

Effect of MgCl_2 and Phosphatidylglycerol on CaCl_2 -Mediated Recovery of Oxygen Evolution in a Photosystem II Complex Depleted of the 17 and 24 kDa Extrinsic Proteins

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Phosphatidylglycerol (PG) is an anionic lipid of the thylakoid membrane of higher plant chloroplasts. PG was shown previously to stimulate the evolution of oxygen in intact photosystem II (PSII) membranes [Fragata, M., Strzałka, K. and Nénonéné, E. K. (1991) J. Photochem. Photobiol. B: Biol **11**, 329–342]. In this work, a study was undertaken of the effect of MgCl_2 and PG on the CaCl_2 -mediated recovery of oxygen evolution in a PSII complex depleted of the extrinsic proteins (EP) of molecular masses 17 kDa (EP17) and 24 kDa (EP24), hereunder designated $d_{17,24}$ PSII. This molecular system is structurally close to the PSII core complex of cyanobacteria and is therefore useful in the comparative analysis of PSII-PG relationships in cyanobacteria and the higher plants. This work reveals a new aspect of the thylakoid lipids role in the PSII function, namely the PG effect on intact PSII is observed as well in $d_{17,24}$ PSII. The results show that phosphatidylglycerol has the ability to compensate for the loss of EP17 and EP24 in the PSII complex. That is, PG restores the oxygen evolution in $d_{17,24}$ PSII incubated in the presence of MgCl_2 and/or CaCl_2 to the levels observed in native PSII. Moreover, the site of H_2O degradation in $d_{17,24}$ PSII, including most probably the pool of calcium and chloride ions, would seem to be protected by phosphatidylglycerol. This suggests that one of the docking sites of PG in the PSII complex is near EP24, inasmuch as this extrinsic protein participates in the regulation of the affinity of the calcium and chloride ions to the water oxidation site. Furthermore, taking into account that in $d_{17,24}$ PSII the PSII core complex is directly exposed to PG, then the phospholipid effect reported here indicates that phosphatidylglycerol might be a functional effector and membrane anchor of the D1 protein in the PSII core complex as was shown recently in the cyanobacterium *Oscillatoria chalybea* [Kruse, O. and Schmid, G. H. (1995) Z. Naturforsch. **50c**, 380–390].

Introduction

The oxidation of water to O_2 in green plants and algae is catalyzed by the photosystem II (PSII)

complex which consists of a cluster of four Mn-atoms, several intrinsic membrane proteins and three extrinsic proteins (Hansson and Wydrzynski, 1990). That is, the D1 and D2 proteins, the subunits α and β of cytochrome *b559*, the two intrinsic Chl *a*-binding proteins (CP47 and CP43), the three extrinsic proteins (EP) of molecular masses 15–18, 23–24 and 32–34 kDa, i.e., EP17, EP24 and EP33 respectively (Miyao and Murata, 1983; Rutherford *et al.*, 1992), a calcium-binding protein with an apparent molecular mass of 13–15 kDa which is thought to be a calmodulin-like protein (Sparrow and England, 1984; Hansson and Wydrzynski, 1990), and a few other proteins of low molecular mass (Ikeuchi and Inoue, 1988; Murata *et al.*, 1988; Schröder *et al.*, 1988; Fragata *et al.*, 1990a; Fotinou *et al.*, 1993). The extrinsic proteins and the Mn-cluster are related to the function of

Abbreviations: Chl, chlorophyll; $d_{17,24}$ PSII, PSII depleted of EP17 and EP24; DCBQ, 2,5-dichloro-p-benzoquinone; EP17, EP24, EP33, extrinsic proteins of respectively 17, 24 and 33 kDa molecular masses; MES, 2-(N-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex of PSII; PG, phosphatidylglycerol; PSII, photosystem II; P680, reaction center of PSII; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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the oxygen-evolving complex (OEC). However, the OEC activity depends essentially on the presence of the Mn-cluster and EP33. EP33 is a protein which is believed to shield or stabilize the Mn-cluster (Hayashi *et al.*, 1993; Leuschner and Bricker, 1996; Rögner *et al.*, 1996; Hankamer *et al.*, 1997) and the binding of the Ca^{2+} and Cl^- ions (Hankamer *et al.*, 1997), whereas the two other extrinsic proteins, that is, EP17 and EP24, serve essentially to optimize the rates of oxygen evolution (Hillier and Wydrzynski, 1993). While the function of EP17 is yet controversial (Hansson and Wydrzynski, 1990), EP24 is known to play a regulatory role in the affinity of the calcium and chloride ions to the water oxidation site (Andersson *et al.*, 1984; Ghanotakis *et al.*, 1984; Hankamer *et al.*, 1997).

