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Calcium binding site(s) of Photosystem II as probed by lanthanides

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Lanthanides and calcium compete for binding sites on the oxidizing side of Photosystem II. When the trivalent ions replace calcium, the oxygen evolving complex is unable to proceed to the higher oxidation states. Oxygen-evolution activity can be reconstituted by removing the lanthanide and adding back calcium. The number of lanthanide atoms bound to PS II preparations with and without the 17 and 23 kDa species is the same. This is taken as evidence that the extrinsic 17 and 23 kD; do not bind lanthanide/calcium ions. A low-temperature EPR study, with both diamagnetic and paramagnetic lanthanide ions, has demonstrated a close interaction between the lanthanides and the tyrosine radical Y_D^+.

Introduction

The oxygen-evolving complex of Photosystem II (PS II) is a highly ordered structure of various polypeptides which provide the binding sites for the inorganic cofactors required for catalytic activity (reviewed in Ref. 1). Although it is now widely accepted that manganese, chloride and calcium are necessary for the sequential four-electron oxidation of water to molecular oxygen, their catalytic action is not well understood. Of the three types of ions involved in the photosynthetic splitting of water, manganese has been extensively characterized. It is now believed that the lower limit for Mn content in Photosystem II is four atoms per reaction center [2], and its direct involvement in the oxygen-evolving complex (OEC) has been clearly demonstrated (reviewed in Ref. 3). Various studies have placed the locus of Cl^- action within the OEC [3–7]. The third ion required for oxygen-evolution activity is Ca^{2+} [8,9]. The possible role of Ca^{2+} in the advancement of the S-state sequence of the OEC has been investigated by a series of different experimental approaches. Although various proposals have been made regarding its role, its binding site(s) has (have) not been characterized. In order to better understand the function of calcium within the OEC, we should determine which protein or proteins provide the ligands to the cation. In addition, we should obtain more information about its location with respect to the other redox components of Photosystem II.

Ca^{2+} interacts specifically with almost 100 proteins, and several reviews describe many cases [10,11]. Ca^{2+} lacks both electronic transitions that give rise to accessible absorption spectra and unpaired electrons for studies through magnetic resonance techniques. Due to these difficulties, various calcium binding enzymes have been studied through selective substitution of Ca^{2+} with other metal ions that elicit physically measurable phenomena. A series of trivalent lanthanide or rare earth cations have been used as suitable probes for Ca^{2+}-binding enzymes. Although there is one unit charge difference between the trivalent lanthanides (denoted in this communication as Ln^{3+}) and Ca^{2+}, the close similarity in their ionic radii seems to be the important factor in substitution experiments. Among the various lanthanides there are a few which are suitable for EPR experiments (e.g. Dy^{3+}, Gd^{3+}), others can be used in NMR experiments (e.g. Eu^{3+}, Yb^{3+})

Abbreviations: Chl, chlorophyll; Ln, lanthanide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen evolving complex; PS II, Photosystem II.

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[12], and, finally, one (Tb^{3+}) can be used as a sensitive probe of Ca^{2+}-binding proteins through luminescence experiments [13].

It was previously shown that calcium and lanthanides compete for binding sites at the oxidizing side of Photosystem II [14]. That particular discovery led Ghanotakis et al. [14] to suggest a Concanavalin A-type structure in which Ca^{2+} binds in close proximity to the Mn complex and affects its structure. Such a structural arrangement is supported by the recent EPR data from three different groups. It has been shown that inhibition of O_2 evolution by extraction of Ca^{2+} from PS II membranes causes a structural change of the manganese cluster as demonstrated by the formation of a new modified multiline signal, which is attributed to an S_2-oxidation state of the OEC. This structural change, generated either by Ca^{2+} extraction with citric acid at pH 3 [15,16] or by NaCl/EDTA washing [17], causes enhanced kinetic stability of the manganese cluster.

In the present work, we carried out a selective substitution of Ca^{2+} with various paramagnetic and diamagnetic lanthanide ions and studied their effect on the structural and catalytic properties of Photosystem II. Using EPR spectroscopy, we have been able to extract information about the relative location of Ca^{2+}-binding sites with respect to other redox species of the oxidizing side of PS II.

