlorophyll)	
	Control	+A 23187
ATP	1 8	6 6
ADP	10 0	7 3
AMP	4 8	2 1
ΣATP+ADP+AMP	16 6	16 0
ATP/ADP	0 18	0 90
NADPH	3 0	6 2
NADP	6 5	2 8
ΣNADPH+NADP	9 5	9 0
NADPH/NADP	0 46	2 21
[P_i]	5 4 mM	3 6 mM
pH stroma	7 94	7 73
[triose-*P*]/[PGA] measured	0 05	5 4
[triose-*P*]/[PGA] calculated*	0 018	1 1

* The calculation is based on the following equilibria

$$\frac{[GAP]}{[PGA]} = \frac{1}{K_1 \; K_2} \frac{[NADPH] \cdot [ATP] \cdot [H^+]}{[NADP] \quad [ADP] \quad [P_i]}$$

$K_1 = 3200$ [25], $K_2 = 7 \;\; 10^{-8}$ [26], $\frac{[DHAP]}{[GAP]} = K_3 = 22\,0$ [27],

[triose-*P*] = [DHAP]+[GAP] = (K_3+1) [GAP]

* where PGA = 3-phosphoglycerate, GAP = glyceraldehyde-3-phosphate, DHAP = $(OH)_2$acetone-*P*

these two compounds are in equilibrium catalyzed by the triosephosphate isomerase (EC 5 3 1 1.), dihydroxyacetonephosphate will represent about 95 % of the triose-phosphate. In chloroplasts performing active CO_2 fixation the amount of phospho-glycerate in the stroma is more than one order of magnitude higher than the amount of triosephosphate [20]. It was of interest if this low metabolite ratio reflects an equili-brium state To answer this the expected triosephosphate/phosphoglycerate ratio was calculated from the measured nucleotide, phosphate and proton concentrations and the published corresponding equilibrium constants obtained with triosephosphate

isomerase, phosphoglycerate kinase (EC. 1.1.1.95) and D-glyceraldehyde-3-phosphate: $NADP^+$ oxidoreductase (phosphorylating) (EC. 1.2.1.13). As shown in Table II the triose-*P*/3-phosphoglycerate ratios calculated in this way are rather similar to the measured values in both metabolic states.

The uncertainties of this calculation with respect to the employed equilibrium constants can be avoided by comparing the quotient of the ratios obtained at the two metabolic conditions:
(triose-*P*/3-phosphoglycerate)$_{A\ 23187}$/(trioseP/3-phosphoglycerate)$_{control}$. The quotient from the calculated ratios (61) differs by less than a factor of two from the quotient of the measured ratios (108). These findings suggest that the phosphoglycerate and triosephosphate are not far from equilibrium with the corresponding adenine- and pyridine nucleotides. The dramatic change of the triose-*P*/3-phophoglycerate ratio in the stroma by a factor of approx. 100, observed after the addition of the ionophore, appears to be due mainly to the change of corresponding nucleotide concentrations. The restoration of CO_2 fixation by Mg^{2+} (Table I) decreases the triose-*P*/3-phosphoglycerate ratio to the normal value, and the subsequent inhibition of CO_2 fixation by Ca^{2+} increases this ratio again.

d. Hexose and heptose phosphates. The metabolic changes following the addition of the ionophore discussed so far are striking, but all seem to be secondary events caused by the overall inhibition of CO_2 fixation. Another dramatic change of the metabolite pattern caused by the ionophore concerns the amounts of hexose -and heptosephosphates. During active CO_2 fixation the amount of fructosebisphosphate is low and the sum of hexose- and heptosemonophosphates (these compounds are usually not well separated by our chromatographic technique and are therefore determined in total) is relatively high. In an experiment similar to that in Table I the composition of the hexose- and heptosemonophosphate peak was further analyzed by thin layer chromatography and electrophoresis (Table III).