The selective removal of the extrinsic proteins from the PS II complex by treatment with high concentrations (1 to 2 M) of mono- or divalent cations, e.g., Na^+ and Ca^{2+} , hinders the OEC activity and may even prevent completely the evolution of oxygen (see discussions in Akabori *et al.*, 1984; Hayashi *et al.*, 1993). Nonetheless, the OEC function is restored in EP-depleted PSII preparations by the addition of low concentrations of CaCl_2 (Miyao and Murata, 1984a; Ono and Inoue, 1984; Boussac *et al.*, 1985). Another aspect of this question is the role of the thylakoid lipids and the combined effect of the thylakoid lipids and the metal ions on the function of the PSII core complex.

There is evidence indicating that the thylakoid lipids affect the activity of various photosynthetic processes (Webb and Green, 1991; Fragata *et al.*, 1994). It was found, for example, that the phospholipids are necessary for promoting the charge separation reactions in PSII (Jordan *et al.*, 1983). However, the description of the structure-function relations that underlie the lipid-protein interactions in the thylakoid membrane has not yet been worked out in sufficient detail (see discussions in Fragata *et al.*, 1993, 1997, and Kruse and Schmid, 1995). In this respect, an interesting matter is the presence of phosphatidylglycerol (PG), an anionic lipid of the thylakoid membrane (Webb and Green, 1991), in the PSII core complex at a ratio of 4 mol/mol P680 (Murata *et al.*, 1990). It is worth noting that the stimulation of evolution of oxygen in PSII by PG is enhanced in the presence of MgCl_2 (Fragata *et al.*, 1990b, 1991). This synergetic

lipid-ion effect was interpreted as the result of a cooperative function of oxygen evolving units in the PSII complex (Fragata *et al.*, 1991). In this same connection, Akabori *et al.* (1984) showed that the oxygen evolution in EP-depleted broken thylakoids is enhanced by reconstituting the membranes with the total thylakoid lipids, thereby emphasizing again the importance of the lipid-protein interactions in the thylakoids function. More recently, Kruse and Schmid (1995) theorized that one of the active sites of PG in the PSII core complex is in a structural groove in the D1 protein. According to this hypothesis the D1 protein is inactive in the absence of PG since the functional conformation of the polypeptide is dependent on the presence of the phospholipid (cf. Fig. 7 of Kruse and Schmid, 1995). This assumption is attractive as it suggests a direct effect of PG in the PSII core complex which would hence affect either the Mn-cluster (see model in Yachandra *et al.*, 1993) or the aromatic amino acid residues and the photosynthetic pigments, i.e., the P680 chlorophylls and pheophytin, which constitute the reaction center of PSII (see model in Roffey *et al.*, 1994). In this context, it is important to emphasize that Kruse and Schmid's experiments were performed with the PSII complex of the cyanobacterium *Oscillatoria chalybea* which does not contain any of the extrinsic peptides of 17 and 24 kDa molecular masses, that is, EP17 and EP24.

Our previous work on the phosphatidylglycerol effect (Fragata *et al.*, 1990b, 1991) was done with the PSII complex from higher plants which therefore contains EP17 and EP24. In the present study, we investigated the effect of PG on the CaCl_2 -mediated recovery of oxygen evolution in a PSII complex depleted of EP17 and EP24, hereinafter designated $\text{d}_{17,24}\text{PSII}$. This molecular system is structurally close to the PSII core complex of the cyanobacterium used by Kruse and Schmid (1995), and may therefore assist in providing new insight into the comparative analysis of PSII-PG relations in cyanobacteria and higher plants. We have also studied the effect of MgCl_2 on the oxygen evolution in the modified PSII complex, i.e., $\text{d}_{17,24}\text{PSII}$. We show hereunder that, in the absence of exogenous Ca-ions in the incubation media, PG restores the oxygen evolution in $\text{d}_{17,24}\text{PSII}$ up to the levels seen in native PSII. Furthermore, in the presence of Ca- and Mg-ions phosphatidylglycerol enhances

the oxygen evolution in $d_{17,24}$ PSII to levels much higher than those observed in native PSII in the absence of the phospholipid. The amplification of the oxygen evolution activity upon interaction of PG with $d_{17,24}$ PSII shall be discussed in the perspective of the cooperative function of phosphatidylglycerol and the divalent metal ions.

