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Effect of Photosynthetic Intermediates on the Magnesium Inhibition of Oxygen Evolution by Barley Chloroplasts[1]

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STEVEN C. HUBER
United States Department of Agriculture, Science and Education Administration, Agriculture Research, Departments of Crop Science and Botany, North Carolina State University, Raleigh, North Carolina 27650

ABSTRACT

Millimolar concentrations of Mg^{2+} inhibited CO_2-dependent O_2 evolution by barley (*Hordeum vulgare* L.) chloroplasts and also prevented the activation of NADP-glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase, and fructose-1,6-diphosphatase by light in intact chloroplasts. When added in the dark, 3-phosphoglycerate prevented the inhibition of O_2 evolution by Mg^{2+} and reduced the Mg^{2+} inhibition of enzyme activation by light. Fructose 1,6-diphosphate and ribulose 5-phosphate also prevented the inhibition of O_2 evolution by Mg^{2+} whereas glucose 1-phosphate, glucose 6-phosphate, ribulose 1,5-diphosphate, and citrate had no effect. Phosphoenolpyruvate gave an intermediate response. Metabolites that prevented the Mg^{2+} inhibition of O_2 evolution shortened the lag phase of CO_2-dependent O_2 evolution in the absence of M^{2+}. Loading chloroplasts in the dark with 3-phosphoglycerate reduced both the lag phase of O_2 evolution and the inhibition of O_2 evolution by Mg^{2+}. The results suggested that Mg^{2+} inhibition was lessened either by external metabolites that compete with inorganic phosphate for transport into the chloroplast or by a high concentration of internal metabolites.

Inhibition of photosynthesis by Mg^{2+} has been documented for spinach (2, 12), lettuce (5), barley, and crabgrass (12) mesophyll chloroplasts. Photosynthesis by spinach chloroplasts has been reported to be both sensitive (2, 3, 12) and relatively insensitive to Mg^{2+} (17, 18). The basis for inhibition by Mg^{2+} is not completely understood. Recent studies (12) have indicated that Mg^{2+} blocks the activation by light of certain Calvin cycle enzymes. The effect of Mg^{2+} may involve a membrane interaction because the chloroplast envelope is impermeable to Mg^{2+} and other divalent cations (8). It has been suggested that Mg^{2+} increases Pi exchange across the chloroplast envelope (11, 17) and that it is a high stromal concentration of Pi which prevents the light activation of photosynthetic enzymes (13).

The postulate predicts that metabolites which compete with Pi for transport on the phosphate translocator should reduce inhibition by Mg^{2+}. The objectives of this study were to determine the effect of various phosphate esters on the Mg^{2+} inhibition of O_2 evolution and the light activation of Calvin cycle enzymes. In barley chloroplasts, metabolites that decreased the lag phase of O_2 evolution prevented, but did not reverse, the inhibition by Mg^{2+} of both O_2 evolution and the light activation of P-ribulokinase, NADP-glyceraldehyde-3-P dehydrogenase and fructose-1,6-diphosphatase. In addition, evidence is presented that the concentration of internal metabolites modulates Mg^{2+} inhibition. This may explain why inhibition of chloroplast photosynthesis by low concentrations of Mg^{2+} is observed in some, but not all, studies.

MATERIALS AND METHODS

Plant Growth and Chloroplast Isolation. Barley (*Hordeum vulgare* L., cv. Trophy) was grown in a growth chamber as previously described (14). Chloroplasts were prepared from enzymically isolated mesophyll protoplasts. Protoplast isolation was as previously described (14) except the protoplasts were purified by flotation on 0.5 M sucrose (200*g*, 6 min). Barley protoplasts were ruptured by passage through a 20-μm nylon net in a medium containing 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, and 2 mM isoascorbate (pH 6.5). After centrifugation (200*g*, 3 min), the chloroplast pellet was resuspended in 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA. Chloroplasts were determined to be 93 to 99% intact on the basis of enzyme retention.

O_2 Evolution. O_2 evolution was followed polarographically using Clark-type electrodes in 2-ml water-jacketed vessels (25 C). The basic reaction mixture contained 0.33 M sorbitol, 50 mM Tricine-NaOH (pH 8.2), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2 mM sodium phosphate, 5 mM $NaHCO_3$ and 15 to 30 μg Chl/ml. In all tables and figures, the given concentration of Mg^{2+} represents the final net concentration (Mg^{2+} concentration minus EDTA concentration).