Materials and Methods

Photosystem II membranes were prepared as described in Ref. 18. Substitution of calcium by various lanthanide ions was carried out by the following procedure: intact PS II membranes (1 mg Chl/ml; 0.4 M sucrose, 15 mM NaCl and 50 mM Mes (pH 6.0)) were exposed for 45 min (in the dark at 4°C) to 2 M NaCl in the presence of 2 mM $LnCl_3$ (Ln: lanthanide in general). This treatment results in a PS II system which has all its calcium replaced by the lanthanide and moreover has been depleted of the extrinsic 17 and 23 kDa polypeptides (denoted as [Ln]'-PS II). In addition, the presence of high concentrations of NaCl during exposure to the lanthanide results in a system which retains all four manganese atoms (see Ref. 14 for details on the protective effect of NaCl). A decrease of the ionic strength by dialysis against a solution containing 50 mM Mes (pH 6.0) and 15 mM NaCl results in rebinding of both the 17 and 23 kDa proteins (denoted as [Ln]-PS II). If the rebinding of the 17 and 23 kDa species was preceded by a 2 mM EDTA treatment (2 h at 4°C, in the dark), a system which retains all three extrinsic proteins but contains neither calcium nor a lanthanide was prepared ([Ln/EDTA]-PS II). Tris treatment of the [Ln]-PS II and [Ln/EDTA]-PS II systems was carried out by exposure of the preparations to 0.8 M Tris (pH 8.2) (20 min at 4°C, at room light) (Tris[Ln]-PS II and Tris[Ln/EDTA]-PS II). All samples were resuspended in a solution containing 20 mM Mes (pH 6.0), 15 mM NaCl and 30% (v/v) ethylene glycol. Gel electrophoresis was carried out as described in Ref. 18. The oxygen-evolution activity of the various PS II preparations was measured by a YSI Clark-type oxygen electrode. The manganese and calcium content of the various preparations was determined by atomic absorption spectroscopy (Perkin-Elmer, model 4000). The number of Dy^{3+} per PS II was estimated by EPR spectroscopy. The peak height of the $g = 17$ Dy^{3+} EPR signal in Dy^{3+}-treated PS II was compared to a Dy^{3+} standard (0.20 mM Dy^{3+} in 15 mM NaCl, 20 mM Mes at pH 6.0, and 30% (v/v) ethylene glycol) to determine the Dy^{3+} concentration in the Dy^{3+}-treated PS II preparations. The data were collected using the following EPR spectrometer conditions: microwave frequency, 9.05 GHz; microwave power, 11 μW; field modulation frequency, 100 kHz; field modulation amplitude, 20 G; temperature, 7.0 K. The PS II concentration was determined by an EPR spin count of $Tyr_D^{\cdot}$ as described by Babcock et al. [25]. EPR scans were performed on a modified JEOL ME-3X X-band EPR spectrometer equipped with an Oxford ESR-900 liquid helium cryostat, as described in Ref. 19.

Results and Discussion

The following PS II preparations were investigated in this study (see Materials and Methods section for notation): intact PS II membranes (denoted as [Ca]-PS II), [La]-PS II, [Dy]'-PS II, [Dy]-PS II, [Dy/EDTA]-PS II, Tris[Dy]-PS II and Tris[Dy/EDTA]-PS II. As shown in Fig. 1, the [Dy]-PS II preparation, in which all of the Ca^{2+} is replaced by Dy^{3+}, retains all three water-soluble proteins (17, 23 and 33 kDa). The various preparations were characterized by spectroscopic and polarographic techniques; the results are summarized in Table I.

