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REQUIREMENT OF DIVALENT CATIONS FOR PHOTOACTIVATION OF THE LATEN WATER-OXIDATION SYSTEM IN INTACT CHLOROPLASTS FROM FLASHED LEAVES

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The effects of divalent cations on photoactivation of the latent water-oxidation system in intact chloroplasts isolated from wheat (*Triticum aestivum* L.) leaves grown under intermittent flash illumination were investigated by using A23187, an ionophore for divalent cations, and the following results were obtained. (a) Photoactivation in the intact chloroplasts was inhibited by A23187, but was restored on addition of a low concentration of Mn^{2+} (10 μM). (b) A high concentration of Mn^{2+} (70 μM) was inhibitory, in contrast, for photoactivation, but the inhibition was restored by the coexistence of a suitable concentration of Ca^{2+} (5 mM). (c) The Ca^{2+}-dependent restoration was inhibited by a high concentration of Mg^{2+} or Sr^{2+}, but the inhibition was restored by the coexistence of Ca^{2+}. (d) Kinetic analyses of these competitive effects between divalent cations revealed that: (i) High concentration of Ca^{2+} inhibits photoactivation in competition with Mn^{2+}. (ii) High concentration of Mn^{2+} inhibits photoactivation in competition with Ca^{2+}. (iii) High concentration of Mg^{2+} affects photoactivation by inhibiting Ca^{2+}-dependent restoration in competition with Ca^{2+}. Based on these results, we propose that the latent water-oxidation center has two binding sites, each specific for Mn^{2+} and Ca^{2+}, and that photoactivation takes place in the center having both Mn^{2+} and Ca^{2+} on their respective binding sites.

Introduction

A number of reports have indicated that manganese is closely associated with the photosynthetic water-oxidation system [1–6]. Cheniae and Martin [2] have demonstrated that algae grown in an Mn-deficient medium have their oxygen-evolving capacity specifically inhibited without affecting other photochemical activities, and restoration of the lost oxygen-evolving activity strictly depends on exogenous Mn^{2+} and light. On the other hand, reactivation of the water-oxidation system in NH_2OH-treated cells of *Anacystis* does not require exogenous Mn^{2+} but is strictly light dependent [7]. Similar photoactivation without exogenous Mn^{2+} is commonly observed for dark-grown algal cells [8], dark-grown gymnosperm leaves [9] and intermittently flashed angiosperm leaves [10]. These reports suggested that light for photoactivation is needed for incorporation of Mn^{2+} into the proper site on the water-oxidation enzyme, but not for the transport of Mn^{2+} into cells or chloroplasts. This view may be supported by the observation that activation of the oxygen-evolving system in Mn-depleted algal cells requires light even after accumulation of Mn^{2+} in cells via dark process [2,11]. Probably, the presence of Mn^{2+} at a suitable con-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Chl, chlorophyll; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

centration in the vicinity of the latent water-oxidation center is a requirement for the subsequent light-requiring process of photoactivation.

Kinetic analyses with multiple flashes applied to the photoactivation in Mn-deficient algal cells [2], dark-grown algal cells [7], intermittently flashed wheat leaves [12] and Tris-treated chloroplasts [13] have shown that the photoactivation requires a multiquantum process. The process is considered to be related to incorporation of manganese into the latent system [14]. However, the biochemical properties of the process remain unknown.

Yamashita and Tomita [4,15] have shown that the oxygen evolution inactivated by Tris-acetone treatment is restored in vitro by DCIP washing followed by illumination in the presence of dithiothreitol, Mn^{2+} and Ca^{2+}; other combinations of metals are ineffective. This is the only case in which Ca^{2+} is reported to be required for the activation of the latent or inactivated water-oxidation system.

Recently, we developed an experimental system to study photoactivation of the latent water-oxidation system by using intact chloroplasts isolated from wheat leaves grown under intermittent flashes [16]. This system enabled us to investigate the effect of various exogenous chemicals on the process. In this study, we investigated the effect of divalent cations on the process using A23187, an ionophore for divalent cations. As opposed to photoreactivation in Tris-inactivated chloroplasts, the photoactivation in the present system is insensitive to uncouplers, so that we could modulate the activity of divalent cations inside the intact chloroplasts by using the ionophore, A23187, which is known to be an effective uncoupler. Kinetic analyses revealed that the latent water-oxidation system has two binding sites for divalent cations specific for Mn^{2+} and Ca^{2+}, and the competitive relationships between several divalent cations on these sites are discussed.

Experimental Procedure

Protoplasts of wheat (*Triticum aestivum* L.) leaves greening under intermittent flash illumination were prepared according to the method previously described [16]. The protoplasts were washed twice with a medium containing 0.5 M sorbitol and 5 mM Mes-KOH, pH 6.1, and then resuspended in a small volume of a medium containing 0.4 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, DNAase (10 μg/ml) and 50 mM Hepes-NaOH, pH 7.5. Intact chloroplasts were prepared as previously described [16], but the sorbitol concentration was increased to 0.4 M from 0.33 M. The separated intact chloroplasts were used for experiments within 1 h after isolation.

Intact chloroplasts were suspended in a small volume of the above-mentioned resuspending medium (DNAase was omitted) at a chlorophyll concentration of 20 μg Chl/ml in a total volume of 0.5 ml. The reaction mixture was incubated in the dark at 20°C for 10 min after addition of various chemicals, and then illuminated with continuous light from an incandescent projector lamp (150 W) passing through an orange filter (Toshiba VO-56) and a 4 cm layer of 4% $CuSO_4$ solution at an intensity of 250 $\mu W/cm^2$.

