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Structure of the oxygen-evolving complex of Photosystem II: calcium and lanthanum compete for sites on the oxidizing side of Photosystem II which control the binding of water-soluble polypeptides and regulate the activity of the manganese complex

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Exposure of Photosystem II membranes to lanthanides produces a destruction of oxygen-evolution activity. This inactivation is characterized by release of water-soluble polypeptides (33, 23 and 17 kDa species) as well as release of functional manganese. If calcium is included during lanthanum treatment, a different effect is obtained. Although 23 and 17 kDa proteins are released from the Photosystem II membranes, manganese and the 33 kDa protein are not released, and these membranes are active in oxygen evolution if calcium is present during assay. If high concentrations (200 mM) of NaCl are present during exposure to lanthanum, manganese and some of the 33 kDa protein are retained by the membranes; higher concentrations (2 M) of NaCl release all of the 33 kDa protein, but no manganese is released. Since the interaction between calcium and lanthanum is competitive, we conclude that displacement of calcium by lanthanum produces structural changes to the oxidizing side of Photosystem II which result in destruction of the manganese complex and the release of the water-soluble 33 kDa protein.

Introduction

The oxygen-evolving complex of PS II is a unique system, comprised in part of manganese, chloride, and calcium, which catalyzes the sequential 4-electron oxidation of water to molecular oxygen. Early studies, reviewed by Radmer and Cheniae [1], indicated that the lower limit for Mn content in PS II was four atoms per reaction center, a number confirmed by Yocum et al. [2] for intact thylakoid membranes and by several investigators who have examined the stoichiometry of Mn in highly active preparations of PS II membranes [3–5]. The implication of Cl^- as a cofactor for oxygen evolution activity by Warburg and Luttgens [6] and later by Bove et al. [7] has been confirmed by Izawa and his colleagues [8,9]. More recent research has placed the locus of Cl^- action within the oxygen evolving complex, perhaps to neutralize charge [10] or as a ligand to manganese [11]. Several investigators [12–15] proposed that Ca^{2+} was a required cofactor for PS II activity and these proposals have been substantiated by recent observations that the cation is specifically required

Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; EPR, electron paramagnetic resonance; HQ, hydroquinone; PS, Photosystem; Mes, 4-morpholineethanesulphonic acid.

to promote optimal rates of oxygen evolution activity in high-salt treated PS II membranes [16–18].

Advances in our understanding of the polypeptide composition of PS II have parallelled the research on inorganic cofactors required for oxygen-evolution activity. Åkerlund et al. [19] were the first to identify certain water-soluble polypeptides as essential elements of the fully competent oxygen-evolving complex. A substantial body of experimental evidence now supports the view that three water soluble proteins (33, 23 and 17 kDa) form part of the structure of the oxygen-evolving apparatus. Studies by many investigators have provided data to indicate that the 33 kDa species is somehow asociated with manganese binding to PS II membranes, although the exact nature of this association is not yet clear, and under certain conditions, the polypeptide can be removed from membranes without concurrent release of manganese [20,21]. For the 23 and 17 kDa water-soluble proteins, our own observations [16] indicate that these species are required for high-affinity binding of Ca^{2+} within the oxygen-evolving complex. Boussac and Etienne [22] have shown that the 23 kDa protein is in fact required for Ca^{2+} binding, and Toyoshima's group [23] has provided evidence to implicate the 17 kDa protein in the Cl^- requirement for oxygen evolution activity.

We have continued our examination of the Ca^{2+} requirement for oxygen evolution activity, since the cation is now widely implicated in a wide variety of biological processes in which specific Ca^{2+} binding proteins have been detected. In many of the studies on Ca^{2+}-linked biological phenomena, a class of trivalent cations, the lanthanides, has been shown to compete effectively for Ca^{2+} binding sites (e.g., Ref. 24). In this communication, we show that La^{3+} competes with Ca^{2+} for binding sites on the oxidizing side of PS II. Displacement of Ca^{2+} by La^{3+} produces a wide range of effects on the oxygen-evolving complex, and as we show here, these effects include destruction of activity, release of water-soluble polypeptides, and perturbation of the manganese complex.