Illumination was provided by a 75-w General Electric floodlamp passed through 4 cm water to give a quantum flux density of 80 nE/cm^2·s between 400 and 700 nm at the face of the cuvette.

To load chloroplasts with PGA[2] (experiment of Fig. 4), plastids were incubated in the dark at 4 C in the resuspension medium containing 10 mM PGA. After 2 min, the chloroplasts were pelleted (200*g*, 3 min) and washed twice, with the walls of the tube being carefully washed prior to resuspension of the pellet. All washes were performed at 4 C using the resuspension medium described above. Chloroplasts were used immediately after the final wash.

Activation by Light and Enzyme Assays. For the light activation experiments, intact chloroplasts were incubated in the O_2 evolution reaction mixtures with other additions as described. Aliquots were removed at various times and injected directly into the enzyme assay mixtures. Enzyme activity was followed spectrophotometrically at 340 nm at 25 C. NADP-glyceraldehyde-3-P dehydrogenase was assayed (16) in 0.1 M Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 5 mM ATP, 2 mM PGA, 0.2 units/ml P-glycerokinase and 0.2 mM NADPH. Fructose-1,6-diphosphatase was assayed in a

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[2] Abbreviations: PGA: 3-phosphoglycerate; R5P: ribulose 5-phosphate; FDP: fructose 1,6-diphosphate; G1P: glucose 1-phosphate; G6P: glucose 6-phosphate; RuDP: ribulose 1,5-diphosphate; PEP: phosphoenolpyruvate.

medium containing 100 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 0.3 mM NADP, 0.6 mM fructose-1,6-diP, 2 units P-glucose isomerase, and 1 unit glucose-6-P dehydrogenase as described by Kelly *et al.* (15). P-ribulokinase was assayed (12) in a CO_2-free mixture containing 100 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 5 mM PEP, 0.4 mM NADH, 0.5 mM ribose-5-P, 6 units of pyruvate kinase, and 9 units of lactate dehydrogenase. Addition of P-ribuloisomerase was not required. For the P-ribulokinase assay, the only bicarbonate present was that introduced by the addition of the chloroplasts (10 μl of chloroplast mixture to give 33 μM $NaHCO_3$ in the assay mixture). Rates were linear for approximately 5 min with this assay. This assay was chosen for the preliminary experiments because it does not rely on coupling to any of the other Calvin cycle enzymes. Each experiment was repeated at least three times. Numerical data are the average of three experiments. All enzymes and biochemicals were obtained from Sigma Chemical Co.[3]

RESULTS AND DISCUSSION

Effects of Metabolites and Mg^{2+} on O_2 Evolution. When intact barley chloroplasts were illuminated and CO_2 was the sole substrate, O_2 evolution began gradually with a lag phase of approximately 5 min (Table I). Addition of PGA, FDP, or R5P in the dark shortened the lag phase and slightly increased the rate of O_2 evolution (Table I). Shortening of the lag phase by these metabolites has been documented previously with spinach chloroplasts (4, 6, 18). The PGA and possibly R5P (4, 6) are directly transported across the chloroplast envelope and FDP is also transported, but probably after conversion to triose-P (18). Shortening of the lag phase by transported metabolites is consistent with the proposal (18) that the lag phase of O_2 evolution reflects the time required to build up the pool sizes of photosynthetic intermediates in the chloroplast stroma.

O_2 evolution by barley chloroplasts with CO_2 as sole substrate was completely inhibited when 2 mM $MgCl_2$ was added in the dark before illumination (Table I). O_2 evolution was substantial in the presence of 2 mM $MgCl_2$ and either PGA, R5P, or FDP (Table I). Mg^{2+} did not significantly affect the length of the lag phase of O_2 evolution in the presence of added substrates but did reduce the rate of O_2 evolution. The ability of a substrate to shorten the lag phase of O_2 evolution in the absence of Mg^{2+} correlated with the rate of O_2 evolution in the presence of Mg^{2+}. PGA was most effective and R5P least effective. Prevention of Mg^{2+} inhibition by metabolites that compete with Pi for transport on the phosphate translocator (9, 10) is consistent with the proposal that Mg^{2+} causes inhibition of O_2 evolution by stimulating phosphate transport (11, 17). Addition of PGA, FDP, or R5P in the light to Mg^{2+}-inhibited chloroplasts did not induce O_2 evolution (data not shown).

