Europium- and Dysprosium-Ions as Probes for the Study of Calcium Binding Sites in Photosystem II

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Trivalent lanthanide cations are suitable probes for Ca^{2+}-binding sites in photosystem II (PS II). PS II membranes prepared from *Nicotiana tabacum*, intact and depleted of the extrinsic polypeptides were exposed to lanthanide ions (Dy^{3+} and Eu^{3+}). Small concentrations of dysprosium and europium ions enhance oxygen evolution under short saturating flashes. Higher concentrations of the rare earth cations cause the release of the three extrinsic peptides (17, 23 and 33 kDa) and reduce O_2 yield. The reactivation of the PS II membranes, thus depleted of the 33 kDa subunit, by Ca^{2+} ions is not possible. Comparing Eu^{3+} with Dy^{3+} in this effect shows that Eu^{3+} is more effective than Dy^{3+}, because a lower Eu^{3+}-concentration in comparison to that of Dy^{3+} inactivates O_2-evolution. The differences between europium and dysprosium can be explained by their different ionic radius. Our results suggest the existence of two Ca-binding regions: one with a low affinity for calcium would be located on the contact surface of the 23 and 33 kDa proteins and the second one with a high affinity, should be located close to the Mn-cluster and to tyrosine-161 (Z). The more tightly-bound calcium would be responsible for the activity of the PS II system.

Introduction

There are three electron transferring complexes in the thylakoid membrane: photosystem II (PS II), photosystem I (PS I) and the cytochrome *b/f* complex (Govindjee and Coleman, 1993; Golbeck and Bryant, 1991; Cramer *et al.,* 1991). Considerable efforts have been directed towards the understanding of the electron transfer reactions, especially within the photosystem II complex which contains the oxygen-evolving complex (OEC).

Electrons removed from water by a transiently oxidized tetranuclear manganese complex are transferred to an intermediate "one-electron carrier" (*Tyr/Z*) which is the electron donor to the oxidized reaction center chlorophyll P680. Light induces charge separation, P680 is oxidized and pheophytin (Pheo) is reduced. Thereafter the reduction of the acceptor-side quinones (Q_A and Q_B) follows (Govindjee and Coleman, 1993; Babcock, 1987). The reaction of photosynthetic water-splitting is located in the luminal side of the thylakoid membrane (Åkerlund and Jansson, 1981). Two intrinsic polypeptides D_1 an D_2 of 32 kDa and 34 kDa respectively have been suggested to constitute the core of the PS II reaction center. The function of several extrinsic polypeptides belonging to a functional PS II complex is not entirely clear yet. However, recent studies gave evidence that three polypeptides 33 kDa, 24 kDa and 17 kDa are involved in the process of transferring electrons from water on the donor side of PS II (Mavankal *et al.,* 1986). The 33 kDa subunit appears to be the most essential extrinsic polypeptide (Homann, 1987). The highly ordered structure of the polypeptides is supposed to provide the binding sites for inorganic cofactors such as Cl^- and Ca^{2+}, required for the catalytic activity of PS II. It is widely accepted now, that manganese, chloride and calcium are necessary for the sequential four-electron water oxidation leading to molecular oxygen. Only the role of the 4 manganese is defined, whereas the mode of functioning of Cl^- and Ca^{2+} is still unknown.

Abbreviations: Hepes, N-(2-hydroxyethyl)-piperazine-N′-2-ethane sulfonic acid.

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The requirement of Ca^{2+} for the reaction was demonstrated by Piccioni and Mauzerall, 1976. They showed that calcium ions are involved in PS II electron transport of cyanobacteria. Ono and Inoue reported (1983, 1984) a Ca^{2+} requiring state of PS II in intact chloroplasts, isolated from wheat. It has been suggested that calcium is very important for the incorporation and function of Mn. The same has been shown by Pistorius and Schmid (1979) in experiments with thylakoid preparations of cyanobacteria. Recent investigations (for a review, see Yocum, 1991) with high-salt treated PS II-membranes support the importance of Ca^{2+} cations in the process of oxygen evolution.