Materials and Methods

Isolation of PS II membranes, free of Photosystem I and having high rates of oxygen evolution, was carried out as described in Ref. 3. Treatment of the PS II complex with $LaCl_3$ and other salts (see Table II) was carried out as described below. PS II membranes (2 mg Chl/ml) were dialyzed overnight at 4°C against a medium containing 0.4 M sucrose/50 mM Mes (pH 6.0), $LaCl_3$ and other salts (1 Vol. of PS II preparation/100 Vols. of dialysis medium). The dialyzed membranes were collected by centrifugation, washed once with the dialysis medium and were finally resuspended in a solution containing 50 mM Mes (pH 6.0)/0.4 M sucrose/20 mM NaCl. Complete release of the 17 and 23 kDa polypeptides was carried out by two high-salt washes; the procedure for high-salt treatment of PS II membranes is described in Ref. 25. Oxygen-evolution activity was followed using a Clark-type electrode and the polypeptide content of the membranes was examined by gel electrophoresis, which was carried out in the presence of 6.5 M urea. Determination of the Mn content of PS II preparations was carried out by use of EPR spectroscopy. Complete release of Mn from the photosynthetic membranes was carried out as follows: the PS II preparation was first incubated for 30 min in the dark in the presence of 5 mM NH_2OH (pH 6.0), and subsequently slightly acidified and heated for 5 min at 50°C. EPR spectroscopy was carried out with a Bruker ER-200D spectrometer operated at X-band and interfaced to a Nicolet 1180 computer.

Results and Discussion

Replacement of calcium by a lanthanide ion has proven to be a very effective method for studying the properties of various calcium binding proteins [24,26]. Cations like La^{3+} or Tb^{3+}, which have ionic radii close to that of Ca^{2+}, effectively substitute for calcium even though in most cases the resulting protein has no physiological activity [26]. As shown in Table I, incubation of PS II membranes with $LaCl_3$ inhibits oxygen-evolution activity (see also Ref. 27); when sufficient calcium is included in the incubation medium, full activity is retained. Of the cations tested, only calcium was effective in protecting activity against inhibition by the lanthanide (Table I). Figs. 1 and 2 present a titration of the lanthanum effect and the time course of inhibition, respectively. Note that when

TABLE I

EFFECT OF LANTHANIDES ON OXYGEN EVOLUTION ACTIVITY IN PS II PREPARATIONS

PS II membranes were incubated for 10 min at 25°C in a solution containing 0.4 M sucrose/10 mM NaCl/50 mM Mes (pH 6.0) (10 μg Chl/ml) and the salts indicated below. After incubation, the membranes were assayed for oxygen-evolution activity with 400 μM DCBQ as an acceptor. Control activity, 680 μmol O_2/h per mg Chl.

Additions	% activity
None	100
$LaCl_3$ (1 mM)	18
$LaCl_3$ (1 mM)+$CaCl_2$ (20 mM)	98
(i) $LaCl_3$ (1 mM); (ii) $CaCl_2$ (20 mM) [a]	16
$LaCl_3$ (1 mM)+$MgCl_2$ (20 mM)	20
$LaCl_3$ (1 mM)+$SrCl_2$ (20 mM)	24
$LaCl_3$ (1 mM)+$MnCl_2$ (20 mM)	14
$LaCl_3$ (1 mM)+NaCl (100 mM)	18
(i) $LaCl_3$ (1 mM)+NaCl (100 mM); (ii) $CaCl_2$ (20 mM) [a]	22

[a] 5 min incubation with $LaCl_3$ (1 mM) (±200 mM NaCl) followed by addition of $CaCl_2$ (20 mM) and another 5 min incubation.