The finding that certain photosynthetic intermediates prevented the inhibition of O_2 evolution by Mg^{2+} suggested that the relative inhibition of O_2 evolution by Mg^{2+} might decrease with increasing Chl concentration. When the concentration of Chl was increased from 10 to 30 μg/ml, the lag phase of O_2 evolution decreased from 4.5 to 3 min without affecting the maximum rate expressed on a Chl basis (70 μmol/mg Chl·h, Fig. 1). One mM $MgCl_2$ added in the dark increased the lag phase of O_2 evolution roughly 50% and inhibited the maximum rate of O_2 evolution nearly 70% at both Chl concentrations. These results were interpreted as follows. The high Chl concentration in the absence of Mg^{2+} increased the rate of build-up of Calvin cycle intermediates in the reaction mixture (7) and shortened the lag, but had no effect on the final velocity when expressed on a Chl basis. In the presence of Mg^{2+} intermediates accumulated after several min in the light, but the increased rate of build-up of intermediates did not lessen the inhibition. Prevention of Mg^{2+} inhibition required that the substrates be present before illumination.

An experiment to explore prevention of Mg^{2+} inhibition as a function of PGA concentration (Fig. 2) was performed with an excess of Mg^{2+} (1 mM above that required to give complete inhibition of O_2 evolution) to eliminate artifacts as a result of Mg^{2+} chelation by the phosphate ester. When plotted in double reciprocal form, the data of Figure 2 yielded an apparent K_m (PGA) of 0.25 mM and an extrapolated V_{max} of 125 μmol/mg Chl·h. These kinetic constants are similar to those obtained previously for transport of PGA by intact spinach chloroplast (9, 10).

The ability of other metabolites to prevent inhibition of CO_2-dependent O_2 evolution by Mg^{2+} was determined. For comparison,

Table I. Effects of substrates and Mg^{2+} on the lag phase and maximum rate of O_2 evolution by intact barley chloroplasts.

Added substrate[1]	Condition			
	minus $MgCl_2$		+2 mM $MgCl_2$	
	lag[2]	rate[3]	lag[2]	rate[3]
None	5.0	130	<15	0
R5P	3.8	148	4.8	56
FDP	2.5	154	2.5	84
PGA	0.8	180	0.8	142

[1] Substrates indicated were added in the dark to give a final concentration of 0.5 mM.

[2] Lag in minutes.

[3] Rates expressed as μmol O_2 evolved/mg Chl·h.

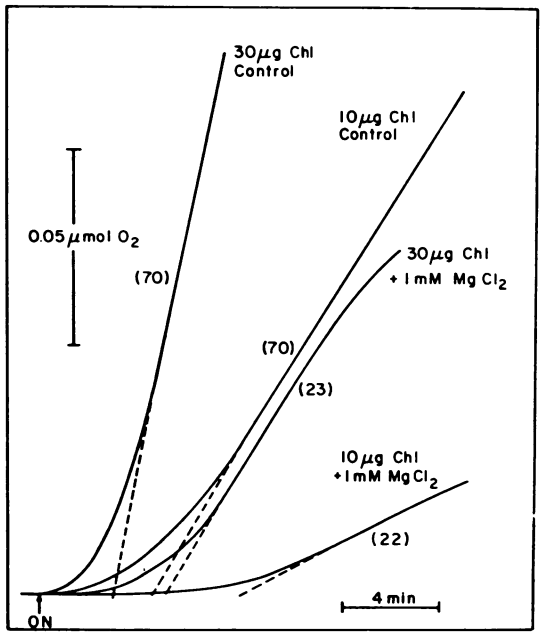

FIG. 1. Effect of Chl concentration on O_2 evolution by barley chloroplasts in the presence and absence of 1 mM $MgCl_2$. Maximum rates of evolution, expressed as μmol O_2 evolved/mg Chl·h, are shown parenthetically. Dashed lines are tangents to the maximum rate portion of the curves.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

