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$CaCl_2$ inhibition of H_2O_2 electron donation to photosystem II in submembrane preparations depleted in extrinsic polypeptides

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The interaction of $CaCl_2$ and H_2O_2 was studied in photosystem II (PSII) enriched submembrane preparations depleted in the extrinsic polypeptides associated with oxygen evolution In PSII preparations, depleted of 16 and 23 kDa polypeptides, the addition of exogenous $CaCl_2$ substantially stimulated the rate of H_2O oxidation but had no effect on the rate of H_2O_2 oxidation In PSII preparations depleted of 16, 23 and 33 kDa polypeptides, addition of $CaCl_2$ strongly inhibited the H_2O_2 oxidation when mediated via exogenous Mn^{2+} The inhibition kinetics of H_2O_2 oxidation by $CaCl_2$ in these PSII preparations were negatively correlated with the retention of native Mn-atoms in the PSII core complex These results suggest that removal of 16 and 23 kDa extrinsic polypeptides from the PSII oxygen-evolving complex causes disorganisation of Ca^{2+} and Cl^- and allows H_2O_2 to undergo oxidation and to donate electrons to P680 via the native Mn-cluster and/or exogenous Mn^{2+} However, readdition of Ca^{2+} and Cl^- to the depleted preparations restores the native conformation of the PSII core complex, consequently inhibiting H_2O_2 oxidation

Calcium chloride, Hydrogen peroxide, Photosystem II, Electron transport, Polypeptide, extrinsic

1. INTRODUCTION

Ca^{2+} and Cl^- are considered to be the essential cofactors for photosynthetic water oxidation (for a review see [1]). Removal of the 16 and 23 kDa extrinsic polypeptides from the PSII-OEC by NaCl washing of PSII particles or inside out thylakoids is considered to disorganize the binding of these two ions within the PSII core complex [2–6]. It has been reported that depletion of the above two polypeptides from PSII particles inhibits 70 to 80% of oxygen-evolving capacity [2], but readdition of Ca^{2+} and Cl^- to the protein-depleted preparations substantially restores the oxygen evolution activity [2,7–10]. Therefore, it was reported that Ca^{2+} and Cl^- can stimulate water oxidation even in the absence of 16 and 23 kDa polypeptides [2,6].

In the oxygen-evolving complex, two water molecules are oxidized to one dioxygen molecule. However, Kelly and Izawa [11] have reported that H_2O_2 can be used as an electron donor in chloride-depleted thylakoid membranes, unable to catalyse the oxidation of H_2O. This was confirmed by Sandusky and Yocum who have shown that H_2O_2 oxidation by these thylakoid membranes is mediated by a pool of free or loosely bound Mn^{2+} [12]. In that respect, several lines of evidence have also indicated that H_2O_2 was able to undergo oxidation in PSII-enriched submembrane fractions, provided that exogenous Mn^{2+} was added [13–15].

Recently, Schroder and Åkerlund [3] have reported from their oxygen flash yield experiments, that H_2O_2 can act as an electron donor only in PSII preparations depleted in 16 and 23 kDa extrinsic polypeptides. They considered these two polypeptides to act as a shielding barrier for H_2O_2 accessibility to the PSII donor side. In the present paper we show that it is not the removal of 16 and 23 kDa polypeptides that enables the H_2O_2 accessibility to the PSII donor side; rather, it is electron donation to P680 mediated by either the native Mn-cluster or by exogenously added Mn^{2+} that is inhibited by Ca^{2+} and Cl^-.

2. MATERIALS AND METHODS

Oxygen-evolving PSII submembrane fractions were isolated from spinach following a modification of [16] Deveined leaves were homogenized in a medium containing 50 mM tricine-NaOH (pH 7 6), 10 mM NaCl, 5 mM $MgCl_2$, 0 4 M sorbitol, 6 mM ascorbate and 1 mM PMSF The homogenate was filtered through 12 layers of cheesecloth and the filtrate was centrifuged for 5 min at 2000 × *g* The pellet was suspended in the same buffer but without sorbitol and PMSF and then recentrifuged under the same conditions The resulting pellet was resuspended in a buffer containing 20 mM Mes-NaOH (pH 6 5), 15 mM NaCl, 10 mM $MgCl_2$ and 4% Triton X-100 with a chlorophyll concentration of 1 mg/ml After an incubation of 20 min in the dark at ice-cold temperature with continuous stirring, the mixture was centrifuged for 10 min at 3600 × *g* The PSII particles