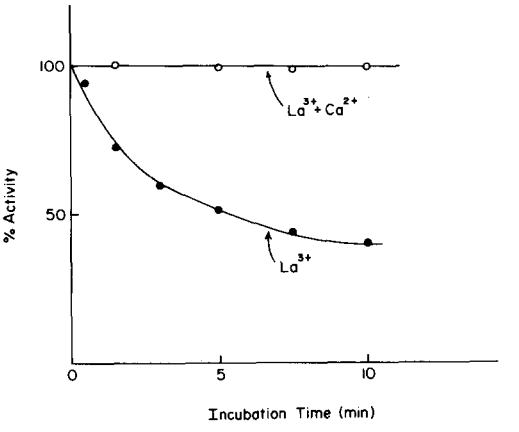

Fig. 2. PS II membranes (10 μg Chl/ml) were incubated for the indicated time at 25°C in a medium containing 0.4 M sucrose/10 mM NaCl/50 mM Mes (pH 6.0) and either 0.5 mM $LaCl_3$ (●———●) or 0.5 mM $LaCl_3$ + 20 mM $CaCl_2$ (○———○). After incubation, the membranes were assayed for oxygen-evolution activity in the presence of 400 μM DCBQ. Control activity: 680 μmol O_2/mg Ch per h.

$CaCl_2$ was added to PS II membranes previously treated with the lanthanide, no reactivation was observed (Table I); this observation indicates that an irreversible inhibition is induced by the lanthanide in the absence of calcium. Two other lanthanides, Ce^{3+} and Tb^{3+}, produced exactly the same effect as shown here for La^{3+} (data not shown).

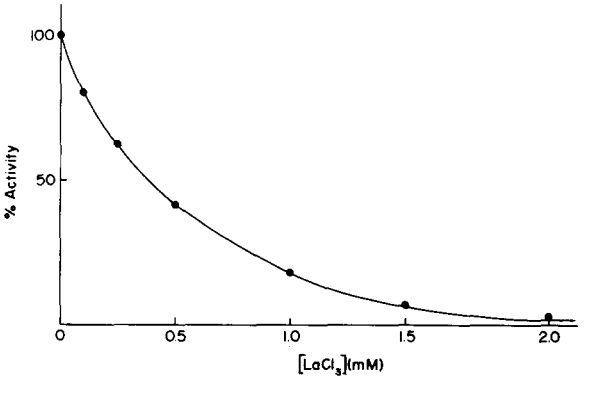

Fig. 1. PS II membranes (10 μg Chl/ml) were incubated for 10 min, at 25°C in the dark, in a medium containing 0.4 M sucrose/10 mM NaCl/50 mM Mes (pH 6.0) and the indicated concentration of $LaCl_3$, and then were assayed for oxygen-evolution activity in the presence of 400 μM DCBQ. Control activity, 680 μmol O_2/mg Chl per h.

In order to understand the mechanism by which lanthanum inhibits oxygen-evolution activity we next examined the manganese and polypeptide content of the PS II complex after it had been subjected to an extensive dialysis against $LaCl_3$. As shown in Fig. 3 and Table II, overnight dialysis against 2 mM $LaCl_3$ produces a system which has been depleted of the three water soluble polypeptides (17, 23 and 33 kDa), as well as 60% of the manganese. The presence of $CaCl_2$ (50 mM) during dialysis against $LaCl_3$ (2 mM) protects against release of the 33 kDa polypeptide and manganese (Table II), but not against release of the 17 and 23 kDa species (Fig. 3 shows that 10 mM calcium only partially protects against release of the 33 kDa species by 2 mM lanthanum); the resulting system behaves like the high-salt-treated PS II complex in that it is very active in the presence of calcium (Table II). We repeated the last dialysis treatment under conditions of low-ionic strength (0.5 mM $LaCl_3$ and 10 mM $CaCl_2$) and again, even though Mn and the 33 kDa polypeptide were retained, the two water-soluble polypeptides (17 and 23 kDa) were released (data not shown). Since

TABLE II

EFFECT OF LANTHANUM ON OXYGEN-EVOLUTION ACTIVITY, MANGANESE AND POLYPEPTIDE CONTENT OF THE PS II COMPLEX

See Materials and Methods section for conditions during treatment as well as techniques for determination of Mn and polypeptide content. Control activity: 680 μmol O_2/h per mg Chl. For assay of oxygen-evolution activity PS II membranes were transferred in a medium containing 0.4 M sucrose/10 mM NaCl/50 mM Mes (pH 6.0) (10 μg Chl/ml). An increase of the NaCl concentration to 200 mM had no appreciable effect on O_2 activity.