TABLE III

EFFECT OF A 23187 ON THE LEVELS OF SUGARMONO- AND SUGARBISPHOSPHATES IN THE STROMA

For details see experiment of Table I The hexose- and heptosemonophosphates were separated by thin layer electrophoresis and chromatography [28] and the radioactivity counted by liquid scintillation Rates of CO_2 fixation control, 104; +A 23187, O μmol/mg chlorophyll per h.

	nmol/mg chlorophyll	
	Control	+A 23187
Fructosemonophosphate	9.8	2 9
Glucosemonophosphate	12 4	11.6
Sedoheptulosemonophosphate	15 4	4 7
Fructosebisphosphate	4 2	44 3
Sedoheptulosebisphosphate	2 4	17.3
Fructosebisphosphate / Fructosemonophosphate	0 43	15 3
Sedoheptulosebisphosphate / Sedoheptulosemonophosphate	0 16	3.7

The addition of the ionophore causes a very strong increase of the fructose- and sedoheptulosebisphosphate and a decrease of the fructose- and sedoheptulosemonophosphate, resulting in a dramatic change of the sugarbisphosphate/sugarmonophosphate ratios. This clearly indicates that the hydrolysis of fructose- and sedoheptulosebisphosphate is blocked under these conditions. The subsequent addition of Mg^{2+} normalizes the pattern of hexose and heptose phosphates, demonstrating that the block of the two hydrolysis reactions is released by Mg^{2+} (Table I). These findings concur with earlier investigations of isolated fructosebisphosphatase from spinach chloroplasts, which showed that the enzyme was strongly activated by low concentrations of Mg^{2+} [29].

The further addition of Ca^{2+} causes a subsequent increase of fructose- and sedoheptulosebisphosphate and a corresponding decrease of the monophosphates. In this respect Ca^{2+} mimics the effect of the ionophore. It has been shown by Gimmler [30] that A 23187 facilitates a rapid influx of Ca^{2+} into chloroplasts in exchange for Mg^{2+}. Although this Ca^{2+} influx would probably result in a decrease of stromal Mg^{2+}, the major effect of the Ca^{2+} may be a direct inhibition of the hexose- and heptosebisphosphatase, as Ca^{2+} has been found to be a very potent inhibitor of isolated fructosebisphosphatase [31].

e. Pentosephosphates. The addition of the ionophore has no marked effect on the amount of ribulosebisphosphate in the stroma. However, that a considerable amount of ribulosebisphosphate is found (possibly bound to the ribulosebisphosphate carboxylase [2]) even though the rate of CO_2 fixation is practically zero may indicate that the carboxylation of ribulosebisphosphate is inhibited under these conditions. This would concur with earlier investigations of the Mg^{2+} requirement of the isolated enzyme (for refs. see 1, 2). After the addition of Mg^{2+} the amount of ribulosebisphosphate is even elevated, suggesting that the activity of the fructosebisphosphatase is increased relatively more than the activity of the ribulosebisphosphate carboxylase.

The subsequent addition of Ca^{2+} strongly decreases the ribulosebisphosphate. The present authors are not aware of any study of the effect of Ca^{2+} on the activity of the isolated carboxylase. Our data suggest that Ca^{2+} has only a minor effect on the activity of this enzyme.

The level of pentosemonophosphate is not markedly changed under the different conditions of our experiment. Assuming that the amount of ribulosemonophosphate in the stroma is proportional to the total pentosephosphates, these findings indicate that the phosphorylation of ribulosemonophosphate is not a major point of Mg^{2+} control.