As shown in Table I, the [Ln]-PS II (Ln: La^{3+} or Dy^{3+}) and [Dy/EDTA]-PS II preparations retain all four atoms of the Mn complex but they are unable to oxidize water. When these preparations were incubated with 10 mM $CaCl_2$, we observed no reactivation of the [Ln]-PS II but the [Ln/EDTA]-PS II system recovered 43% of its oxygen-evolving capacity. The reactivation of the [Ln/EDTA]-PS II preparation was a slow process and it required an incubation for at least 10 min, at 25°C. The fact that external calcium failed to reactivate the [Ln]-PS II preparations is in agreement with the results of Ghanotakis et al. [14], which demonstrated that lanthanides bind to the PS II membrane much more strongly than calcium. In addition, the slow reactivation of the [Ln/EDTA]-PS II preparation (calcium-depleted/17 kDa + 23 kDa-re-

taining system) by external calcium was also observed in a previous study [20]; it was shown in that particular study that in the presence of the extrinsic 17 and 23 kDa species reactivation by external calcium required a relatively long time [20].

The various preparations were studied by low-temperature EPR spectroscopy in order to characterize their electron transport properties. As shown in Fig. 2, the Ln-treated systems ([La]-PS II and [Dy]-PS II) were unable to generate the S_2-state, as demonstrated by the absence of either the $g = 4.1$ or the multiline signal. Since cytochrome *b*-559 was low potential in the [La]-PS II and the [Dy]-PS II, and, thus, oxidized in the dark (see Fig. 2), illumination of these systems at 200 K resulted in the photooxidation of a Chl (Fig. 3). The chlorophyll radical was also generated during low-temperature illumination of the [Dy/EDTA]-PS II system (data not shown). Since the calcium reactivated [Dy/EDTA]-PS II system showed a significant fraction of oxygen evolution activity (Table I), we studied this system by low-temperature EPR spectroscopy. As shown in Fig. 2, after rebinding of calcium the reactivated system is able to proceed to the S_2 state, as demonstrated by the formation of the multiline signal (54% of the control). These results clearly demonstrate that depletion of the PS II membrane of all its calcium results in a system unable to proceed to higher S-states.

La^{3+} is a diamagnetic ion which is not detectable by EPR spectroscopy, but Dy^{3+} is a paramagnetic one which gives a characteristic EPR signal (Fig. 4). Using this signal, we estimated the number of the dysprosium atoms in the [Dy]-PS II samples (see Materials and Methods). As summarized in Table I, all calcium atoms were quantitatively replaced by the lanthanide.

It is interesting to note that the lanthanide-treated PS II membranes [Ln]-PS II (presence of the 17 and 23 kDa polypeptides) and [Ln]'-PS II (absence of the 17 and 23 kDa species) contain the same number of Dy^{3+} atoms/PS II reaction center (Table I), suggesting that the 17 and 23 kDa polypeptides do not bind Ca^{2+}.

Although the preparations [La]-PS II and [Dy]-PS II have basically the same properties, there is a major difference in the EPR signal of the dark-stable Tyr radical (due to Tyr_D^+) [21]. As shown in Fig. 3, in the presence of the ion La^{3+}, the EPR signal of Tyr_D^+ is the same as the one observed in control PS II membranes. In contrast, when calcium has been replaced by the paramagnetic ion Dy^{3+}, the EPR signal of the tyrosine radical is different; more specifically the hyperfine structure of the signal is lost, when observed under the same conditions. This observation shows that there is a strong interaction between the paramagnetic Dy^{3+} and Tyr_D^+ [22]. As expected, the power saturation of Tyr_D^+ signal, is affected by the interacting cation. In

TABLE I

Characterization of the PS II preparations (see Materials and Methods)

Preparation	Description [a] (extrinsic polypeptides)	Ca/PSII	Mn/PSII	Ln/PSII	V_{O_2}		S_2-state multiline		$P_{1/2}$ [c] (μW)
					$-CaCl_2$ [d]	$+CaCl_2$ [e]	$-CaCl_2$	$+CaCl_2$	(for Tyr_D^+)
Untreated PS II ([Ca]-PS II)	+17/+23/+33	15	4	–	100%	100%	100%	n.d.	8
[La]-PS II	+17/+23/+33	0	4	n.d. [f]	0	0	0	0	7
[Dy]-PS II	+17/+23/+33	0	4	14–15	0	0	0	0	–
[Dy]'-PS II	−17/−23/+33	0	4	13–14	0	0	0	0	–
[Dy/EDTA]-PS II	+17/+23/+33, EDTA-washed	0	4	0	0	43%	0	54% [g]	–
Tris[Dy]-PS II	−17/−23/−33	½	0	1–3	0	0	0	0	97
Tris[Dy/EDTA]-PS II	−17/−23/−33	0	0	0	0	0	0	0	3

[a] Extrinsic polypeptide content of each PS II preparation: ±17/±23/±33 refers to the presence (+) or absence (−) of the 17, 23, and 33 kDa polypeptides, respectively.