The activity of DCIP photoreduction with water as electron donor was assayed spectrophotometrically as previously described [16]. Chlorophyll concentration was determined by the method of Arnon [18]. Trifluoperazine hydrochloride was a gift from Yoshitomi Seiyaku Co., Tokyo, Japan.

Results

Requirement of Mn^{2+} and Ca^{2+} for photoactivation

Table I shows the effects of various divalent cations on photoactivation in intact chloroplasts in the presence of A23187, an ionophore for divalent cations. Illumination of intact chloroplasts induced water-oxidation activity without any exogenous factors as previously reported [16]. The activity generation proceeded almost linearly during the first 8 min of illumination and reached a saturation level of about 300–600 μmol DCIP/mg Chl per h after 20 min illumination.

The presence of A23187 inhibited the generation of water-oxidation activity almost completely. The inhibition by A23187 was fully restored, however, when 10 μM Mn^{2+} was present, and the rate after restoration was notably higher than that of the control. This stimulation by exogenous Mn^{2+} was reproducibly observed, although the extent of stimulation fluctuated depending on the preparation. Other divalent cations tested, such as Mg^{2+},

TABLE I

EFFECT OF VARIOUS CATIONS ON PHOTOACTIVATION IN INTACT CHLOROPLASTS

Water-oxidation activity generated in the first 8 min of illumination is listed. The arrow denotes addition of $MnCl_2$ 10 min after the addition of A23187.

Conditions	Water-oxidation activity (μmol DCIP/mg Chl per h)
Nonilluminated	7
Illuminated (8 min)	
control	84
+ A23187 (10 μM)	7
+ A23187 + $MnCl_2$ (10 μM)	107
+ A23187 + $MgCl_2$ (10 μM)	8
+ A23187 + $CaCl_2$ (10 μM)	7
+ A23187 + $SrCl_2$ (10 μM)	7
+ A23187 + $BaCl_2$ (10 μM)	8
+ A23187 $\overset{10\ min}{\rightarrow}$ $MnCl_2$ (10 μM)	100

Ca^{2+}, Sr^{2+} and Ba^{2+} having an appreciable affinity for A23187, could not restore the inhibition by A23187. The restoration effect of Mn^{2+} was also observed when Mn^{2+} was added after 10 min preincubation with A23187. These results clearly indicate that Mn^{2+} is required for photoactivation and the inhibition by A23187 is mainly due to the release of Mn^{2+} from the intact chloroplasts by the action of this ionophore. The inhibition by A23187 did not require the coexistence of a chelating agent such as EDTA to trap the released metals. This implies that the content of Mn^{2+} in the intact chloroplasts is such that it is diluted enough by the action of A23187 to be no longer functional under the present experimental conditions. The stimulation of the photoactivation rate by exogenous Mn^{2+} may imply that the content of Mn^{2+} in intact chloroplasts is not always sufficient or partly lost during isolation procedure.

Table II shows the effect of divalent cations added to the A23187 system. As shown in Table I, photoactivation proceeded normally in the presence of A23187, when 10 μM Mn^{2+} was exogenously supplied. However, on increasing Mn^{2+} concentration up to 70 μM, an inhibition of DCIP rate was observed. This inhibition was overcome when 10 mM Ca^{2+} was present in the medium.

TABLE II

EFFECT OF VARIOUS CATIONS IN THE PRESENCE OF $MnCl_2$ ON PHOTOACTIVATION IN INTACT CHLOROPLASTS

Water-oxidation activity generated in the first 8 min of illumination is listed. The arrow denotes addition of $CaCl_2$ 10 min after the addition of A23187, $MnCl_2$ and $MgCl_2$.

Conditions	Water-oxidation activity (μmol DCIP/mg Chl per h)
Nonilluminated	7
Illuminated (8 min)	
control	84
+ A23187 (10 μM)	7
+ A23187 + $MnCl_2$ (10 μM)	107
+ A23187 + $MnCl_2$ + $MgCl_2$ (10 mM)	31
+ A23187 + $MnCl_2$ + $CaCl_2$ (10 mM)	117
+ A23187 + $MnCl_2$ + $SrCl_2$ (10 mM)	40
+ A23187 + $MnCl_2$ + $BaCl_2$ (10 mM)	84
+ A23187 + $MnCl_2$ + $MgCl_2$ (10 mM) $\overset{10\ min}{\rightarrow}$ $CaCl_2$ (10 mM)	107
+ A23187 + $MnCl_2$ (70 μM)	23
+ A23187 + $MnCl_2$ + $MgCl_2$ (10 mM)	17
+ A23187 + $MnCl_2$ + $CaCl_2$ (10 mM)	140
+ A23187 + $MnCl_2$ + $SrCl_2$ (10 mM)	18
+ A23187 + $MnCl_2$ + $BaCl_2$ (10 mM)	19

TABLE III

EFFECT OF EGTA ON PHOTOACTIVATION IN INTACT CHLOROPLASTS

Water-oxidation activity generated in the first 8 min illumination is listed.

Conditions	Water-oxidation activity (μmol DCIP/mg Chl per h)
Nonilluminated	0
Illuminated (8 min)	
control	91
+ A23187 (10 μM)	4
+ A23187 + $MnCl_2$ (10 μM)	143
+ A23187 + $MnCl_2$ + $CaCl_2$ (1 mM)	153
+ A23187 + $MnCl_2$ + EGTA (200 μM)	0
+ A23187 + $MnCl_2$ + EGTA + $CaCl_2$ (1 mM)	154

This restoration effect was specific for Ca^{2+}, whereas Mg^{2+}, Sr^{2+} and Ba^{2+} did not show any restoration, but were rather inhibitory. The inhibitory effect was pronounced for Mg^{2+} and was appreciable for Sr^{2+} and Ba^{2+}. The inhibition by 10 mM Mg^{2+} was completely restored by subsequent addition of 10 mM Ca^{2+}.