Materials and Methods

Isolation of PSII particles

PSII membranes were obtained from chloroplasts of eight-day-old barley seedlings grown on vermiculite (continuous light, 25 °C) according to Berthold *et al.* (1981) with the modifications described in Nénonéné and Fragata (1990). First, chloroplast thylakoids were isolated and suspended in a buffer containing 20 mM MES-NaOH (pH 6.0), 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$ (buffer A) to give a final chlorophyll (Chl) concentration of 2 mg ml⁻¹. Then, the PSII membranes were obtained upon incubation (20 min, 277 K, darkness) of an aliquot of the thylakoid suspension with buffer A containing 4% Triton X-100 to give a final Chl concentration of 1 mg ml⁻¹. Upon centrifugation, the final pellet (PSII membranes) was suspended in buffer A containing 0.1% digitonin and stored at 143 K in the presence of 30% (v/v) ethylene glycol. The Chl concentration in these preparations was determined according to Arnon (1949) in 80% acetone. Before use, the PSII samples were washed twice in a buffer containing 20 mM MES-NaOH (pH 6.5), 0.4 M sucrose and 15 mM NaCl (buffer B). The molecular masses of the polypeptides contained in the PSII samples were determined by SDS-PAGE (see details hereunder).

Preparation of $d_{17,24}$ PSII particles

The EP-depleted PSII membranes, i.e., $d_{17,24}$ PSII, were obtained by treating the PSII preparations with buffer B containing 1 M NaCl to give a final Chl concentration of 1 mg ml⁻¹. This NaCl concentration assures a complete removal of EP17 and EP24 without elimination of EP33 (cf. Fig. 1). After 30 min incubation at 273 K in the dark, the suspension was centrifuged at 29000 × g for 30 min at 277 K. The resultant pellet was washed once with buffer B by resuspension and

recentrifugation. Then, the final pellet was suspended in buffer B for later use. Untreated PSII membranes were held in the same conditions in buffer B and were used as reference.

Interaction of PG with PSII or $d_{17,24}$ PSII

The interaction of PG with PS II or $d_{17,24}$ PS II were studied as follows. First, PG vesicles were prepared according to the method described in L'Heureux and Fragata (1988). In short, PG was dried under a nitrogen current and then dispersed in buffer B to give a final lipid concentration of 10 mg ml⁻¹. This suspension was subject to sonication in a capped tube for 13 min at 160 W output in a Heat System-Ultrasonics Sonicator, model W-225R (Plainview, IL). Then, the PS II or $d_{17,24}$ PS II preparations were gently mixed in a vortex with PG vesicles and incubated for 20 min in the dark at 273 K. After this incubation period, the preparations were used for determinations of oxygen evolution activity as described below. We note that the lipid:Chl ratio was maintained at 20:1 (w:w) throughout this work, and that the PS II concentration, here given as Chl concentration, was kept constant in all experiments.

Measurement of oxygen evolution

To measure oxygen evolution the samples (PSII and $d_{17,24}$ PSII complexed or not with PG vesicles) were first pre-incubated in buffer B at 298 K (2 min, darkness). The oxygen evolution measurements were then performed at 298 K using a Clark-type electrode (Hansatech D.W. Oxygen Electrode Unit, King's Lynn, Norfolk, U. K.). The samples were irradiated with white light at saturation intensity for a period of 2 min. The artificial electron acceptor was 2,5-dichloro-p-benzoquinone (DCBQ). The reaction media contained 20 mM MES-NaOH (pH 6.5), 15 mM NaCl, 0.4 M sucrose, and 350 μM DCBQ and various salt concentrations as needed. The chlorophyll concentration in the samples was 10 μg Chl ml⁻¹. The pH was adjusted by addition of NaOH to avoid changes in the chemical composition of the reaction media. Every experiment was repeated six times using different preparations of $CaCl_2$ - and $MgCl_2$ -treated and untreated PSII and $d_{17,24}$ PSII membranes, and also different PG vesicles preparations.

SDS-polyacrylamide gel electrophoresis

The polypeptide composition in PSII and $d_{17,24}$ PSII was analyzed by SDS-PAGE following the method described by Chua (1980) with slight modifications. That is, a sucrose gradient (5–17.5%) was used in the separating gel which was composed of 10–20% acrylamide. The stacking gel contained 7.5% sucrose and 5% acrylamide. Staining was performed with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA). Destaining of the gel was done in aqueous solutions containing 3% glycerol, 20% methanol and 70% acetic acid (Chua 1980). The molecular masses and relative amounts of the polypeptides were determined using a LKB Ultrosan XL Laser Densitometer, model 2222–020, equipped with a LKB Recording Integrator, model 2220, by comparing the unknown proteins with standard molecular markers obtained from Sigma Chemical Company (St-Louis, MO). A typical SDS-PAGE diagram of the protein composition of the PSII membranes is given in Fig 1A. It displays eight major proteins with molecular masses of approximately 17, 19, 22, 23, 24, 26, 30 and 33 kDa. This analysis was performed for every series of experiments and the final result was essentially the same. This is therefore a clear indication that the PSII preparations used in the present work were not contaminated with the photosystem I proteins.