Additions in the dialysis medium	Bound polypeptides			Mn content (%)	% activity	
	17 kDa	23 kDa	33 kDa		+ $CaCl_2$ (10 mM)	− $CaCl_2$
None	+	+	+	100	100	100
$LaCl_3$ (2 mM)	−	−	−	42	8	6
$LaCl_3$ (2 mM)+$CaCl_2$ (50 mM)	−	−	+	94	80	30
$LaCl_3$ (2 mM)+NaCl (200 mM)	−	−	± [a]	86	8	4
$LaCl_3$ (2 mM)+NaCl (2 M)	−	−	−	98	6	0

[a] After this treatment, the PS II complex retains about 50% of the 33 kDa species.

the above experiments utilized a long dialysis period (overnight, 4°C), we next determined whether the experimental conditions of Table I (10 min incubation at 25°C with $LaCl_3 + CaCl_2$) would produce a release of the 17 and 23 kDa proteins. Using our previous observation that after removal of the 17 and 23 kDa prpoteins exogenous reductants, such as HQ, will reduce and destroy the manganese complex [28], it can be shown that incorporation of 0.5 mM HQ during the incubation with La^{3+} plus Ca^{2+} inhibits oxygen evolution (Table III); this finding suggests that the La^{3+}-plus-Ca^{2+} treatment releases the 17 and 23 kDa polypeptides, and in doing so exposes the Mn complex to the destructive effect of the reductant (reduction and subsequent release as free Mn^{2+}). Productive binding of functional manganese has been correlated with the presence of the 33 kDa

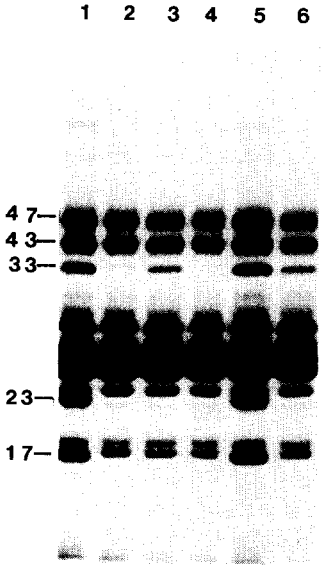

Fig. 3. Polypeptide content of the PS II complex after various treatments. (1) The untreated PS II complex; (2) PS II complex dialyzed overnight against 2 mM $LaCl_3$ (pH 6.0), (3) PS II complex dialyzed overnight against 2 mM $LaCl_3$/200 mM NaCl (pH 6.0); (4) PS II complex dialyzed against 2 mM $LaCl_3$/2 M NaCl (pH 6.0); (5) as (1), (6) PS II complex dialyzed against 2 mM $LaCl_3$/10 mM $CaCl_2$. Residual bands seen in the 17 kDa region are unrelated to the water-soluble species removed by La^{3+} (or high salt) treatment, and can be more clearly separated from the water-soluble protein if 2.5 M urea is included in the gel [16,28].

TABLE III

EFFECT OF HYDROQUINONE ON LANTHANIDE TREATED PS II MEMBRANES

PS II membranes were incubated for 10 min, at 25°C in the dark, in a buffer solution containing 0.4 M Sucrose/10 mM NaCl/50 mM Mes (pH 6.0) (10 μg Chl/ml) and the chemicals shown below (see Table I). After incubation, the membranes were assayed for oxygen-evolution activity in the presence of 800 μM DCBQ. Control activity: 680 μmol O_2/h per mg Chl.