Calcium has been shown to play a role in controlling proteins by switching on and off their activity and by maintaining their three dimensional structure (Gerady *et al.*, 1988). In the study of Ca^{2+}-binding sites lanthanides have been successfully used, having a high affinity for the specific binding sites of calcium. The substitution of Ca^{2+} ions by other cations has already been introduced into OEC studies. Displacement of calcium by lanthanides produces destruction of activity and perturbation of the manganese complex (Ghanotakis *et al.*, 1985; Bakou and Ghanotakis, 1993).

In the present work, we substituted Ca^{2+} by Eu^{3+} or Dy^{3+} in intact and high-salt treated PS II membranes. The 17 kDa and 23 kDa polypeptides were removed by NaCl washing and the 33 kDa subunit is removed by $CaCl_2$ or $MgCl_2$ washing. The present investigation on oxygen evolution under short saturating flash illumination allowed us to localize sites with high and low affinity for calcium binding, which influence the functionality of the Mn-complex. The reason for the choice of dysprosium and europium is that they have isotopes (^{161}Dy and ^{151}Eu) suitable for Mössbauer spectroscopy. The method will allow a future investigation to observe directly valence states of the ions and to distinguish different binding sites of lanthanides.

Materials and Methods

Photosystem II particles were isolated from *Nicotiana tabacum* var. John William's Broadleaf (JWB) according to the method of Berthold *et al.*, 1981 (with minor modifications), using a Triton X-100 to chlorophyll (Chl) ratio of 20:1 (omitting the second Triton washing). Tetra-methylammonium was used instead of sodium chloride in the reaction buffers in order to avoid the inhibition of the calcium-binding site responsible for the activation of the oxygen-evolving complex (Waggoner *et al.*, 1989). The PS II membranes were suspended in a buffer containing 400 mM sucrose, 20 mM Hepes (N-(2-hydroxyethyl)-piperazine-N′-2-ethane sulfonic acid), 5 mM $MgCl_2$ and 15 mM $(CH_3)_4NCl$ (pH 6.5).

The 17 kDa and 23 kDa proteins were removed from PS II membranes by incubation (30 min in darkness, on ice) with a buffer containing 1.5 M NaCl, 400 mM sucrose, 40 mM Hepes and 5 mM $MgCl_2$ (pH 6.5) in samples containing 1 mg Chl/ml. The washed particles were resuspended in a buffer containing 400 mM sucrose, 20 mM Hepes, 5 mM $MgCl_2$ and 15 mM $(CH_3)_4NCl$ (pH 6.5), after two washings with the same medium.

In other experimental series the 17 kDa, 23 kDa and 33 kDa proteins were removed from the PS II particles by incubation (30 min. in darkness, on ice) in a buffer containing 1.5 M $CaCl_2$ (or $MgCl_2$), 400 mM sucrose, 40 mM Hepes and 5 mM $MgCl_2$ (pH 6.5) with samples containing 1 mg Chl/ml. After two washings in a buffer containing 400 mM sucrose, 20 mM Hepes, 5 mM $MgCl_2$ and 15 mM $(CH_3)_4NCl$ (pH 6.5) the particles were resuspended in the same medium. Only freshly prepared PS II particles were used for experiments.

Amperometric measurements of oxygen evolution as the consequence of short saturating light flashes were carried out with the "Three Electrode System" described by Schmid and Thibault (1979). Light flashes were produced by a xenon lamp (Stroboscope 1539A of General Radio). The flash duration at half intensity was 8 μs. 15 flashes, spaced 300 ms apart, were given.

The reaction buffer (400 mM sucrose, 10 mM Hepes, 5 mM $MgCl_2$ and 15 mM $(CH_3)_4NCl$; pH 6.5) contained, in a total volume of 0.6 ml, about 26 μg chlorophyll. Cations of lanthanides in the form of $EuCl_3$ or $DyCl_3$ in solution and calcium as $CaCl_2$ were added as indicated in the legends of the figures. The samples were incubated for 2 min on ice and then 5 min at room temperature in darkness before putting them on the electrode. The dark adaptation period (on the electrode) was 10 min.

Results

It has been shown that lanthanides can selectively replace calcium in PS II. The substitution of lanthanides causes an inhibition of oxygen evolution as reported by Bakou *et al.* (1992). PS II membranes, untreated as well as depleted of the extrinsic 17 kDa, 23 kDa polypeptides or of all three extrinsic proteins (17 kDa, 23 kDa and 33 kDa), then exposed to lanthanide ions (Dy^{+3} and Eu^{+3}), were studied for oxygen evolution under short saturating flashes by using the "Three Electrode System" of Schmid and Thibault (1979).