Chemicals

L- α -phosphatidylglycerol was purchased from Serdary Research Laboratory (London, Ontario). The analysis of its fatty acid chains was done by gas chromatography of the methyl esters formed from methanolysis of the lipid (0.1 mg) with 1 mL of BF_3 /methanol 14% (w/v) (Pierce Chemical Company) and 40 ml hexane. The analysis was performed in a Varian gas chromatograph, model 3700, equipped with a Shimadzu integrator, model C-R3A. The fatty acid chains composition in mol% (in parenthesis) was shown to be: 16:0 (36.5), 16:1 (0.6), 18:0 (12.7), 18:1 (32.6), 18:2 (11.6), and 18:3 (3.0). All other compounds were obtained from Sigma Chemical Company (St-Louis, MO).

Results

Fig. 1 shows the SDS-PAGE densitograms of intact PSII membranes (A) and PSII treated with 1 M NaCl (B), 1 M MgCl_2 (C) and 1 M CaCl_2 (D). We note first that the PSII preparations used in the present work were not contaminated with the photosystem I proteins (see Materials and Methods). The densitogram traces show clearly the depletion of the extrinsic proteins of molecular masses 17 and 24 kDa in (B), (C) and (D). The densitogram also reveals the gradual depletion of EP33 which is observed from (B) to (D). To deplete the PSII complex of EP17 and EP24, we used in this work 1 M NaCl as these conditions are milder comparatively to the much stronger effect of CaCl_2 or MgCl_2 . This therefore permits to keep the EP33 attached to the PS II core complex (cf. Fig. 1).

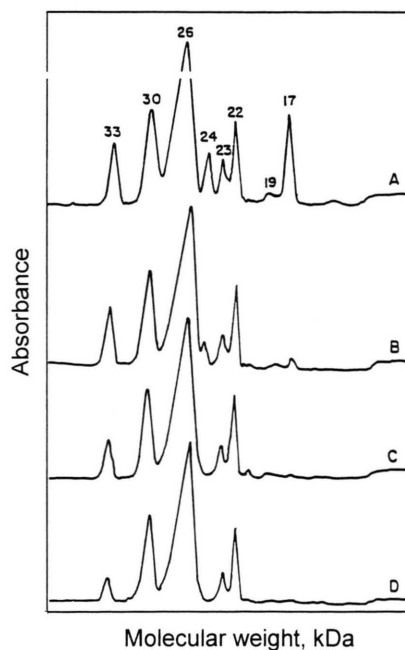


Fig. 1. SDS-PAGE densitogram of intact photosystem II (PSII) membranes (A) and PSII membranes treated with 1 M NaCl (B), 1 M MgCl_2 (C) and 1 M CaCl_2 (D). The molecular weight of the PSII proteins is indicated in kDa. Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

The efficiency of MgCl_2 and PG to enhance the CaCl_2 -mediated recovery of oxygen evolution in PSII depleted of the 17 and 24 kDa proteins, i.e., $d_{17,24}$ PSII, is studied in Table I and Fig. 2. Table I

Table I. Effect of phosphatidylglycerol (PG) and MgCl_2 on the CaCl_2 -mediated stimulation of oxygen evolution in intact photosystem II (PSII) and PS II depleted of the extrinsic proteins of 17 and 24 kDa molecular masses ($d_{17,24}\text{PSII}$)^a.

CaCl_2 [mM]	PSII ^b	$d_{17,24}\text{PSII}$	$d_{17,24}\text{PSII}$ + 5 mM MgCl_2	$d_{17,24}\text{PSI}$ + 5 mM MgCl_2 + PG
0	100 ^a	37	52	98
5 ^c	131 m ^d	101	117	158
10	130	134	150	178
15	127	139 m	155 m	180 m

^a The oxygen evolution data are given in percentage to facilitate the interpretation of the results. 100% is the oxygen evolution observed in intact PSII in the absence of MgCl_2 and/or CaCl_2 , i.e., about $390 \mu\text{M O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$.

^b The PSII data was taken from Fig. 3.

^c 5 and 15 mM are the CaCl_2 concentrations which induce the maximum oxygen evolution activity in respectively PSII and $d_{17,24}\text{PSII}$.

^d m, maximum oxygen evolution activity (see c).