Effects of Mg^{2+} concentration on metabolite levels

In an attempt to resolve the concentration dependency of the Mg^{2+} sensitive steps, we examined the effect of varying the rate of CO_2 fixation by adding back suboptimal Mg^{2+} concentrations after CO_2 fixation had been inhibited by the ionophore. In the experiment shown in Fig. 1 the chloroplasts were first illuminated until a constant rate of CO_2 fixation was reached, and A 23187 was then added. When complete inhibition of CO_2 fixation was assured, various amounts of Mg^{2+} were added and the illumination of the samples was terminated by silicon layer filtering centrifugation after a constant rate of restored CO_2 fixation was obtained As has been shown before [9], very low concentrations of Mg^{2+} in the external medium are sufficient for partial

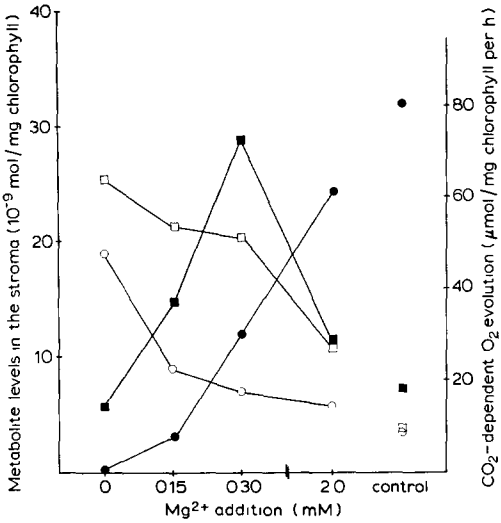

Fig. 1. Restoration of CO_2 fixation by the addition of various Mg^{2+} concentrations to the chloroplasts inhibited by A 23187. The chloroplasts (0.1 mg chlorophyll/ml) were illuminated in the presence of 10 mM $NaHCO_3$, 0 1 mM EDTA, 0.5 mM [^{32}P]$KH_2F_3PO_4$ (specific activity 80 Ci/mol) in parallel samples 150 s after the start of illumination 2 μM A 23187 were added. 90 s after the addition of the ionophore, when CO_2 fixation was totally inhibited, the indicated concentrations of Mg^{2+} were added. At this time the control sample containing no ionophore and a sample containing only ionophore were terminated by silicone layer filtering centrifugation. The other samples were terminated when a linear rate of CO_2 fixation was reached (Mg 0.15 mM, 330 s, Mg 0 3 mM, 270 s, Mg 2 mM, 120 s after the addition of A 23187) The rates of CO_2 fixation were measured in parallel samples as O_2 evolution ●, O_2 evolution, ○, Sed-1,7-P_2, ■, Ru-1,5-P_2; □, Fru-1,6-P_2.

restoration of CO_2 fixation. The restoration of CO_2 fixation is accompanied by a decrease of the amounts of fructose- and sedoheptulosebisphosphate, but distinct differences between the changes of the two metabolites were observed. The addition of 0.15 mM Mg^{2+} (in the presence of 0.1 mM EDTA) which permitted 9 % of the initial rate of CO_2 fixation was accompanied by a 42 % decrease of the sedoheptulosebisphosphate level, but only a 16 % decrease of the fructosebisphosphate. Even the addition of 0.3 mM Mg^{2+} which allowed 38 % recovery of the rate of CO_2 fixation, decreased the level of fructosebisphosphate by only 19 % whereas the level of sedoheptulosebisphosphate was decreased by 63 %. Only the addition of 2 mM Mg^{2+}, which restored CO_2 fixation to 76 %, caused a large decrease of the fructosebisphosphate level. These differences suggest that the inhibition of sedoheptulosebisphosphate hydrolysis is released with a lower Mg^{2+} concentration than the inhibition of fructosebisphosphatase. It should be also noted that at low Mg^{2+} concentrations there is a marked rise of the ribulosebisphosphate. This may reflect the fact that at such low Mg^{2+} concentrations the fructose- and sedoheptulosebisphosphatase are activated relatively more than the ribulosebisphosphate carboxylase. Under these conditions the activity of the latter enzyme may be limiting the overall process of CO_2 fixation.