Additions	% activity
$CaCl_2$ (20 mM)	100
$LaCl_3$ (2 mM)	4
$LaCl_3$ (2 mM)+$CaCl_2$ (50 mM)	92
$LaCl_3$ (2 mM)+$CaCl_2$ (50 mM)+HQ (0.5 mM)	14
$CaCl_2$ (50 mM)+HQ (0.5 mM)	94

protein and vice versa. Since Cl^- has been shown to protect against manganese release under conditions which perturb binding of the 33 kDa species [20,21], we repeated the dialysis against $LaCl_3$ in the presence of 200 mM NaCl. After such a treatment the PS II complex is missing only the 17 and 23 kDa polypeptides; partial release of the 33 kDa has also occurred. This preparation has very little activity even in the presence of calcium (see Table II and Fig. 3). Thus, even though the presence of 200 mM NaCl protects against complete release of the 33 kDa species and manganese is retained, replacement of calcium by lanthanum has resulted in a modified form of the PS II complex. Treatment of this modified PS II complex with a high-ionic strength solution (2 M NaCl) releases the 33 kDa polypeptide, but not Mn (Fig. 3 and Table II), an effect which is not observed in the intact PS II complex (see Ref. 16).

The experimental data presented above suggest that lanthanum has two discrete effects at the oxidizing side of Photosystem II. First, it causes release of the 17 and 23 kDa polypeptides; this inhibitory effect is observed even at low concentrations of the lanthanide ($\lesssim 1$ mM) and the presence of calcium during the treatment does not protect against release of the two proteins. The second effect of the lanthanide is destruction of the manganese complex with concomitant release of the 33 kDa species; in contrast to the first mode of inhibition, calcium completely protects the system against this inhibitory effect of the lanthanide. In order to examine further the mechanism of this calcium sensitive inhibitory effect of lanthanum, we used membranes which had been depleted of the 17 and 23 kDa species by two successive high-salt treatments. The oxygen evolving capacity of such a preparation is shown in Fig. 4, where the need for the presence of calcium in mM concentrations is demonstrated. In Fig. 5A we present a titration of the inhibition by lanthanum in the presence of various concentrations of $CaCl_2$; the

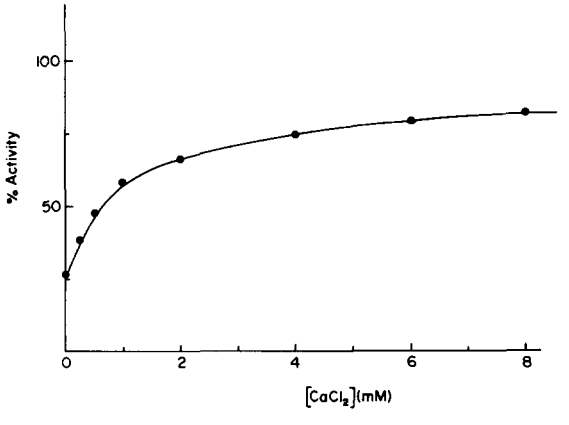

Fig. 4. Percent activity of PS II membranes, treated twice with 2 M NaCl, in the presence of various concentrations of $CaCl_2$. Salt-washed PS II membranes (10 μg Chl/ml) were incubated for 10 min (25°C) in a medium containing 0.4 M sucrose/10 mM NaCl/50 mM Mes (pH 6.0) and the indicated concentration of $CaCl_2$. After incubation, the membranes were assayed for oxygen-evolution activity in the presence of 400 μM DCBQ. Control activity: 640 μmol O_2/mg Chl per h.