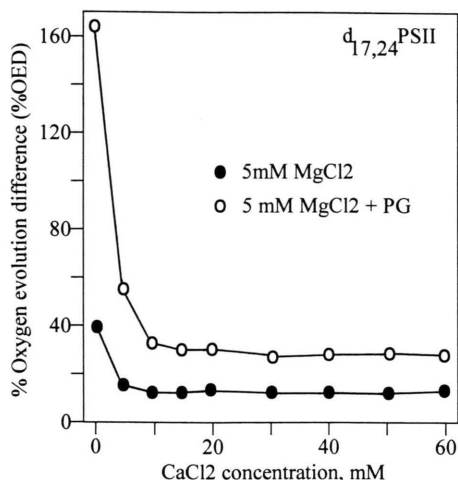


Fig. 2. Effect of MgCl_2 and phosphatidylglycerol (PG) on the CaCl_2 -mediated recovery of oxygen evolution in PSII depleted of the extrinsic proteins of 17 and 24 kDa molecular masses ($d_{17,24}\text{PSII}$). Each data point is the average of six experiments. The oxygen evolution (in percentage) is expressed in relation to the activity observed in PSII preparations incubated in the absence of PG and MgCl_2 and/or CaCl_2 , i.e., about $390 \mu\text{M O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$ (=100%). The relative oxygen evolution difference (in percentage) is given as $\% \text{OED} = (d_{17,24}\text{PSII}_x - d_{17,24}\text{PSII}_0)100/d_{17,24}\text{PSII}_0$, where $d_{17,24}\text{PSII}_0$ is the oxygen evolution of $d_{17,24}\text{PSII}$ in the absence of PG and MgCl_2 , and $d_{17,24}\text{PSII}_x$ the oxygen evolution in the presence of 5 mM MgCl_2 , or 5 mM MgCl_2 plus PG. Abbreviations: Chl, chlorophyll; PG, phosphatidylglycerol; PSII, photosystem II.

contains also data obtained with intact PSII (cf. Fig. 3). It is seen at first (Table I) that the addition of 5 mM CaCl_2 to the $d_{17,24}\text{PSII}$ preparations causes the oxygen evolution to increase from 37% (in the absence of salt) to the level observed in intact PSII, i.e., 100% activity, which in the present experiments is approximately $390 \mu\text{M O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$. Then, a saturation of oxygen evolution in the $d_{17,24}\text{PSII}$ preparations is attained around 139% at CaCl_2 concentrations in the incubation medium of the order of 15 mM. Furthermore, the addition of 5 mM MgCl_2 to the $d_{17,24}\text{PSII}$ preparations in the absence of CaCl_2 causes the rate of oxygen evolution to rise from 37 to 52%, that is, an increase of about 15%. This result may be explained by the propensity of MgCl_2 to function as a Cl^- -ion donor. The result suggests also a possible additive effect of the divalent Mg and Ca cations in the mixtures of MgCl_2 with CaCl_2 . The latter assumption is consistent with the effect of MgCl_2 and CaCl_2 on the oxygen evolution in intact PSII incubated in the same conditions as those used in the experiments with $d_{17,24}\text{PSII}$ (cf. Fig. 3). Fig. 3 shows that there is no significant difference between the effects of MgCl_2 and CaCl_2 in the concentration range from 0 to 30 mM. These data warrant further investigation. Namely, it will be interesting to examine whether the effect described here denotes a similar ionic accessibility or affinity in the $d_{17,24}\text{PSII}$ complex or, more importantly, whether MgCl_2 and CaCl_2 share the same mechanism of action in the PSII core complex.

The effect of PG on $d_{17,24}\text{PSII}$ displayed in Table I indicates that, in contrast with the CaCl_2 and MgCl_2 data, the mode of action of the phospholipid does not appear to follow an additive mechanism. At least at low calcium concentration, that is, up to about 15 mM CaCl_2 . This is better seen in Fig. 2 which gives the relative oxygen evolution difference (in percentage), i.e., %OED, in the $d_{17,24}\text{PSII}$ preparations, that is,

$$\% \text{OED} = (d_{17,24}\text{PSII}_x - d_{17,24}\text{PSII}_0)100/d_{17,24}\text{PSII}_0,$$

where $d_{17,24}\text{PSII}_0$ is the oxygen evolution in $d_{17,24}\text{PSII}$ in the absence of PG and MgCl_2 , and $d_{17,24}\text{PSII}_x$ the oxygen evolution in the presence of 5 mM MgCl_2 , or 5 mM MgCl_2 plus PG. The major observations are as follows.