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Abbreviations PSII, photosystem II, PMSF, phenylmethyl-sulfonyl fluoride, Mes, 2-(*N*-morpholino)ethanesulfonic acid, Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; OEC, oxygen-evolving complex

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were collected from the supernatant by centrifugation for 30 min at 36 000 × g and resuspended in the same buffer (without Triton X-100) at a Chl concentration of 2 mg/ml Chlorophyll was determined according to [17]

The treatment of PSII particles with NaCl or with Tris-NaCl was carried out according to [2] $CaCl_2$-treatment was done as described by Ono and Inoue [18] After the treatments, the particles were washed twice with 20 mM Mes-NaOH (pH 6 5) and finally suspended in the same medium

DCIP photoreduction was measured at 600 nm with a UV/VIS-spectrophotometer (Perkin-Elmer, model 553) The reaction medium (30 μM DCIP and 5 μg Chl/ml) was illuminated in a 3 ml cuvette with the maximum intensity of a 150 W quartz halogen projector lamp This activating light beam was passed through a 3 cm water filter and through Schott RG665, and Ealing 35-6857 cut-off filters The phototube was protected by a red cut-off filter (Ealing 35-5396 RTB)

3. RESULTS AND DISCUSSION

In order to elucidate the relationships of the 3 extrinsic polypeptides (16, 23 and 33 kDa) with Ca^{2+}, Cl^- and Mn^{2+} in the PSII-OEC and their interactions with H_2O_2, the following 3 types of PSII preparations were used: (1) NaCl-treated PSII particles in which 16 and 23 kDa polypeptides are depleted [2,7,8]. This preparation retains about 20–30% oxygen-evolving capacity. However, readdition of high concentration of Ca^{2+} and Cl^- are necessary in order to stimulate the electron transport activity; (2) $CaCl_2$-treated PSII particles in which the 16, 23 and 33 kDa extrinsic polypeptides are depleted [18,20,21]. These do not retain oxygen-evolving capacity. Addition of either H_2O_2 or H_2O_2 plus Mn^{2+} is necessary to stimulate electron transport activity; (3) Tris-NaCl-treated PSII particles in which the 3 extrinsic polypeptides are depleted [2,22,23]. These particles also do not retain oxygen-evolving capacity. Addition of H_2O_2 plus Mn^{2+} is necessary in order to stimulate electron transport activity.

Table 1 shows the effects of various additions on the stimulation of electron transport in the above 3 types of PSII preparations. It was observed that in the NaCl-treated PSII particles, where 16 and 23 kDa polypeptides are depleted and the Ca^{2+} and Cl^- interaction is disorganized, H_2O_2 alone can stimulate electron transport. This indicates that H_2O_2 can undergo oxidation and donate electrons to the native Mn-cluster (native Mn-cluster catalyses the oxidation of H_2O) in this type of preparation. However, addition of exogenous Mn^{2+} together with H_2O_2 greatly stimulated the electron transport. Likewise, addition of exogenous $CaCl_2$ to such a preparation substantially stimulated the rate of water oxidation. On the other hand, when $CaCl_2$ together with H_2O_2 were added, electron transport was not accelerated to the same extent as with $CaCl_2$ alone Therefore, in the presence of H_2O_2, stimulation of water oxidation by $CaCl_2$ is inhibited.

In order to understand further the $CaCl_2$ interaction with H_2O_2, we compared the rate of electron transport in the NaCl-treated preparations with the presence of either exogenous $MnCl_2$ (or $Mn(NO_3)_2$) together with

Table 1

Effects of various additives on stimulation of DCIP photoreduction in PSII submembrane preparations depleted in extrinsic polypeptides

Addition	DCIP photoreduction (μmol/mg Chl h)		
	NaCl-treated	$CaCl_2$-treated	Tris-NaCl-treated
None	29	5	5
H_2O_2	80	74	5
NaCl	58	10	5
NaCl + H_2O_2	80	57	5
$CaCl_2$	115	20	5
$CaCl_2$ + H_2O_2	86	20	5
$MnCl_2/Mn(NO_3)_2$	29	7	5
$MnCl_2/Mn(NO_3)_2$ + H_2O_2	160	126	109
$MnCl_2$ + $CaCl_2$	100	–	–
$MnCl_2$ + H_2O_2 + $CaCl_2$	98	57	29

The assay medium contained 20 mM Mes-NaOH (pH 6 5) The concentrations of different additions are H_2O_2 (3 mM), NaCl (10 mM), $CaCl_2$ (10 mM), and $MnCl_2$ (3 μM) Variations in the rates shown were within 5%

H_2O_2, or exogenous $MnCl_2$ together with H_2O_2 and $CaCl_2$ (table 1). It was found that electron transport was inhibited to about 50% in the latter reaction compared to the former one. $CaCl_2$ together with $MnCl_2$ also showed inhibitory effect on electron transport which is not understood at this moment.