Table III shows the effect of EGTA on photoactivation. The photoactivation supported by 10 μM Mn^{2+} was again inhibited completely by 200 μM EGTA. The addition of 1 mM Ca^{2+} again restored the inhibition induced by EGTA. Judging from the high specificity of EGTA for Ca^{2+} within the pH range of this experiment, the EGTA inhibition must have resulted from trapping of endogenous Ca^{2+}. The complete inhibition by EGTA and marked recovery by exogenous Ca^{2+} may indicate that Ca^{2+} is one of the factors required for photoactivation as well as Mn^{2+}. In the absence of EGTA, a trace amount of endogenous Ca^{2+} may have supported the photoactivation, e.g., in the A23187 plus 10 μM Mn^{2+} system.

Kinetic analysis of the competition between divalent cations

Dependence on Mn^{2+} concentration. When the initial rate of photoactivation was determined at various concentrations of exogenous Mn^{2+} in the presence and absence of Ca^{2+}, the curves in Fig. 1 were obtained. In the absence of exogenous Ca^{2+}, the rate increased with increasing Mn^{2+} concentration to show the maximal rate near 5 μM Mn^{2+}, and then gradually dropped at higher concentrations (curve A). In the presence of 5 mM Ca^{2+}, however, the rate kept increasing up to higher Mn^{2+} concentrations of about 10 μM to show a saturation rate of about twice the maximal rate in the absence of Ca^{2+} (curve B). In the presence of higher concentration (25 mM) of Ca^{2+}, the rate was markedly suppressed, but kept increasing linearly with increasing Mn^{2+} concentration, showing no saturation in the concentration range examined (curve C).

The drop of curve A at high Mn^{2+} concentrations in the absence of exogenous Ca^{2+} corresponds to the inhibition by high Mn^{2+} concentrations, and the stimulation in the presence of 5 mM Ca^{2+} corresponds to Ca^{2+}-dependent restoration of the inhibition induced by high Mn^{2+} concentration (Table II). Besides these phenomena, suppression of the rate shown by curve C seems to indicate that higher concentration of Ca^{2+} is also inhibitory to the process and the inhibition is restored gradually by increasing Mn^{2+} concentration. It appears, hence, that both Mn^{2+} and Ca^{2+} function not only as the factors essential for the process but also effect inhibition and restoration mutually.

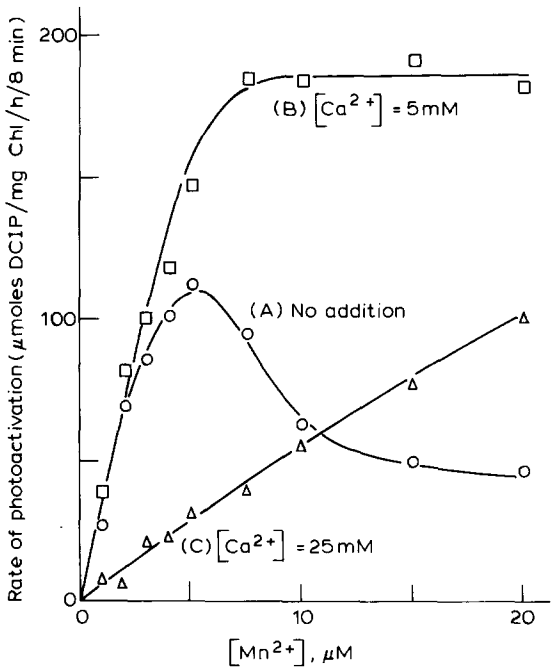

Fig. 1. Dependence of the initial rate of photoactivation on Mn^{2+} concentration in the presence of various concentrations of Ca^{2+}. (○) 25 mM Ca^{2+}, (□) 5 mM Ca^{2+}, (△) no addition. The initial rates were estimated from the activity increments in the first 8 min of continuous illumination of isolated intact chloroplasts. The reaction mixture contained 10 μM A23187

In order to explain these phenomena as a whole, we postulated that the process of photoactivation is made up of the following reactions:

$$\bigcirc E\bullet + Ca^{2+} \overset{K_{Ca}}{\rightleftharpoons} \bigcirc ECa \quad (1)$$

$$\bigcirc E\bullet + Mn^{2+} \overset{K_{Mn}}{\rightleftharpoons} MnE\bullet \quad (2)$$

$$\bigcirc ECa + Ca^{2+} \overset{K_{Ca\cdot Ca}}{\rightleftharpoons} CaECa \quad (3)$$

$$MnE\bullet + Mn^{2+} \overset{K_{Mn\cdot Mn}}{\rightleftharpoons} MnEMn \quad (4)$$

$$\circ ECa + Mn^{2+} \overset{K_{Ca\cdot Mn}}{\rightleftharpoons} MnECa \xrightarrow[\text{light}]{k} MnE^{*}Ca$$

$$(\text{or } MnE^{*}\bullet + Ca^{2+}) \quad (5)$$

$$MnE\bullet + Ca^{2+} \overset{K_{Mn\cdot Ca}}{\rightleftharpoons} MnECa \xrightarrow[\text{light}]{k} MnE^{*}Ca$$

$$(\text{or } MnE^{*}\bullet + Ca^{2+}) \quad (6)$$

where $\circ E\bullet$ is the latent system in which open and solid circles represent the binding sites for Mn^{2+} and Ca^{2+}, respectively; the K terms with various subscripts are the dissociation constants between the latent system and respective cations; k is the rate constant of the final activation step driven by light; and E* is the activated system. It is assumed in this scheme that: (i) the latent system has two binding sites, each specific for Mn^{2+} and Ca^{2+}; (ii) both cations have affinity for the two binding sites; and (iii) photoactivation proceeds independently only in the latent centers having both cations on their respective sites.