First, Fig. 2 (cf. also Table I) shows that in $d_{17,24}\text{PSII}$ preparations incubated in the presence

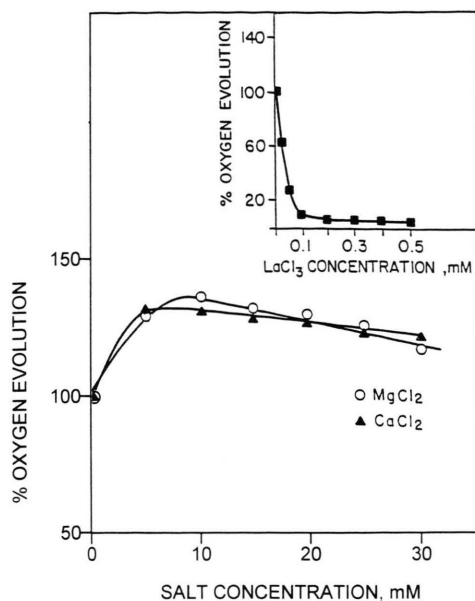


Fig. 3. Effect of MgCl_2 and CaCl_2 on oxygen evolution in photosystem II. Each data point is the average of six experiments. The oxygen evolution (in percentage) is expressed in relation to the value obtained in PSII preparations incubated in the absence of MgCl_2 or CaCl_2 , i.e., about $390 \mu\text{M O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$ (=100%). Abbreviations: Chl, chlorophyll; PSII, photosystem II. Inset: LaCl_3 effect on the oxygen evolution in PSII. Note the strong inhibitory effect of LaCl_3 at concentrations as low as 0.1 mM.

of 5 mM MgCl_2 the %OED ratio decreases from about 40% oxygen evolution enhancement in the absence of CaCl_2 to an average enhancement value of $(12.6 \pm 0.8)\%$ at CaCl_2 concentrations higher than 10 mM.

Secondly, Fig. 2 (cf. also Table I) reveals the important finding that in the presence of PG and 5 mM MgCl_2 the %OED ratio is about 165% oxygen evolution enhancement in the absence of CaCl_2 and decreases rapidly to attain an average enhancement value of $(28.7 \pm 1.4)\%$ at CaCl_2 concentrations higher than 10 mM.

Discussion

Ionic effect on the $d_{17,24}\text{PSII}$ activity

We note at first that the upper limit of 139% observed in $d_{17,24}\text{PSII}$ incubated with 15 mM CaCl_2 in the absence of MgCl_2 and PG (cf. Table I and Fig. 2), i.e., the O_2 evolution saturation level, may stem from limitations in the Ca^{2+} -binding capacity

in the PS II core complex which is thought to be 1 or 2 Ca atoms per P680 (Ono and Inoue, 1988, Pauly *et al.*, 1992). In this respect, a calculation indicates that at concentrations in the reaction media of $10 \mu\text{g Chl ml}^{-1}$ (see Materials and Methods) and 15 mM CaCl_2 (i.e., $m=139\%$ in the absence of MgCl_2), one might expect a ratio of about 75 mol Ca^{2+} /mol P680 provided that most of the Ca-ions in the incubation medium bind to the PSII core complex and assuming, in addition, an average of 50 Chl per P680 (Murata *et al.*, 1984). As well, at 5 mM CaCl_2 in the reaction medium, that is, the concentration that restores 100% of the O_2 evolution activity in $d_{17,24}\text{PSII}$ (Table I), the Ca^{2+} /P680 ratio is about 25. These molar ratios are obviously out of proportion with 1–2 Ca atoms/P680 (Ono and Inoue, 1988, Pauly *et al.*, 1992). This thereby indicates that a large part of the Ca-ions which interact with $d_{17,24}\text{PSII}$ might have an alternative function in the core complex, in addition to the role which is usually ascribed to them (see, e.g., Murata *et al.*, 1984).

Moreover, an effect which is clearly visible in Table I is the greater capacity of the ions to stimulate higher levels of oxygen evolution in $d_{17,24}\text{PSII}$ than in the intact PSII particles. In brief, Table I shows on the one hand that the oxygen evolution maximum (m) is 131% in PSII in the presence of 5 mM CaCl_2 and then decays. For example, at 15 mM CaCl_2 the oxygen evolution rate decreases to 127%. However, at this same CaCl_2 concentration in the incubation medium one observes $m=139\%$ in $d_{17,24}\text{PSII}$ preparations, and $m=155\%$ in $d_{17,24}\text{PSII}$ preparations incubated in the presence of 5 mM MgCl_2 . This observation is interesting as it may clarify further the molecular mechanisms of EP17 and/or EP24 in the control of the number of ions that reach the PSII core complex.