[b] The amount of S_2-state multiline EPR signal produced in untreated PS II after 5 min, 200 K continuous illumination period was assumed to be 100%.

[c] Comparison of power saturation data ($P_{1/2}$ values) for EPR signal IIs. See ref. 22 for the analysis procedure of the power saturation data.

[d] The control O_2-evolution activity was 720 μmol O_2/mg Chl per h, in the absence of additional $CaCl_2$.

[e] Prior to the O_2-evolution measurement, the PS II membranes (7 mg Chl/ml) were incubated with 10 mM $CaCl_2$ for 10 min (in the dark at 25°C). The assay medium consisted of 0.4 M sucrose, 15 mM NaCl, 50 mM Mes (pH 6.0) and 250 μM DCBQ (as an exogenous electron acceptor).

[f] n.d. = not determined.

[g] After the removal of Dy^{3+} by EDTA treatment (see Materials and Methods), the PS II membranes were incubated with 10 mM $CaCl_2$ for 90 min at 4°C in the dark. Subsequently, a 5 min, 200 K continuous illumination period was used to produce the S_2-state multiline EPR signal.

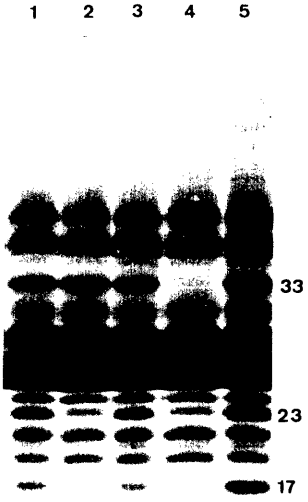

Fig. 1. Gel electrophoresis patterns of PS II membranes. Lane 1, [Dy]-PS II; lane 2, $[Dy]^{3+}$-PS II; lane 3, [Dy/EDTA]-PS II; lane 4, Tris[Dy/EDTA]-PS II; lane 5, control PS II (see Materials and Methods).

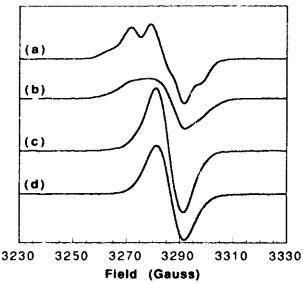

Fig. 3. Comparison of the $g = 2$ radical EPR signals in dark-adapted and illuminated [La]-PS II and [Dy]-PS II: (a) dark-adapted [La]-PS II, (b) dark-adapted [Dy]-PS II, (c) illuminated (200 K, 3 min) minus dark difference spectrum of [La]-PS II, and (d) illuminated (200 K, 3 min) minus dark difference spectrum of [Dy]-PS II. Spectra are scaled to a chlorophyll concentration of 5 mg Chl/ml. Tabulated below are the areas of the $g = 2$ EPR signals before and after illumination, as determined by signal integration of spectra (a)–(d) and normalized for the chlorophyll concentration.

Sample	Area (a.u.)	Peak-to-peak linewidth (G)
[La]-PS II dark-adapted	1.00	
[Dy]-PS II dark-adapted	0.74	
[La]-PS II illum.-dark	0.84	10.6
[Dy]-PS II illum.-dark	0.65	10.6

EPR spectrometer conditions: microwave frequency, 9.05 GHz; microwave power,0.72 μW; field modulation frequency, 100 kHz; field modulation amplitude, 4.0 G; temperature, 15.0 K.

the presence of the paramagnetic Dy^{3+}, the EPR signal of the tyrosine radical saturates at higher microwave power, when compared to the control and the