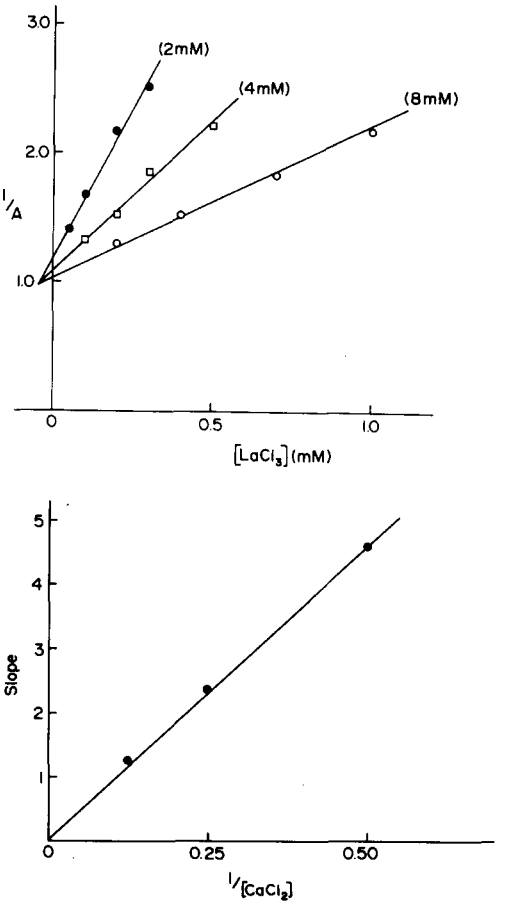

Fig. 5. (A) Inhibitory effect of $LaCl_3$ in the presence of 2 mM (u), 4 mM (□——□) and 8 mM $CaCl_2$ (O——O). Experimental conditions are the same as those in Fig. 1. (B) Graphic representation of the slope obtained from Fig. 5A vs. the inverse of the $CaCl_2$ concentration used.

data of Fig. 5A and B reveal a competitive inhibition by La^{3+} of the Ca^{2+} effect; the estimated constants are $K_I \approx 50$ μM for lanthanum and $K_M \approx 0.6$ mM for calcium.

We next examined the question of whether, after release of the 17, 23 and 33 kDa polypeptides by the $LaCl_3$ (2 mM)/NaCl (2 M) treatment, manganese remains at its native binding site. It has been previously shown that the microwave power saturation properties of Z^+, the donor to P-680, depend on the state of the manganese complex [3,29]. In intact systems or in inhibited systems in which the manganese complex has not been perturbed drastically (NH_3 or high-salt treated PS II preparations) Z^+ saturates at high microwave power (more than 100 mW), whereas when the manganese complex has been destroyed (for example, by Tris or NH_2OH) the manganese–Z^+ magnetic interaction no longer exists and Z^+ saturates at a lowered microwave power (approx. 25 mW). Fig. 6B shows the microwave power saturation properties of Z^+ after release of the 17, 23 and 33 kDa species, but not manganese; for comparison, its properties when all three water-soluble polypeptides and manganese have been removed are also shown. The fact that after treatment with $LaCl_3$ (2 mM)/NaCl (2M) manganese still interacts magnetically with Z^+ in a manner

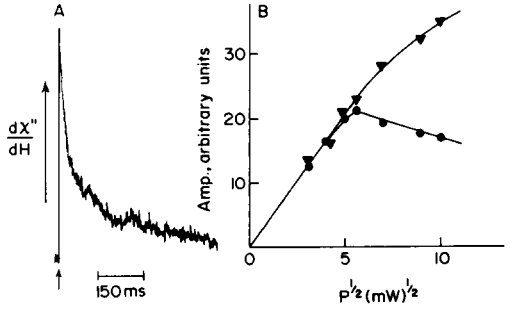

Fig. 6. (A) Kinetic trace of Z^+ rereduction in PS II membranes treated with 2 mM $LaCl_3$ plus 2 M NaCl (17, 23 and 33 kDa-depleted, but Mn-retainingf system). Instrument conditions; microwave power, 50 mW; time constant, 1 ms: time between flashes, 3 s; number of flashes averaged, 80. Acceptor system, 250 μM DCBQ plus 1 mM $Fe(CN)_6^{3-}$. (B) Microwave power saturation properties of Z^+ (a) after release of the 17, 23 and 33 kDa species but not manganese, by treatment with 2 mM $LaCl_3$/2 M NaCl (▲———▲); (b) after release of the 17, 23 and 33 kDa species as well as functional manganese, by treatment with 2 mM $LaCl_3$ (●———●).

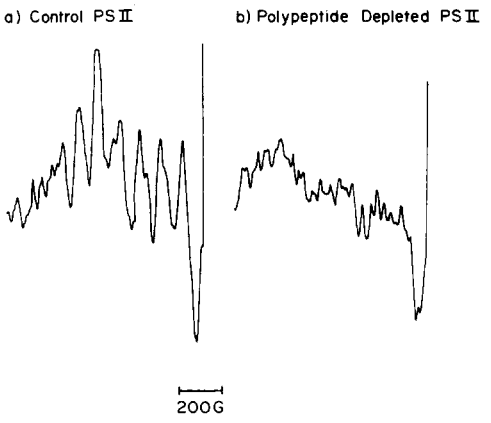

Fig. 7. EPR spectra of (a) intact PS II complex; (b) 2 mM $LaCl_3$ plus 2 M NaCl-treated PS II membranes, recorded at 10 K. PS II membranes (4 mg Chl/ml) were incubated for 4 h in the dark on ice, then illuminated for 3 min at 200 K and finally transferred at the EPR cavity. Microwave power, 100 mW; modulation amplitude, 15 gauss (peak-to-peak).