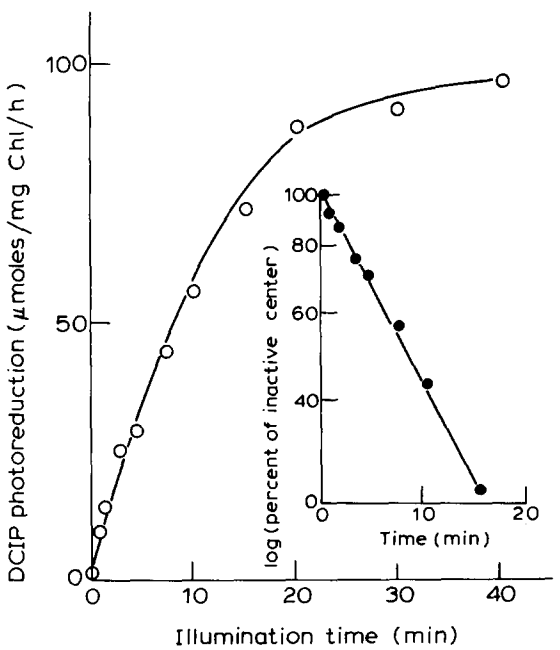

Fig. 2. Time course of photoactivation induced by continuous light illumination of the isolated intact chloroplasts. The inset shows a semilogarithmic plot of the time course.

The last assumption may be reasonable from the result of the experiment of Fig. 2, which shows the time course of photoactivation. On illumination, the water-oxidation activity was rapidly induced and reached saturation after 20 min. The inset in Fig. 2 shows a semilogarithmic plot of the process in which the logarithm of the portion of the water-oxidation system remaining latent was plotted against illumination time. The resulting straight line implies that photoactivation is a first-order process, indicating that the above assumption is reasonable.

Based on these assumptions, the initial rate of photoactivation, v, is expressed as follows:

$$v = Ak[\text{MnECa}] \quad (7)$$

where [MnECa] is the concentration of the latent system ready to be activated by illumination, and A is an independent constant.

When Eqns. 1–7 are resolved as a function of Mn^{2+} concentration under the conditions that Ca^{2+} concentration is relatively high while Mn^{2+} concentration is not so high as to undergo inhibition, the following equation is derived (Eqn. 4 is neglected):

$$\frac{1}{v_0} = \frac{K_{Ca\cdot Mn}}{V_1}\left(1 + \frac{K_{Ca}}{[Ca^{2+}]} + \frac{[Ca^{2+}]}{K_{Ca\cdot Ca}}\right)\frac{1}{[Mn^{2+}]} + \frac{1}{V_1}\left(\frac{K_{Mn\cdot Ca}}{[Ca^{2+}]} + 1\right) \quad (8)$$

provided:

$$V_1 = Ak([\circ E\bullet] + [\circ ECa] + [MnE\bullet] + [CaECa] + [MnECa])$$

where square brackets denote the concentration of respective cations and those of the latent systems with cations, and v_0 is the observed initial velocity of photoactivation. This equation suggests that at high Ca^{2+} concentrations where K_{Ca} and $K_{Mn\cdot Ca}$ are negligible compared to $[Ca^{2+}]$, the double-reciprocal (Lineweaver-Burk) plot will result in a set of straight lines with different inclinations depending on $[Ca^{2+}]$ with an intersecting point on the ordinate.

The plot of Fig. 1 data is shown in Fig. 3. In the presence of exogenous Ca^{2+}, the plot resulted in two straight lines (B and C) having different

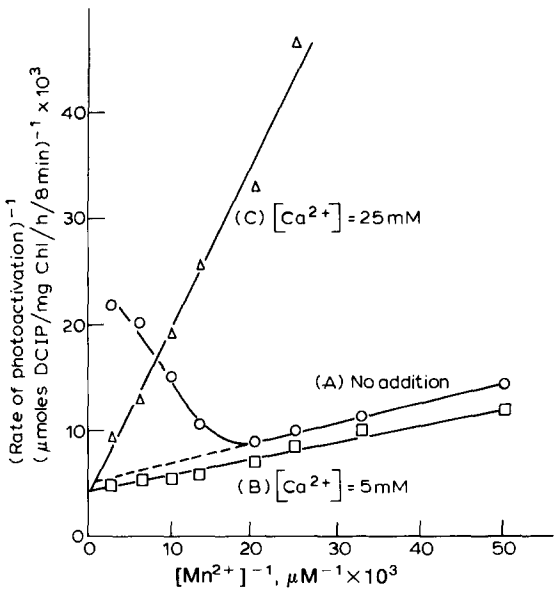

Fig. 3. Double-reciprocal plot of Fig. 1 data. (○) 25 mM Ca^{2+}, (□) 5 mM Ca^{2+}, (△) no addition.

inclinations dependent on Ca^{2+} concentration with a common intersecting point on the ordinate. $K_{Ca \cdot Mn}$ and $K_{Ca \cdot Ca}$ values estimated under this condition with some assumptions (see Discussion) were about 40 μM and 3.0 mM, respectively. These results are in good agreement with the above suggestion and indicate that Ca^{2+} inhibits photoactivation in competition with Mn^{2+} with an apparent inhibition constant of 3.0 mM ($K_{Ca \cdot Ca}$).