From the above experiments, it appears that added $CaCl_2$ inhibits H_2O_2 electron donation in NaCl-treated PSII preparations. Therefore, we also tested this effect in $CaCl_2$-treated and Tris-NaCl-treated PSII particles (table 1). In these preparations, presence of exogenous $CaCl_2$ also greatly inhibited the Mn^{2+}-mediated H_2O_2 electron donation. However, the relationship of H_2O_2 with Mn^{2+}, Ca^{2+} and Cl^- is clarified in fig.1. It is shown that in all the 3 types of PSII preparations, the percentage of DCIP photoreduction increases almost linearly as a function of increasing H_2O_2 concentration if only $MnCl_2$ (3 μM) is present in the reaction media. On the other hand, if $CaCl_2$ (10 mM) was used together with $MnCl_2$, this accelerating tendency was greatly inhibited. The observed higher control rate of DCIP photoreduction in NaCl-treated submembrane fractions (fig.1A), compared to the other two types of preparations (fig.1B and C), is due to the acceleration of H_2O oxidation by $CaCl_2$ in presence of endogenous Mn-complex. The double reciprocal plots in fig.2 show an uncompetitive interaction of $CaCl_2$ with substrate complex, if $MnCl_2$ and H_2O_2 were used together.

Finally, the inhibition of H_2O_2 electron donation by $CaCl_2$ was investigated. It was seen that in NaCl-treated PSII preparations, where Mn-complex is entirely present, addition of increasing concentrations of exogenous $CaCl_2$ raises the percentage of DCIP photoreduction with H_2O as an electron donor (no H_2O_2 present) (fig.3A). However, the increase was slowed down

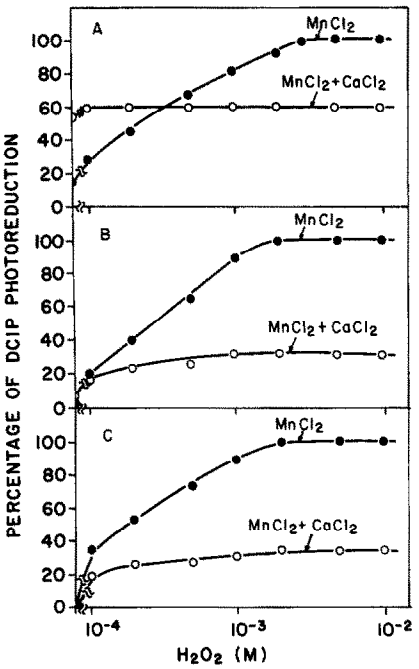

Fig.1. DCIP photoreduction as a function of increasing H_2O_2 concentrations in PSII preparations depleted of extrinsic polypeptides (A) NaCl-treated, (B) $CaCl_2$-treated, (C) Tris-NaCl-treated Either only 3 μM $MnCl_2$ (●), or 3 μM $MnCl_2$ together with 10 mM $CaCl_2$ (○), were added Data are presented as a percentage of the rate observed in the presence of $MnCl_2$ and optimal H_2O_2 concentrations

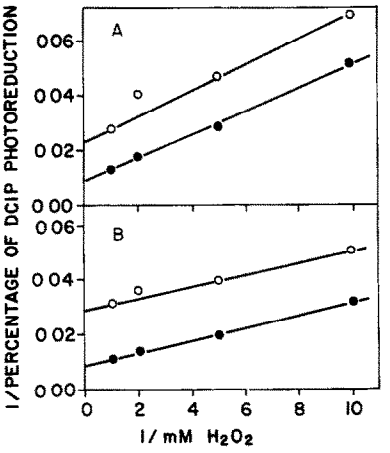

Fig 2 Double reciprocal plots obtained from fig 1B and C showing uncompetitive inhibition H_2O_2 electron donation by $CaCl_2$ Either 3 μM $MnCl_2$ alone (●), or 3 μM $MnCl_2$ together with 10 mM $CaCl_2$ (○), were added (A) $CaCl_2$-treated, (B) Tris-NaCl-treated PSII preparations