Kinetics of metabolite changes upon the addition of A 23187

In order to further characterize the effect of the ionophore, we followed the

time course of the changes in the metabolite levels and O_2 evolution upon the addition of the ionophore. As shown in Fig. 2, 2 μM A 23187 was sufficient to completely inhibit O_2 evolution within 60 s. The result of the metabolite analysis gives a clear picture of the sequence of the overall reaction of CO_2 fixation. 15 s after the addition of the ionophore the level of fructosebisphosphate already reaches its maximum value, and there is also a rapid response of the level of sedoheptulosebisphosphate. The decrease of the levels of hexose- and heptosemonophosphates and phosphoglycerate follow the time course of O_2 evolution and are complete only after approx. 60 s. In

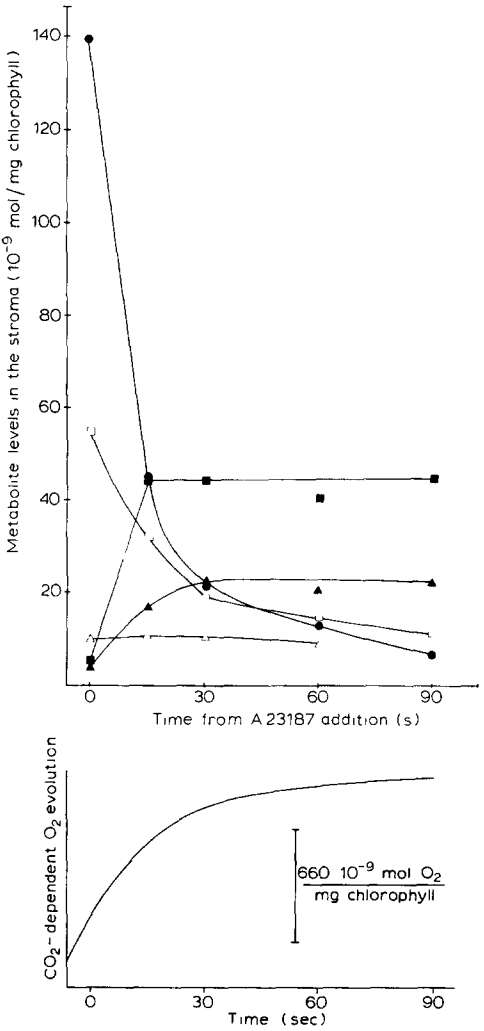

Fig 2 Kinetics of the inhibition of CO_2 fixation by A 23187. Chloroplasts were incubated in the light according to the legend of Fig 1 210 s after the beginning of illumination, when the rate of CO_2 dependent O_2 evolution reached 124 μmol O_2/mg chlorophyll per h, 2 μM A 23187 were added and samples were terminated at the times indicated ●, 3-Phosphoglycerate; □, HMP; ◿, Ru-1,5-P_2, ■, Fru, 1,6-P_1, ▲, Sed-1,7-P_2