As opposed to the plot in the presence of exogenous Ca^{2+}, the plot in the absence of exogenous Ca^{2+} showed a straight line at low Mn^{2+} concentrations with a slightly higher inclination and intersecing point on the ordinate compared to the plot in the presence of 5 mM Ca^{2+}, but showed a marked deviation from the straight line at higher Mn^{2+} concentrations (curve A). It must be noted that the absence of exogenous Ca^{2+} does not necessarily mean the complete depletion of Ca^{2+} from the reaction mixture; unless EGTA is present endogenous Ca^{2+} might support photoactivation as shown by the experiment of Table III. Possibly, the slightly higher inclination and intersecting point obtained by extrapolating the straight part of curve A are attributable to the contributions of $K_{Ca}/[Ca^{2+}]$ and $K_{Mn \cdot Ca}/[Ca^{2+}]$, respectively, at low Ca^{2+} concentrations, and the deviation from Eqn. 8 at higher Mn^{2+} concentrations implies that Eqn. 4 can no longer be negligible if Mn^{2+} concentration is higher than 5 μM in the absence of exogenous Ca^{2+}.

Dependence on Ca^{2+} concentration. As shown by Table II and Fig. 1, Ca^{2+} restored the photoactivation inhibited by high Mn^{2+} concentration. In the presence of inhibitory concentrations of Mn^{2+} and a suitable concentration of Ca^{2+} not so high as to undergo inhibition, the dependence of photoactivation rate on Ca^{2+} concentration, namely, the extent of restoration, is expressed by the following equation (Eqn. 3 is neglected):

$$\frac{1}{v_0} = \frac{K_{Mn \cdot Ca}}{V_2}\left(1 + \frac{K_{Mn}}{[Mn^{2+}]} + \frac{[Mn^{2+}]}{K_{Mn \cdot Mn}}\right)\frac{1}{[Ca^{2+}]} + \frac{1}{V_2}\left(\frac{K_{Ca \cdot Mn}}{[Mn^{2+}]} + 1\right) \quad (9)$$

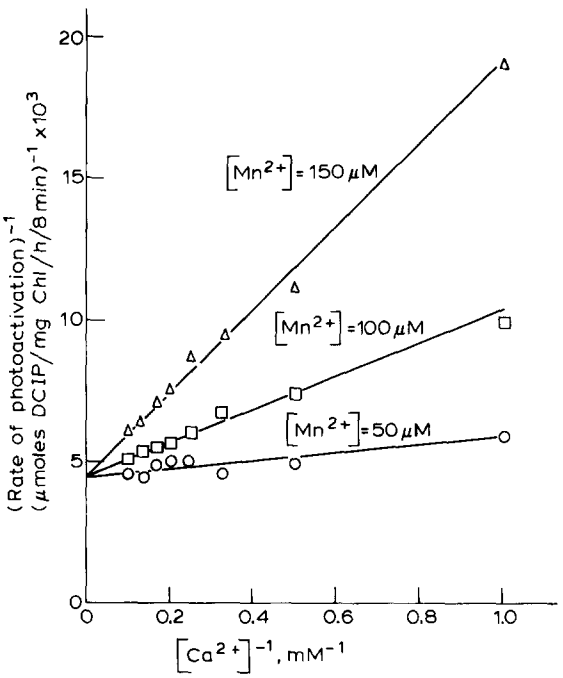

Fig. 4. Linear relationship between the reciprocal of Ca^{2+} concentration and the reciprocal of the initial rate of photoactivation in the presence of various concentrations of Mn^{2+}. (○) 50 μM Mn^{2+}, (□) 100 μM Mn^{2+}, (△) 150 μM. The initial rates were estimated from the activity increments in the first 8 min of continuous illumination of the isolated intact chloroplasts. The reaction mixture contained 10 μM A23187.

provided:

$$V_2 = Ak([\bigcirc E\bullet] + [\bigcirc ECa] + [MnE\bullet] + [MnEMn] + [MnECa])$$

This equation suggests that at high Mn^{2+} concentrations where K_{Mn} and $K_{Ca \cdot Mn}$ are negligibly small compared to $[Mn^{2+}]$, the double-reciprocal plot (Lineweaver-Burk) of v_0 versus Ca^{2+} concentration will result in a set of straight lines having different inclinations dependent on Mn^{2+} concentration with a common intersecting point on the ordinate.

Fig. 4 shows the result of such examination, in which the restoration by Ca^{2+} was measured by changing Ca^{2+} concentration. The double-reciprocal plot resulted in a set of straight lines having different inclinations dependent on Mn^{2+} concentration but with a common intersecting point on the ordinate independent of Mn^{2+} concentration. Since Ca^{2+}-dependent restoration could be observed only in the presence of high concentration of Mn^{2+}, calculation of $K_{Ca \cdot Mn}$ and $K_{Mn \cdot Mn}$ values was impossible. However, these results indicate that high Mn^{2+} concentration inhibits photoactivation in competition with Ca^{2+}.

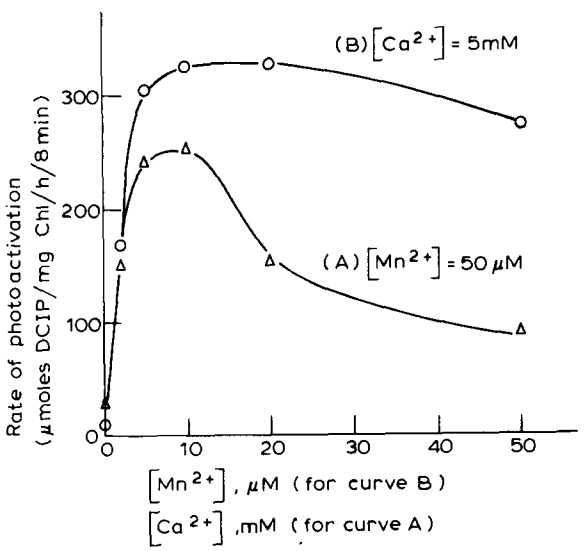

Fig. 5. Mutual effect of Mn^{2+} and Ca^{2+} concentration on photoactivation in isolated intact chloroplasts. The initial rate of photoactivation during the first 8 min of illumination was measured by changing Ca^{2+} concentration in the presence of 50 μM Mn^{2+} (curve A), and by changing Mn^{2+} concentration in the presence of 5 mM Ca^{2+} (curve B). The reaction mixture contained 10 μM A23187.