As has been reported already earlier and as will be shown again in a forthcoming paper (Burda and Schmid, in preparation), external acceptors can siginificantly change the oscillation pattern of oxygen evolution, especially when the pH of the medium is changed. The lack of acceptor causes in our measurements, as already reported by Kok 25 years ago, the typical decrease of oxygen evolution with increasing flash numbers (Fig. 1). Figure 1 gives the sequence of oxygen yield detected in untreated PS II membranes and in preparations depleted of the extrinsic peptides. Depletion of two extrincic polypeptides (17 and 23 kDa) was achieved by washing with NaCl and depletion of all three extrinsic peptides (17, 23 and 33 kDa) by washing of the PS II particles with $CaCl_2$ or $MgCl_2$. The pH of the reaction buffer was always 6.5. It is clearly seen that oxygen evolution in the $CaCl_2$-washed preparations is very low, whereas washing with $MgCl_2$ leaves the signal pattern at a slightly higher level. Oxygen evolution is 2.4 times higher in the latter case, but still only about 25% of the oxygen yield of intact particles. The PS II membranes washed with NaCl exhibit oxygen evolution which is about 75% of the unwashed sample. In this case the pattern of O_2 yield is unchanged when compared to the untreated photosystem II particle, whereas the oscillations are practically fully damped in the system depleted of all three extrinsic proteins. Moreover, the ratio of the fourth peak to the third one increases.

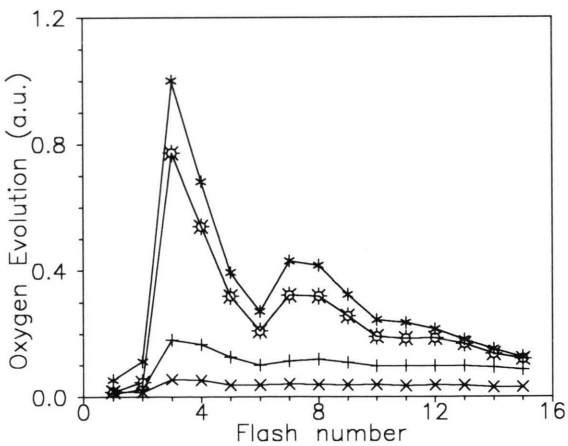

Fig. 1. Flash-induced oxygen evolution measured with photosystem (PS) II membrane preparations from *Nicotiana tabacum* John William's Broadleaf. *, Untreated PS II membranes; ✳, PS II membranes depleted of the 17 kDa, 23 kDa polypeptides by washing with NaCl; +, PS II membranes depleted of the 17 kDa, 23 kDa and 33 kDa polypeptides by washing with $MgCl_2$; ×, PS II membranes depleted of the 17 kDa, 23 kDa and 33 kDa polypeptides by washing with $CaCl_2$. All amplitudes are normalized to the amplitude of O_2-evolution after the third flash of untreated PS II membranes.

Treatment with Ca^{2+} ions

The dependence of oxygen evolution on different Ca^{2+} concentrations in intact PS II membranes and that in particles depleted of the 17 kDa, 23 kDa or the 33 kDa peptides is shown in Fig. 2. The untreated PS II particles have the highest amplitudes of O_2 evolution after incubation in darkness (on ice) with 0.8 μM $CaCl_2$. The optimal condition for the photosystem II particle preparation washed with 1.5 M NaCl was apparently found when 0.4 μM $CaCl_2$ were added. In the case of preparations washed with $MgCl_2$ the highest concentration of $CaCl_2$ leads to the highest oxygen yield, whereas the addition of calcium did not significantly change the amount of released oxygen of PS II membranes, washed with $CaCl_2$. With increasing concentrations of calcium ions, the ratio of the third to the fourth flash amplitude increases. The general observation is that an increasing amount of $CaCl_2$ in the reaction medium improves oscillations (they are less damped) and increases the ratio of the third peak to the fourth one.