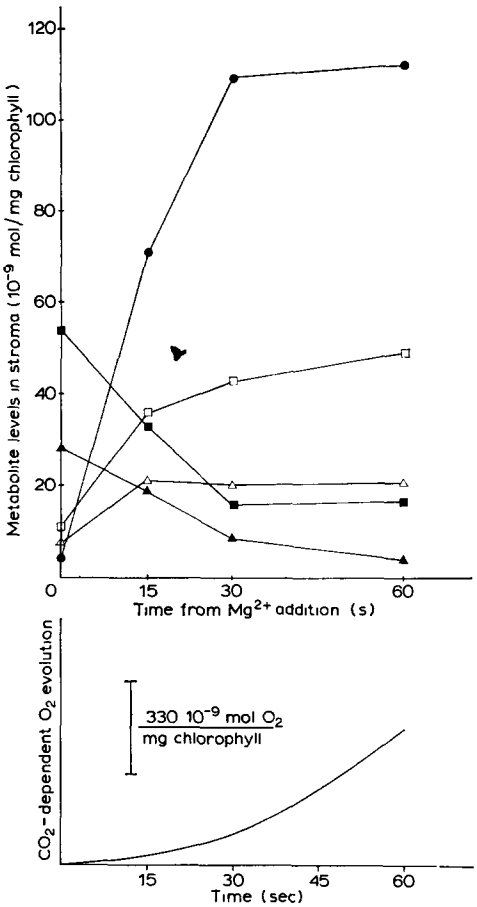

Fig. 3. Kinetics of the restoration of A 23187 inhibited CO_2 fixation by Mg^{2+}. The chloroplasts were incubated in the light according to the legend of Fig. 1. 210 s after the beginning of illumination, when the rate of CO_2-dependent O_2 evolution reached 114 μmol O_2/mg chlorophyll per h, 2 μM A 23187 were added 120 s later, when CO_2 fixation was totally inhibited, 2 mM Mg^{2+} were added Samples were then terminated at the indicated times. The restored rate of CO_2-dependent O_2 evolution at 60 s was 73 μmol O_2/mg chlorophyll per h Symbols as per Fig 2.

agreement with the data of Table I the level of ribulosebisphosphate remains almost unchanged. Reversed kinetics are obtained when the inhibition of CO_2 fixation by A 23187 is reversed by the addition of Mg^{2+} (Fig. 3). There is a rapid decrease of fructose- and sedoheptulosebisphosphate observed and an increase of hexose- and heptosemonophosphate. During the first 15 s there is also observed an increase of ribulosebisphosphate. The large increase of the phosphoglycerate is in close relationship with the increase of oxygen evolution.

CONCLUSION

At the beginning of this work, the question was asked which enzymatic steps in

the metabolic pathway of CO_2 fixation are responsible for the observed inhibition of CO_2 fixation caused by a decrease of the stromal Mg^{2+} concentration. In principle these should be identifiable from an accumulation of the substrates of these particular steps after the addition of the inhibitor. A release of the inhibition should also reverse this accumulation. As has been shown here, such changes of the metabolite levels have been observed only for ATP, NADPH, fructose- and sedoheptulosebisphosphate. Whereas the increases of ATP and NADPH levels can be explained as secondary events following the overall inhibition of CO_2 fixation, the rise of the sugarbisphosphate levels clearly demonstrates that the observed inhibition of CO_2 fixation is ultimately due to inhibition of the fructose- and sedoheptulosebisphosphatase. Participation of the ribulosebisphosphate carboxylase in the overall inhibition of CO_2 fixation by a decrease of the stromal Mg^{2+} concentration could not be shown definitely, although the effects of low Mg^{2+} concentration suggest that this enzyme might be also involved. It should be also noted that our model experiments have been carried out with saturating CO_2 concentrations. It is possible that with air levels of CO_2 the carboxylation reaction may be relatively more affected by lowering the stromal Mg^{2+} concentration.

Investigations of the metabolite levels in *Chlorella* performing CO_2 fixation have shown that the reactions catalyzed by the fructose- and sedoheptulosebisphosphatase are far from equilibrium, indicating that the activity of these enzymes was limiting the overall reaction of CO_2 fixation [32]. These findings and also the known properties of the isolated fructosebisphosphatase (for ref. see 2) led to the speculation that these enzymes are major control points for the regulation of CO_2 fixation.

Our data on the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts [9] and the identification of the Mg^{2+} dependent steps shown here indicate that the activity of the fructose- and sedoheptulosebisphosphatase can be controlled by light-dependent changes of the Mg^{2+} concentration in the stroma. Likewise, the activities of fructose- and sedoheptulosebisphosphatase in intact chloroplasts are also very effectively controlled by changes of H^+ concentration in the stroma, as will be shown in a future publication. Activation of stromal fructosebisphosphatase by increases in pH and the Mg^{2+} concentration has been found also with the isolated enzyme [29] and with reconstituted chloroplasts [33]. These experimental findings show that the light induced decrease of H^+ concentration and increase of Mg^{2+} concentration in the stroma both stimulate CO_2 fixation in a similar way, by increasing the activities of the sugarbisphosphatases. Thus, light-dependent cation transport across the thylakoid membrane appears to have an important function for the regulation of CO_2 fixation.