The above competitive relationship between Mn^{2+} and Ca^{2+} is more clearly demonstrated by Fig. 5. In the experiment, the initial rate of photoactivation in the presence of exogenous Mn^{2+} was measured by changing Ca^{2+} concentration, and the rate in the presence of exogenous Ca^{2+} was measured by changing Mn^{2+} concentration. As shown by curve A, the rate in the presence of 50 μM Mn^{2+} was very low when Ca^{2+} was not exogenously supplied. On addition of Ca^{2+}, the rate steeply increased to show the maximum value around 10 mM Ca^{2+}, and then decreased with further increasing Ca^{2+} concentration. The steep rise of curve A at low Ca^{2+} concentrations implies the restoration by Ca^{2+} of the inhibition by high Mn^{2+} concentration on the binding site for Ca^{2+}, and the gradual drop at higher Ca^{2+} concentrations implies the manifestation of competitive inhibition by Ca^{2+} on the binding site for Mn^{2+}. Curve B shows the result of similar titration against Mn^{2+} concentration in the presence of 5 mM Ca^{2+}. When Mn^{2+} was not present, the rate was very low because of Mn^{2+} deficiency. On addition of Mn^{2+}, the rate steeply increased to show the maximum at Mn^{2+} concentration of 10 μM. On further increasing Mn^{2+} concentration, a slight but significant decrease in the rate occurred at 50 μM Mn^{2+}. This is probably caused by the competitive inhibition by Mn^{2+} on the binding site for Ca^{2+}.

Dependence on Mg^{2+} concentration. As was also shown by Table II, high concentration of Mg^{2+} was inhibitory for photoactivation when exogenous Mn^{2+} was present but exogenous Ca^{2+} was absent, and the inhibition was restored by addition of Ca^{2+}. This suggested that Mg^{2+} affected photoactivation by competitively binding to the site for Ca^{2+}. In order to analyze this competition, three more reactions as follows were assumed:

$$\bigcirc E\bullet + Mg^{2+} \overset{K_{Mg}}{\rightleftharpoons} \bigcirc EMg \quad (10)$$

$$MnE\bullet + Mg^{2+} \overset{K_{Mn \cdot Mg}}{\rightleftharpoons} MnEMg \quad (11)$$

$$\bigcirc EMg + Mn^{2+} \overset{K_{Mg \cdot Mn}}{\rightleftharpoons} MnEMg \quad (12)$$

When these equations are resolved together with Eqns. 1–7 as a function of Mg^{2+} concentration under the condition that Mn^{2+} concentration is high enough to undergo inhibition whereas Ca^{2+} concentration is suitable to undergo restoration, the following equation is derived (Eqn. 3 is neglected):

$$\frac{1}{v_0} = \frac{1}{V_3}\left[1 + \frac{K_{Mn\cdot Ca}}{[Ca^{2+}]}\left(1 + \frac{K_{Mn}}{[Mn^{2+}]} + \frac{[Mn^{2+}]}{K_{Mn\cdot Mn}}\right) + \frac{K_{Ca\cdot Mn}}{[Mn^{2+}]}\right] + \frac{1}{V_3}\cdot\frac{K_{Mn\cdot Ca}}{[Ca^{2+}]}\left(\frac{1}{K_{Mn\cdot Mg}} + \frac{K_{Mn}}{[Mn^{2+}]\cdot K_{Mg}} + \frac{K_{Mn}}{K_{Mg}\cdot K_{Mg\cdot Mn}}\right)[Mg^{2+}] \quad (13)$$

provided

$$V_3 = Ak([\bigcirc E\bullet] + [\bigcirc ECa] + [MnE\bullet] + [MnEMn] + [MnECa] + [\bigcirc EMg] + [MnEMg])$$

This equation suggests that the reciprocal of v_0 plotted against Mg^{2+} concentration (Dixon plot) will result in a set of straight lines having different inclinations and different intersecting points with the ordinate both dependent on Ca^{2+} and Mn^{2+} concentrations, but the lines cross each other at one point.

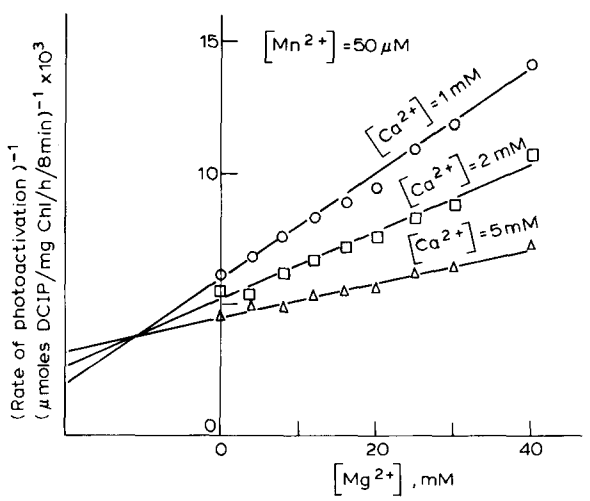

Fig. 6. Linear relationship between Mg^{2+} concentration and the reciprocal of the initial rate of photoactivation in the presence of 50 μM Mn^{2+} and various concentrations of Ca^{2+}. (○) 1 mM Ca^{2+}, (□) 2 mM Ca^{2+}, (△) 5 mM Ca^{2+}. The initial rates were estimated from the activity increments in the first 8 min of continuous illumination of the isolated intact chloroplasts. The reaction mixture contained 10 μM A23187