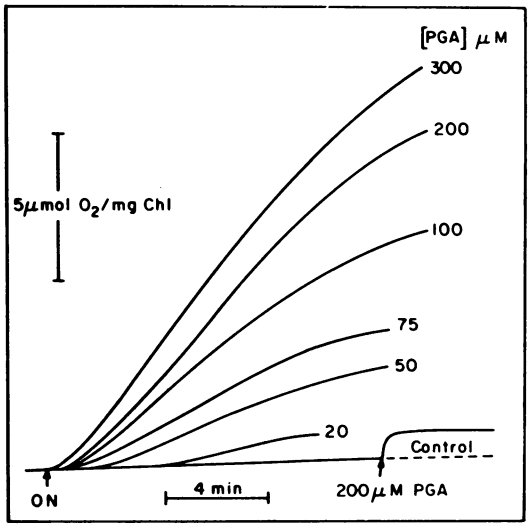

FIG. 2. Effect of PGA concentration on O_2 evolution by barley chloroplasts in the presence of 2 mM $MgCl_2$. After approximately 13 min, 200 μM PGA was added to the control, as indicated by the arrow.

O_2 evolution in the presence of PGA plus $MgCl_2$ is also shown. G1P, G6P, RuDP, and citrate were not effective (Fig. 3). These compounds also had no effect on O_2 evolution in the absence of Mg^{2+} (data not shown). These metabolites are not thought to be transported across the chloroplast envelope (9, 10) and did not affect O_2 evolution by spinach chloroplasts (4, 6). PEP, in the absence of Mg^{2+}, decreased the lag phase of O_2 evolution slightly without affecting the maximum rate of evolution (data not shown) and partially prevented Mg^{2+} inhibition (Fig. 3). The intermediate effect observed with PEP is consistent with the reported low affinity of the phosphate translocator of spinach chloroplasts for PEP (9).

Inhibition by Mg^{2+} was prevented only by metabolites that shortened the lag phase of O_2 evolution. This suggested that transport of the substrate across the chloroplast envelope was required to protect the chloroplast from inhibition by Mg^{2+}. To determine whether protection involved internal or external substrate levels, chloroplasts were preloaded with PGA. As demonstrated by Heldt *et al.* (9, 10), incubation of chloroplasts in the dark with PGA resulted in an apparent accumulation in the stroma because of uptake via a strict exchange-diffusion translocator (the phosphate translocator, ref. 10). Loading barley chloroplasts with PGA decreased the lag phase of O_2 evolution from 3 to 1 min and significantly reduced the inhibition of O_2 evolution caused by addition of 3 mM $MgCl_2$ in the dark (Fig. 4). The results suggested that internal metabolite pools prevented Mg^{2+} inhibition, which was consistent with the observation that inhibition was lessened only by those metabolites that could be transported across the envelope.

Effects of Mg^{2+} and PGA on the Activation of Calvin Cycle Enzymes by Light. Previous work (12) suggested that Mg^{2+} inhibits O_2 evolution by blocking the light activation of certain Calvin cycle enzymes. The results were consistent with the earlier proposal of Lilley *et al.* (17) that Mg^{2+} may produce its effects indirectly by influencing the rate of formation of cycle intermediates such as hexose monophosphates. Prevention of Mg^{2+} inhibition raised the question of whether protective substrates allowed enzyme activation by light in the presence of Mg^{2+}, or whether increased metabolite pool sizes allowed greater flux through the Calvin cycle even though total enzyme activity was reduced. Illumination of intact barley chloroplasts in the absence of Mg^{2+} and substrates caused a 3.6-fold increase in activity of NADP-glyceraldehyde-3-P dehydrogenase (Table II). Light activation of this and other enzymes has been previously demonstrated *in vitro*

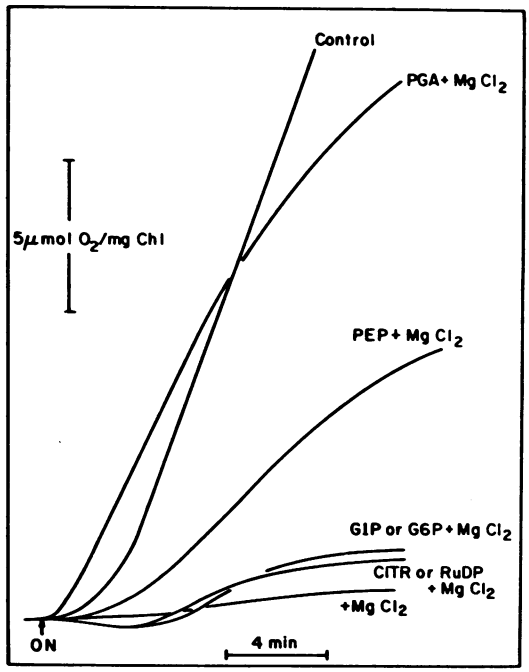

FIG. 3. Effect of various metabolites (0.5 mM) on O_2 evolution by barley chloroplasts in the presence of 2 mM $MgCl_2$.