at around 5 mM $CaCl_2$ and became stabilized reaching the optimum from 10–25 mM $CaCl_2$. Beyond this range, the activity was decreased. These results indicate that in the NaCl-treated PSII preparations where native Ca^{2+} and Cl^- are disorganized, addition of increasing concentrations of $CaCl_2$ (up to 10 mM), partially restores the native conformation of the PSII core complex and thus accelerates the water oxidation as well. However, the inhibitory effect of $CaCl_2$ beyond 25 mM (fig.3A) is comparable to the effect of high concentration of $CaCl_2$ on the native PSII, where it was reported to have inhibitory effect on oxygen evolution [23,24]. In fig.3B, it is shown that the $CaCl_2$ inhibition kinetics of H_2O_2 electron donation was comparable among the 3 types of PSII preparations, at concentrations up to 1 mM. Visual analysis of the curves indicates 3 types of inhibition kinetics of H_2O_2 electron donation by $CaCl_2$ in NaCl-treated preparations. The first is the linear inhibition of H_2O_2 electron donation by $CaCl_2$ up to 1 mM (fig.3B). It sharply corresponds to the linear activation of water oxidation up to about 2 mM $CaCl_2$ (fig.3A). The second is the lag phase in the range of 2–10 mM $CaCl_2$, corresponding to the slow activation of water oxidation by $CaCl_2$ in the same range of concentrations. The third is the rapid inhibition of H_2O_2 oxidation by $CaCl_2$ beyond 50 mM. It corresponds with the inhibition of water oxidation by high concentration of $CaCl_2$ (fig.3A). In the other two preparations ($CaCl_2$-treated and Tris-NaCl-treated), only a linear inhibition was observed in the whole range of $CaCl_2$ concentrations. At $CaCl_2$ concentrations above 5 mM the inhibition became increasingly pronounced in $CaCl_2$-treated and Tris-NaCl-treated preparations, respectively, compared to the NaCl-treated one. This indicates that $CaCl_2$ inhibition of electron donation by H_2O_2 is enhanced by the release of native Mn-atoms from the oxygen-evolving complex. The above is consistent with the conclusions of Sandusky and Yocum to the effect that H_2O_2 oxidation by Cl^- depleted thylakoids is mediated by Mn^{2+} [12].

In conclusion, our results are in disagreement with [3,7], who considered the 16 and 23 kDa polypeptides as a shielding barrier for H_2O_2 accessibility to the PSII

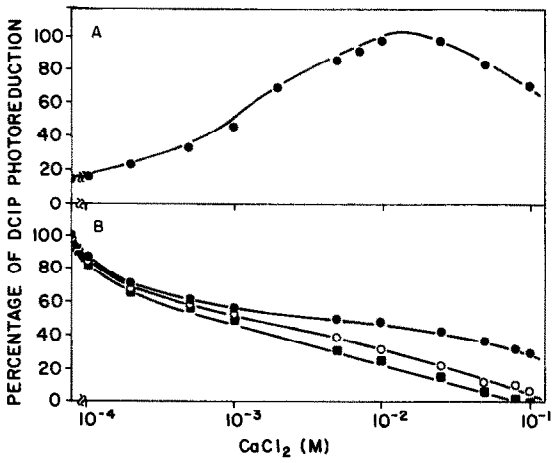

Fig 3. DCIP photoreduction as a function of (A) increasing $CaCl_2$ concentrations in NaCl-treated PSII preparations, $H_2O \rightarrow$ DCIP, and (B) in PSII preparations treated with either NaCl (●), or $CaCl_2$ (○) or Tris-NaCl (■) $MnCl_2$ (3 μM) and H_2O_2 (3 mM) were added to the reaction media Data presented as a percentage of the maximum rate as in fig 1

donor side. Schroder and Åkerlund [3], however, suspected the possible involvement of Ca^{2+} and Cl^- in the shielding effect. The data presented here clearly show that disorganization of Ca^{2+} and Cl^- within the oxygen-evolving complex due to the depletion of 16 and 23 kDa extrinsic polypeptides, allows H_2O_2 to undergo oxidation and to donate electrons to P680 via the native Mn-cluster or via exogenously added Mn^{2+}. Readdition of extra Ca^{2+} and Cl^- to the depleted PSII preparations results in the ions occupying their functional sites in the vicinity of the PSII core complex. This probably helps in the concomitant partial restoration of the native conformation of the PSII core complex and thereby inhibits the H_2O_2 oxidation in these preparations. It is also apparent from our data that H_2O_2 cannot serve as electron donor in polypeptide-depleted PSII preparations if Mn^{2+} is absent in the PSII particles. This is in line with prior reports that H_2O_2 does not undergo oxidation in native PSII particles unless exogenous Mn^{2+} is added [13–15].

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