similar to that of the intact system indicates that manganese has remained in its native site even after removal of the 17, 23 and 33 kDa polypeptides. A kinetic study of Z^+ (Fig. 6A) in the above system revealed a decay time of approx. 30ms; this time probably reflects a back reaction between Q_A and Z^+, as is the case in Tris-treated PS II preparations (Ghanotakis D.F., unpublished data). Since the 30 ms reduction time in our experiments was obtained by averaging 80 successive flashes, we cannot exclude the possibility that Z^+ is initially reduced by some of the S-states before it starts back-reacting with Q_A. To examine this possibility the multiline ESR signal which has been correlated with the S_2-state was analyzed [30]. Fig. 7 shows that after removal of the 17, 23 and 33 kDa proteins (but not manganese) no multiline signal is observed under our experimental conditions; this suggests either that the system does not proceed to the S_2-state, or, alternatively, that S_2 does form, but the complex which gives rise to the multiline signal has been drastically modified (e.g., ligands, coupling, temperature of formation).

Conclusion

Our results may be summarized by the following points.

(1) Calcium and lanthanides compete with one another for binding site(s) on the oxidizing side of PS II. Replacement of calcium by a lanthanide under standard conditions of ionic strength (15 mM NaCl) releases the 33 kDa polypeptide and functional manganese, and oxygen evolution activity is inactivated.

(2) At elevated ionic strength (200 mM NaCl), exposure of the PS II complex to lanthanum inactivates the oxygen-evolving complex and releases the 17 and 23 kDa polypeptides; functional manganese and some of the 33 kDa protein are not released. At even higher ionic strength (2M NaCl), the 33 kDa protein is released from the PS II complex, but the pool of functional manganese remains EPR silent, bound either at its native site or at closely adjacent sites where the ion can affect the microwave power saturation properties of Z^+.

(3) Although coincident exposure of the PS II complex to calcium and lanthanum prevents the release of the 33 kDa polypeptide and functional manganese, and leaves activity intact (in the presence of added calcium), the presence of calcium during lanthanum exposure cannot prevent release of water-soluble 23 and 17 kDa polypeptides. It is possible that lanthanides compete with calcium for binding sites which affect the manganese complex and the 33 kDa protein, and in addition affect the interaction of the 17 and 23 kDa polypeptides with the PS II complex by a mechanism which at this point is not clear.

It is apparent from the results in this work that even though lanthanides, which have ionic radii similar to that of calcium, can replace calcium in the photosynthetic apparatus the Ca^{2+} requirement for O_2 evolution activity is highly specific. Speculating on the role of calcium at the oxidizing side of Photosystem II, it is very attractive to suggest a concanavalin-A-type organization; according to such a model calcium binds close to the manganese complex and affects its structure [31]. The presence of two closely interacting binding sites at the water-oxidation center, each specific for Mn^{2+} and Ca^{2+}, was also proposed by Ono and Inoue [15]. Replacement of calcium by lanthanum would result in dramatic cnformational changes in the system leading to destruction of the Mn complex. At this point it is not clear whether the site for which calcium and the lanthanide compete is on the 33 kDa protein or on another protein in the hydrophobic core complex, which is close to the 33 kDa polypeptide; further work must be done in order to answer this question. The preparation of a 17, 23 and 33 kDa-depleted PS II complex which retains manganese at or near its native site argues against the 33 kDa protein, being the only protein present in the PS II complex which is associated with the stability and function of the Mn complex. Using this preparation, we are investigating the protein(s) which bind(s) the manganese after release of the three water soluble polypeptides (17, 23 and 33 kDa).

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