The stimulation by MgCl_2 and CaCl_2 of the oxygen evolution in PSII and $d_{17,24}\text{PSII}$ (Figs. 2 and 3) may be ascribed to the increase of the chloride ions concentration in the vicinity of the Mn-cluster (see, e.g., Miyao and Murata, 1984b; Homann, 1988; Rashid and Carpentier, 1990; Hayashi *et al.*, 1993). However, we recall in this context that Waggoner *et al.* (1989) showed that the monovalent cations Na^+ , K^+ and Cs^+ can alter the calcium-mediated enhancement of oxygen evolution in the PS II complex depleted of EP17 and EP24 by inhibiting the Ca-binding site responsible for the ac-

tivation of the OEC. Another argument is provided by the work of Ghanotakis *et al.* (1985) showing that a concentration as low as 2 mM LaCl_3 causes the release of EP17, EP23, EP33 and about 60% of the functional Mn-atoms from the PS II core complex. Moreover, this is accompanied by the loss of 94% of the oxygen-evolving activity that could not be recovered by addition of 10 mM CaCl_2 . Yet in this connection, it is worth noting that Ca^{2+} is replaceable by the Sr^{2+} cation (Ghanotakis *et al.*, 1984; Boussac and Rutherford, 1988) which, moreover, is able to bind to the Mn-cluster at the calcium site (see model in Yachandra *et al.*, 1993). The LaCl_3 data given in Fig. 3 inset supports further this assumption inasmuch as the LaCl_3 effect is observed at very low salt concentration. The inset shows that concentrations as low as 0.1 mM LaCl_3 or less, that is, 0.3 mM of chloride ions or less, inhibit almost totally, i.e., more than 92%, the oxygen-evolving activity in PSII. It is obvious that such small concentrations are out of proportion with the much higher concentrations, i.e., up to 120 or 200 mM Cl^- , used in the CaCl_2 experiments reported in this work and elsewhere in which a stimulation of oxygen evolution has been systematically observed. Fig. 3 inset indicates, therefore, that we must be in presence of a La^{3+} -induced effect. The above discussions indicate that the ionic effects described here (cf. Figs. 2 and 3, and Table I) may have their origin in mechanisms induced by the Mg^{2+} and Ca^{2+} ions. We emphasize, in this respect, that Mg^{2+} can replace Ca^{2+} in cyanobacterial PS II (see discussions in Pauly *et al.*, 1992). Taking into account, in addition, that the mechanisms of water oxidation in higher plants and cyanobacteria are quite similar (Pauly *et al.*, 1992), then the assumption of a contribution of the Mg-ion to the evolution of oxygen in the PS II core complex in higher plants seems plausible.

Finally, it should be emphasized that the drastic loss of oxygen evolution enhancement at high CaCl_2 concentrations which is observed in the MgCl_2 and MgCl_2 +PG curves displayed in Fig. 2 may stem from the disruption of cooperative function between PSII units (see Fragata *et al.*, 1991, 1994). We hypothesize that this is likely the result of loss of PSII dimerization (see, in this respect, Hankamer *et al.*, 1997; Kruse *et al.*, 1997). That is, a PSII dimer to monomer transition at high CaCl_2 concentration.

Phosphatidylglycerol effect on the $d_{17,24}$ PSII activity

As was observed with MgCl_2 and CaCl_2 , the levels of oxygen evolution in $d_{17,24}$ PSII complexed with phosphatidylglycerol are higher than those observed in the intact PSII particles (Table I). The table indicates that the oxygen evolution maximum is as high as 180% in $d_{17,24}$ PSII preparations which are incubated concomitantly with 5 mM MgCl_2 and 15 mM CaCl_2 . That is to say, in the conditions that, in PSII particles incubated in the presence of 5 mM CaCl_2 , cause already a decay of oxygen evolution to values below the observed maximum, i.e., $m=131\%$. The data in Table I and Fig. 2 illustrate conclusively that phosphatidylglycerol has the capacity of compensating for the loss of EP17 and EP24 in the PSII complex. That is, PG is able to restore in $d_{17,24}$ PSII, in the absence of salts, the normal levels of oxygen evolution which are usually observed in native PSII particles. Besides, one observes a substantial PG-mediated enhancement of oxygen evolution in $d_{17,24}$ PSII particles incubated in the presence of MgCl_2 or CaCl_2 . Summarizing, the PG data (Table I, Fig. 2) demonstrate the important point that the PG effect is a more-than-additive mechanism. In fact, it was consistently observed that

i. in the absence of CaCl_2 , PG heightens the maximum oxygen evolution from $m=37\%$, in the absence of phospholipid and salts, to $m=98\%$ in the presence of 5 mM MgCl_2 , whereas the increase is from 37 to only 52% in the absence of the phospholipid; and

ii. in the presence of 15 mM CaCl_2 , PG increases the maximum oxygen evolution in $d_{17,24}$ PSII from $m=139\%$, in the absence of phospholipid and salts, to $m=180\%$ in the presence of 5 mM MgCl_2 , whereas the increase is from 139 to only 155% in the absence of the phospholipid.