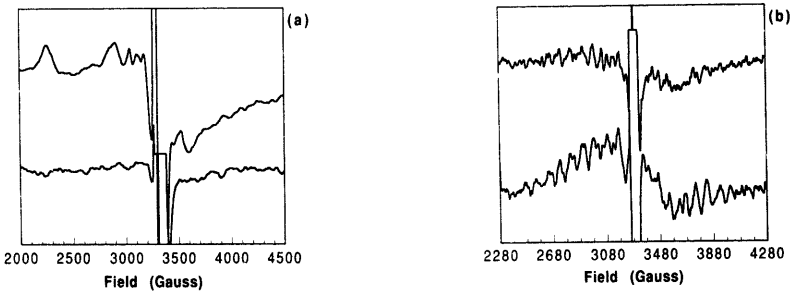

Fig. 2. The effect of Dy^{3+} substitution and Ca^{2+} reconstitution on the S_2-state multiline EPR signal in PS II membranes. Shown in (a) are the dark-adapted spectrum (top) and illuminated (200 K, 5 min) minus dark difference spectrum (bottom) of [Dy]-PS II membranes. Shown in (b) are the illuminated (200 K, 5 min) minus dark difference spectra of [Dy/EDTA]-PS II membranes reconstituted with 10 mM $CaCl_2$ (top spectrum; see Materials and Methods) and of a control PS II sample (bottom spectrum). EPR spectrometer conditions: microwave frequency, 9.05 GHz; microwave power, 7.2 μW and 18.1 μW for (a) and (b), respectively;; field modulation frequency, 100 kHz; field modulation amplitude, 20 G; temperature, 7.0 K. Spectra in (b) are scaled to a chlorophyll concentration of 5 mg Chl/ml.

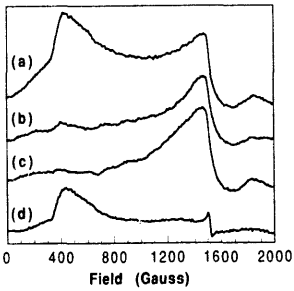

Fig. 4. Comparison of the $g = 17$ Dy^{3+} EPR signal in dark-adapted, Dy^{3+}-substituted (Ca^{2+}-depleted) PS II membranes: (a) [Dy]-PS II with 14–15 Dy^{3+} atoms/PS II reaction center, (b) Tris[Dy]-PS II with 1–3 Dy^{3+} atoms/PS II reaction center, (c) Tris[Dy/EDTA]-PS II after removal of Dy^{3+} by EDTA, and (d) Dy^{3+} standard (0.20 mM Dy^{3+}; see Materials and Methods for determination of the Dy^{3+} concentration in [Dy]-PS II preparations). EPR spectrometer conditions: microwave frequency, 9.05 GHz; microwave power, 11.4 μW; field modulation frequency, 100 kHz; field modulation amplitude, 20 G; temperature, 7.0 K. Spectra (a)–(c) are scaled to a chlorophyll concentration of 5 mg Chl/ml.

[La]-PS II preparation (see Table I). Apparently, Dy^{3+} acts as a relaxing species and, thus, affects the power saturation of the neighboring radical. A similar effect was observed on the power saturation of Tyr_Z^+ in intact PS II membranes, because of the magnetic coupling between Tyr_Z^+ and the manganese complex [23].