Fig. 6 shows the result of such examination, in which the extent of Mg^{2+}-induced inhibition was measured by changing Mg^{2+} concentration in the presence of a certain high concentration of Mn^{2+} (50 μM) and three different concentrations of Ca^{2+} to restore the inhibition induced by Mn^{2+}. The plot resulted in a set of straight lines having three different inclinations dependent on Ca^{2+} concentration, each crossing the ordinate at different point but crossing each other at a single intersecting point. The apparent inhibition constant estimated from the crossing point was about 10 mM. The results completely coincide with the content of Eqn. 13, and indicate clearly that Mg^{2+} inhibits, in competition with Ca^{2+}, the Ca^{2+}-dependent restoration of the inhibition induced by high concentration of Mn^{2+}.

Discussion

The present study revealed that photoactivation of the latent water-oxidation system in intact chloroplasts isolated from wheat leaves grown under intermittent flashes requires Ca^{2+} in addition to Mn^{2+} and light. The crucial evidence for the involvement of Ca^{2+} is: (i) Ca^{2+} restores photoactivation inhibited by high concentration of Mn^{2+} and (ii) chelation of Ca^{2+} by EGTA inhibits photoactivation and the inhibition is restored by addition of exogenous Ca^{2+}. Based on these observations and other competitive relations observed between Ca^{2+}, Mn^{2+} and Mg^{2+}, we considered that the process of photoactivation consists of the reactions represented by Eqns. 1–6 and 10–12.

Mutual competition between Mn^{2+} and Ca^{2+}

The double-reciprocal plot of v_0 vs. [Mn^{2+}] resulted in striaght lines with an intersecting point on the ordinate (Fig. 3), and that of v_0 vs. [Ca^{2+}] also resulted in straight lines with an intersecting point on the ordinate (Fig. 4). These results indicated clearly that both Mn^{2+} and Ca^{2+} are the factors essential for the process but are simultaneously competitive inhibitors for each other.

In Eqn. 8, $K_{Ca\cdot Mn}$ and $K_{Ca\cdot Ca}$ represent the

dissociation constants of Mn^{2+} and Ca^{2+}, respectively, on the site for Mn^{2+}. Determination of these values from the plot in Fig. 3 is difficult; the plot in the complete absence of the inhibitor (Ca^{2+}) is not available, since Ca^{2+} is an essential factor, and both the intersection point and inclination are dependent on [Ca^{2+}] at low Ca^{2+} concentrations. However, assuming that 5 mM Ca^{2+} practically does not affect the process in the experiment of Fig. 1, we can expediently calculate $K_{Ca \cdot Mn}$ to be about 40 μM (at most), and using this value $K_{Ca \cdot Ca}$ can be calculated to be about 3.0 mM (at least). The $K_{Ca \cdot Ca}$ value is not always consistent with the above assumption, but we can determine that the Mn^{2+} site has a stronger affinity for Mn^{2+} than Ca^{2+} by a factor of more than 10^2.

In Eqn. 9, $K_{Mn \cdot Ca}$ and $K_{Mn \cdot Mn}$ represent the dissociation constants of Ca^{2+} and Mn^{2+}, respectively, on the site for Ca^{2+}. Estimation of these values from the plots in Fig. 4 is much more difficult, since Mn^{2+} inhibition occurs at much lower concentrations than Ca^{2+} inhibition, so that all the measurements in the presence of Mn^{2+} are more or less affected by the inhibition effect of Mn^{2+}. In fact, the inhibition by Mn^{2+} is appreciable even at 5 μM Mn^{2+}; the photoactivation rate in the absence of exogenous Ca^{2+} was appreciably lower than that in the presence of 5 mM Ca^{2+} (Fig. 1). Judging from this fact, we may assume that the affinity of the Ca^{2+} site for Mn^{2+} is comparable with that for Ca^{2+}.

Although the quantitative analysis is difficult, the specificities of the two sites are thus qualitatively different; the Mn^{2+} site has a high specificity for Mn^{2+} with less than 1% affinity for Ca^{2+}, while the Ca^{2+} site has a low specificity, almost comparable affinity to both Ca^{2+} and Mn^{2+}. The inhibition induced by high concentration of Mn^{2+} and its restoration dependent on rather high concentration of Ca^{2+} are in good agreement with the view of two sites having different specificity. By assuming the presence of two binding sites for Mn^{2+} and Ca^{2+} on the latent water-oxidation system, both specific for respective cations but cross-reacting mutually, we can thus explain the mutual competitive behavior of the two divalent cations. The mutual competitive relations are demonstrated by the two curves in Fig. 5.

Competition between Ca^{2+} and Mg^{2+}

Mg^{2+} and Sr^{2+} showed inhibition in the presence of high concentration of Mn^{2+} and low concentration of Ca^{2+} (Table II). This is the condition under which Mn^{2+} inhibition is predominant and Ca^{2+}-dependent restoration is expected. Since Mg^{2+} inhibition was observed only under such conditions, Mg^{2+} must have affected the process through inhibition of Ca^{2+}-dependent restoration by binding to the Ca^{2+} site in competition with Ca^{2+}. This view is supported by the fact that the inhibition by Mg^{2+} was restored by the coexistence of Ca^{2+} but not by that of Mn^{2+}.