The amplification of the oxygen evolution activity upon interaction of phosphatidylglycerol with $d_{17,24}$ PSII points to the function of cooperative interactions between the phospholipid and the divalent metal ions. This viewpoint is supported by the finding that the Mg-ions form a coordination complex with phosphatidylglycerol in a lipid-ion molecular network (see discussions in Fragata *et al.*, 1993, 1997). In brief, a new aspect of the thylakoid lipids role in the PSII function which the present

study discloses is first the demonstration that the phosphatidylglycerol effect that was demonstrated previously in intact PSII (Fragata *et al.*, 1990b, 1991) is observed as well in PSII depleted of the extrinsic proteins of 17 and 24 kDa masses, i.e., EP17 and EP24. Secondly, the present study gives evidence indicating that the site of H₂O degradation in the PSII core complex, including most probably the pool of calcium and chloride ions, is protected by phosphatidylglycerol. A straightforward corollary is that one of the docking sites of PG in the PSII complex is near EP24, inasmuch as EP24 participates in the regulation of the affinity of the calcium and chloride ions to the water oxidation site (see, e.g., Andersson *et al.*, 1984; Ghanotakis *et al.*, 1984; Hankamer *et al.*, 1997).

Cooperativity of structure and function in PSII

Two matters arise in the present study in relation to the question of the oligomeric structure of PSII (see recent review in Hankamer *et al.*, 1997). The first is whether the PG-induced enhancement of oxygen evolution in d_{17,24}PSII is related to the dimeric structure of the PSII complex. This conjecture is sustained by the experimental procedures used in this work (see Materials and Methods). These procedures assured that the d_{17,24}PSII preparations were incubated with PG in conditions that favor the cooperative function of PSII units, i.e., in media containing MgCl₂ at concentrations of 5 mM or higher (see discussions in Fragata *et al.*, 1991, and cf. also their Fig. 2). We note, in this respect, that the CF model was developed essentially on the basis of the enhancement by phosphatidylglycerol and digalactosyldiacylglycerol of the oxygen-evolving activity in PSII (Fragata *et al.*, 1990b, 1991, 1994). It predicts that the optimum oxygen evolution in PSII is dependent on the state of aggregation of the PSII units which was shown to be dependent on the lipid/protein ratio and is enhanced by the presence of MgCl₂ (Fragata *et al.*, 1991).

The second question relates to the activation of oxygen evolution mediated by phosphatidylglycerol and MgCl₂ (Table I and Fig. 2) in relation to

the recently discovered stabilization of PSII dimers by phosphorylation (Kruse *et al.*, 1997). This could be thought of as the result of PSII phosphorylation heightened by the presence of Mg-ions, thus favoring the dimeric state of PSII with concomitant augmentation of the activity of the oxygen-evolving complex. This is only possible in the framework of the phosphorylation mechanism described by Kruse *et al.* (1997) if the enzyme phosphorylating the D1, D2, CP43 and PsbH proteins is Mg²⁺-dependent. Although attractive, this assumption does not seem to be applicable in the present study since the d_{17,24}PSII preparations might not contain the appropriate phosphorylation enzymes. However, an interesting hypothesis emerges in this connection. That is to say, if it is proved that the phosphorylation enzymes are not present in the d_{17,24}PSII preparations, then the afore discussed data support further the new concept that the stabilization of the PSII dimers can be induced by the interaction of phosphatidylglycerol with the PSII core proteins.

Conclusion

In conclusion, the d_{17,24}PSII data discussed above strengthen the view that the PG molecules intervene in the cooperative function of oxygen evolving units in photosystem II, as our previous work sustained (see Fragata *et al.*, 1990b, 1991, 1994). Moreover, taking into account the studies of Kruse and Schmid (1995) on the PSII complex from the cyanobacterium *Oscillatoria chalybea*, the experimental results obtained with d_{17,24}PSII are interpreted as an indication that PG is essential for the function of the D1 protein in the PSII core complex which, in d_{17,24}PSII, might be directly exposed to the phosphatidylglycerol molecules as is predicted in Kruse and Schmid's model (1995).

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