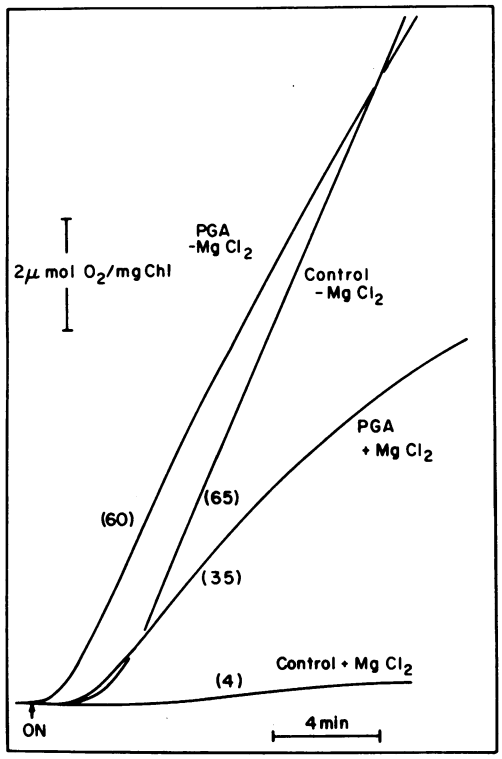

FIG. 4. Effect of preloading barley chloroplasts with PGA on O_2 evolution in the presence and absence of 3 mM $MgCl_2$. Maximum rates, expressed as μmol O_2 evolved/mg Chl·h, are shown parenthetically.

(1, 11, 12). Addition of 2 mM $MgCl_2$ in the dark to intact barley chloroplasts completely blocked the increase of enzyme activity by light. When chloroplasts were incubated in the dark with 2 mM $MgCl_2$ + 0.2 mM PGA, enzyme activity increased in the light and reached a maximum 2-fold stimulation after 12 min. Qualitatively similar results were obtained with P-ribulokinase and fructose-1,6-diphosphatase (Table II). With these enzymes, light activation

Table II. Effects of PGA and Mg^{2+} on the light activation of certain photosynthetic enzymes in intact barley chloroplasts.

Chloroplast treatment	Enzyme activity: NADP-glyceraldehyde-3-P dehydrogenase	Ribulose-5-P kinase	Fructose 1,6-diphosphatase
	(μmol/mg Chl·h)		
Dark	140	180	<2
Light[1]	520	590	22
Light[1] +4 mM $MgCl_2$	160	190	3
Light[1] +4 mM $MgCl_2$ +0.3 mM PGA	280	440	25

[1] 12 min illumination.

was approximately 4- and 20-fold, respectively, in the absence of PGA and Mg^{2+}. When $MgCl_2$ was added in the dark, light activation was blocked whereas in the presence of PGA + $MgCl_2$, activation was substantial. In the absence of Mg^{2+}, PGA had no effect on the maximum level of enzyme activity in the light (data not shown). With the chloroplasts used in the light activation experiments, the addition of 4 mM $MgCl_2$ in the dark decreased O_2 evolution from 140 to 4 μmol O_2/mg Chl·h and addition of 0.3 mM PGA increased the rate to 90 μmol O_2/mg Chl·h. The results clearly indicated that PGA prevents the Mg^{2+} blockage of enzyme activation by light.

The data did not explain how Mg^{2+} and substrates affected the process of enzyme activation. The results are consistent with the postulate that Mg^{2+} activates Pi exchange across the chloroplast envelope (11, 17) and new light was shed on the apparent anomaly that Mg^{2+} is required by various cytoplasmic enzymes yet is inhibitory to chloroplast photosynthesis (12). The results suggested that both needs could be met by maintaining sufficient pools of metabolites in the chloroplasts in the dark. Modulation of the level of cytoplasmic Mg^{2+} and chloroplast metabolite pools may regulate the activity of cytoplasmic enzymes and chloroplast photosynthesis by affecting the light activation of certain Calvin cycle enzymes.

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