Although it has been proposed that only two calcium atoms per reaction center are required for oxygen- evolution activity [24], spinach PS II membranes which have not been treated with chelators such as EGTA or EDTA contain a high number of calcium atoms (10–15/PS II reaction center). Thus, it is not surprising that the [Dy]-PS II preparation contains 14–16 Dy atoms/PS II reaction center. The number of Dy atoms/PS II was drastically decreased when the [Dy]-PS II system was treated with a high concentration of Tris buffer. Tris treatment not only destroyed the Mn complex and released the three extrinsic polypeptides, but also removed most of the Dy^{3+} atoms. A Dy^{3+} quantitation revealed the presence of 1–3 atoms/PS II in the Tris[Dy]-PS II preparation. At this point, it is not clear whether the decrease in the number of Dy^{3+}/PS II is due to the release of the extrinsic polypeptides or the destruction of the manganese complex. As shown in Fig. 5, the interaction between the paramagnetic Dy^{3+} and the Tyr_D^+ radical is also observed in the Tris[Dy]-PS II system. Although we can not say with certainty which calcium site is responsible for the effect on Tyr_D^+, our results clearly show that the site exists in Tris-washed PS II and has a high affinity for the lanthanides. Since it has been demonstrated that removal of the manganese complex destroys the low-affinity calcium site [26], and as stated above we still observe the Dy^{3+} effect in Tris-washed [Dy]-PS II mambranes, the effect of Dy^{3+} on Tyr_D^+ is most likely due to Dy^{3+} binding at the high affinity calcium site. The effect on Tyr_D^+ by Dy^{3+} indicates that there is a magnetic interaction between the two species, which could occur over a distance of up to 40 Å; at this point we are working to obtain a good estimate of this distance.

Using various lanthanide ions as a probe for the calcium binding site(s) of Photosystem II, we have been able to obtain new information regarding the involvement of Ca^{2+} in the photosynthetic process. The main conclusions of the experimental work presented above are the following: trivalent lanthanide ions bind very tightly to Photosystem II at Ca^{2+}-binding sites without displacing functional Mn; substitution of calcium by lanthanides results in a PS II system which does not evolve oxygen and O_2-evolution activity can be reconstituted by removing the Ln^{3+} and adding back Ca^{2+}. The size of the ion seems to be important for its binding on Photosystem II [20], but its charge is probably very important for the proper function of the OEC. Although it has been shown that another divalent ion,

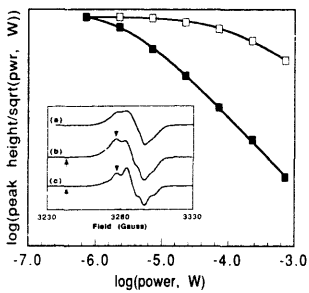

Fig. 5. Comparison of the continuous microwave power saturation curves of EPR signal II_s in Tris[Dy]-PS II, containing 1–3 Dy^{3+} atoms/PS II reaction center (open squares), and Tris[Dy/EDTA]-PS II containing 0 Dy^{3+}/PS II reaction center (closed squares). The data points are fit as described in Ref. 22. EPR spectrometer conditions: microwave frequency, 9.05 GHz; field modulation frequency, 100 kHz; field modulation amplitude, 4.0 G; temperature, 15 K. The data are normalized to the peak height of EPR signal II_s at the data point at lowest power. Inset: Comparison of Signal II_s EPR signals observed in dark-adapted, spinach PS II membranes, showing the effect of Dy^{3+}-binding in Ca^{2+}-depleted PS II: (a) [Dy]-PS II with 14–16 Dy^{3+} atoms/PS II reaction center, (b) Tris[Dy]-PS II with 1–3 Dy^{3+} atoms/PS II reaction center, and (c) Tris[Dy/EDTA]-PS II after removal of Dy^{3+} by EDTA with 0 Dy^{3+} atoms/PS II reaction center. The intensity of Signal II_s was measured as peak-to-baseline height as indicated by the arrows. EPR spectrometer conditions: microwave frequency, 9.05 GHz; microwave power, 0.72 μW; field modulation frequency, 100 kHz; field modulation amplitude, 4.0 G; temperature, 15.0 K. Spectra are scaled to a chlorophyll concentration of 5 mg Chl/ml.

Sr^{2+}, is able to support some activity [20], trivalent lanthanide ions, which effectively bind in place of calcium, inhibit the advancement of the enzyme to the higher S states. At this point, it is not clear whether this inhibition is the effect of the trivalent lanthanide ions on the manganese complex or on the tyrosine radical $Y_Z^{\cdot}$. An effect on the structure of the manganese complex would be an attractive model, but we cannot exclude the possibility of an $Ln^{3+} \leftrightarrow Tyr_Z^{\cdot}$ interaction which could influence the redox properties of the tyrosine species. More experiments are now in progress in order to investigate further the interaction of the lanthanide ions with the manganese complex and the other components of Photosystem II.

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