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REFERENCES

1 Walker, D. A (1976) in Current Topics in Cellular Regulation (Horecker, B L., ed), Vol. 11, pp 203–241, Academic Press, New York
2 Kelly, G J , Latzko, E. and Gibbs, M (1976) Ann Rev Plant Physiol 27, 181–205
3 Werdan, K , Heldt, H W and Milovancev, M (1975) Biochim Biophys Acta 396, 276–292
4 Lilley, R McC , Holborow, K and Walker, D A (1974) New Phytol 73, 657–662
5 Dilley, R A and Vernon, L P (1965) Arch Biochem Biophys 111, 365–375
6 Hind, G , Nakatani, H Y and Izawa, S (1974) Proc Natl. Acad Sci U S 71, 1484–1488
7 Krause, G H. (1974) Biochim Biophys. Acta 333, 301–313
8 Barber, J , Mills, J. and Nicolson, J (1974) FEBS Lett 49, 106–110
9 Portis, A R and Heldt, H. W. (1976) Biochim Biophys. Acta 449, 434–446
10 Krause, G H. (1977) Biochim Biophys Acta 460, 500–510
11 Pfeiffer, D R., Reed, P W. and Lardy, H A. (1974) Biochemistry 13, 4007–4014
12 Lilley, R. McC and Walker, D A (1974) Biochim Biophys Acta 368, 269–278
13 Cockburn, W , Walker, D A and Baldry, C. W (1968) Biochem J 107, 89–95
14 Heldt, H W and Sauer, F (1971) Biochim. Biophys Acta 234, 83–91
15 Delieu, T and Walker, D A (1972) New Phytol., 71, 201–216
16 Whatley, F. R and Arnon, D J. (1963) Methods Enzymol 8, 308–313
17 Heldt, H W , Werdan, K , Milovancev, M and Geller, G (1973) Biochim Biophys. Acta 314, 224–241
18 Werdan, K , Heldt, H W and Geller, G (1972) Biochim Biophys Acta 283, 430–441
19 Heldt, H W. and Klingenberg, M. (1967) Methods in Enzymology, 10, 482–487
20 Lilley, R McC , Chon, C J., Mosbach, A and Heldt, H. W (1977) Biochim Biophys Acta 460, 259–272
21 Roy, H and Moudrianakis, E N (1971) Proc Natl Acad Sci. U S 68, 466–468
22 Harris, D. A and Slater, E. C (1975) Biochim Biophys Acta 387, 335–348
23 Hall, D O (1976) in The Intact Chloroplast (Barber, J ed), pp 135–170, Elsevier/North-Holland Biomedical Press, Amsterdam
24 Heldt, H. W , Klingenberg, M. and Papenberg, K (1965) Biochem Z. 342, 508–517
25 Bucher, Th (1947) Biochim Biophys Acta 1, 292–314
26 Yonuschot, G R , Ortwerth, B. J and Koeppe, O. J (1970) J Biol Chem 245, 4193–4198
27 Oesper, P. and Meyerhof, O (1950) Arch Biochem. 27, 224–233
28 Schurmann, P (1969) J. Chromatog 39, 507–509
29 Baier, D and Latzko, E. (1975) Biochim Biophys. Acta 396, 141–147
30 Gimmler, H (1977) Berichte Dtsch Botan Ges in the press
31 Baier, D (1976) Doktor Dissertation, Fakultat fur Landwirtschaft und Gartenbau Weihenstephan der Technischen Universitat, Munchen
32 Bassham, J A and Krause, G. H (1969) Biochim Biophys Acta 198, 207–221
33 Walker, D A., Slabas, A R and Fitzgerald, M P (1976) Biochim Biophys Acta 440, 147–162