The plot in Fig. 6 resulted in straight lines with a singel intersection point, which indicates clearly that Mg^{2+} inhibits Ca^{2+}-dependent restoration of the inhibition induced by high Mn^{2+} concentration. Similar effects were found for Sr^{2+} and Ba^{2+} as well. The apparent inhibition constant was calculated to be about 10 mM under the present experimental condition. It is of note, however, that the constant is dependent also on Mn^{2+} concentration, and experiments at different Mn^{2+} concentrations will give different values.

Mode of action of Ca^{2+}

It was thus shown in the present study that both Mn^{2+} and Ca^{2+} are required for photoactivation. Of the two divalent cations, Mn^{2+} is known to be closely related to the function of water oxidation, and is considered to be associated with the water-oxidizing enzyme. In fact, Mn^{2+} loosely bound to the latent system before photoactivation becomes tightly bound to the system, becoming resistant to A23187 after photoactivation [16]. As for Ca^{2+}, however, there is no experimental evidence that it plays some role in water oxidation. Yamashita and Tomita [15] have reported the requirement of Ca^{2+} for the photoreactivation of Tris-inactivated oxygen-evolving activity. They interpreted that Ca^{2+} is required for generating a high-energy state in thylakoids [21]. This view, however, may not fit the photoactivation we discussed in this paper, since the photoactivation in flashed leaves was not inhibited by uncouplers or inhibitors of ATP formation [16]. The role of Ca^{2+} in photoactivation of the latent system in flashed leaves appears to be somewhat different from that in photoreactivation in Tris-inactivated chloro-

plasts. As far as the present system is concerned, the site of action of Ca^{2+} seems to be more closely or directly related to that of Mn^{2+}. The competitive interaction of Ca^{2+} with Mn^{2+} may suggest that Ca^{2+} is incorporated in the water-oxidation enzyme as well as Mn^{2+}.

In order to obtain more clues as to the role of Ca^{2+} in photoactivation, the effect of calmodulin antagonists was investigated. As shown in Table IV, the presence of trifluoperazine, chloropromazine or *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) during illumination considerably inhibited photoactivation, while addition of these chemicals after photoactivation did not affect the activity generated beforehand. These chemicals have been reported to inhibit preferentially the calmodulin-dependent processes [19,20], so that the data shown in Table IV might suggest that calmodulin is involved in the Ca^{2+}-dependent process. The inhibitions observed here may not have resulted from the inhibitory action of these chemicals on Photosystem II electron transport, such as reported by Barr et al. [24], since, post-addition of these chemicals did not affect the generated activity at all.

It is too premature to conclude only from these observations that calmodulin is involved in the process. It is of note, however, that the properties of the Ca^{2+}-binding site of the latent water-oxidation system discussed above are somewhat similar to those reported for calmodulin; having a high affinity for Mn^{2+} and medium affinity for Mg^{2+} and Sr^{2+} [22,23].

TABLE IV

EFFECT OF CALMODULIN ANTAGONISTS ON PHOTOACTIVATION IN INTACT CHLOROPLASTS

Water-oxidation activity generated in the first 8 min of illumination is listed. Pre- and post-addition: the chemicals were added before and after illumination for photoactivation, respectively. The result of a separate experiment is shown in parentheses.

Conditions	Water-oxidation activity (μmol DCIP/mg Chl per h)	
	Pre-addition	Post-addition
Nonilluminated	0 (6)	
Illuminated (8 min)		
control	68(65)	–
+ trifluoperazine (100 μM)	46	93
+ chloropromazine (100 μM)	31	69
+ *N*-(6-aminohexyl)-5-chloro-naphthalenesulfonamide (W-7) (100 μM)	(28)	(66)

Concluding remarks

Summarizing the above results and discussion, we would propose a model scheme for the role of divalent cations in photoactivation of the latent water-oxidation system as shown by Fig. 7. The essential points of the scheme are: (i) The latent water-oxidation system possesses two distinguishable binding sites for divalent cations, one for Mn^{2+} and the other for Ca^{2+}; (ii) photoactivation proceeds only when the latent system having both Mn^{2+} and Ca^{2+} on their respective specific binding sites is illuminated; (iii) Mn^{2+} and Ca^{2+} cross-interact with the two sites in competition with each other, while Mg^{2+} interacts with the Ca^{2+} site but not with the Mn^{2+} site.

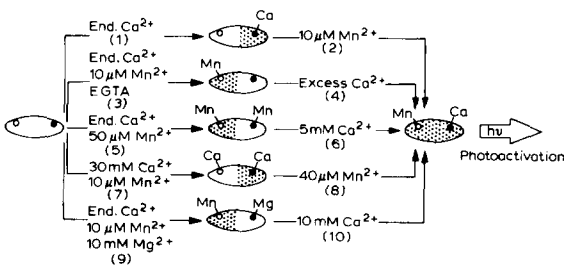

Fig. 7. Possible scheme for the role of divalent cations during photoactivation of the latent water-oxidation system. The open ellipse with open and solid circles represents the latent water-oxidation system with binding sites for Mn^{2+} and Ca^{2+}, respectively, the fully shadowed ellipse for the system with both Mn^{2+} and Ca^{2+} ready to be activated by illumination. The ellipse partly shadowed stand for the system having either Mn^{2+} or Ca^{2+} on its proper binding site. The numbers in parentheses are the following phenomena observed in this study: [1, 2] Photoactivation supported by endogenous (End.) Ca^{2+} and low concentration of Mn^{2+}; [3, 4] inhibition by EGTA and its restoration by excess Ca^{2+}; [5, 6] inhibition by high concentration of Mn^{2+} and its restoration by Ca^{2+}; [7, 8] inhibition by high concentration of Ca^{2+} and its restoration by Mn^{2+}; [9, 10] inhibition by Mg^{2+} and its restoration by Ca^{2+}.

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