Treatment with Dy^{3+} ions

The assays were incubated during 2 min (in darkness, on ice) with the following concentrations of dysprosium chloride: 0.2 μM, 0.4 μM, 0.8 μM and 1.6 μM. Fig. 3 shows the oscillatory pattern of oxygen evolution in amplitudes which are normalized

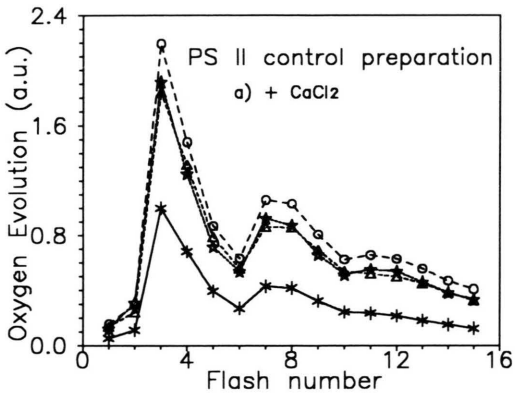

Fig. 2A

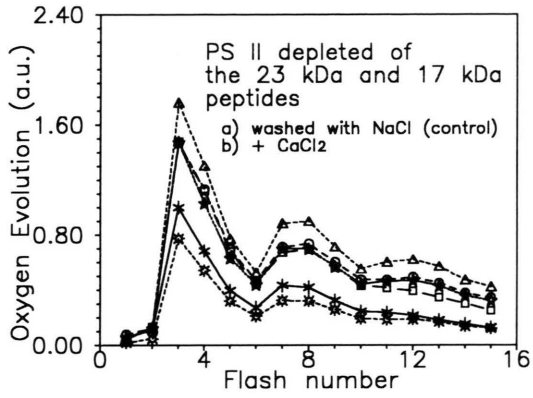

Fig. 2B

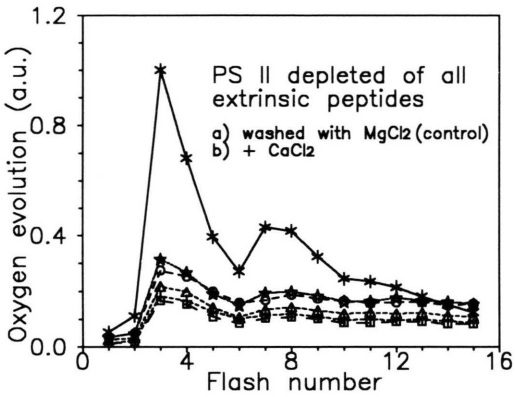

Fig. 2C

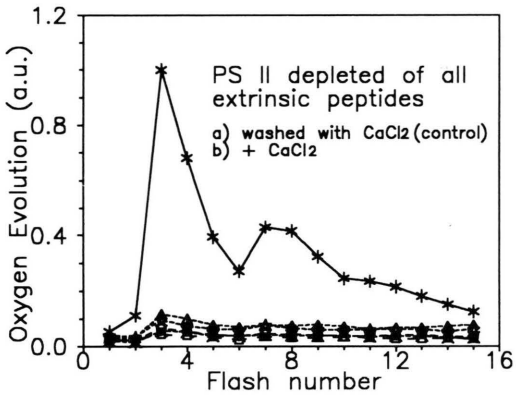

Fig. 2D

Fig. 2. Dependence of oxygen evolution as consequence of short saturating flashes in: **A,** intact photosystem (PS) II membranes (*), data shown in Fig. 2A, 2B, 2C and 2D; **B,** membranes washed with 1.5 M NaCl (✳), data shown in Fig. 2B; **C,** membranes washed with 1.5 M $MgCl_2$ (+), data shown in Fig. 2C; **D,** membranes washed with 1.5 M $CaCl_2$ (×), data shown in Fig. 2D. Conditions **A , B, C** and **D** were treated with the following $CaCl_2$ concentrations: 0.2 μM $CaCl_2$ (□), 0.4 μM $CaCl_2$ (△), 0.8 μM $CaCl_2$ (○) and 1.6 μM $CaCl_2$ (☆). Data are shown in Fig. 2A, 2B, 2C and 2D respectively. All amplitudes are normalized to the amplitude of O_2-evolution after the third flash of untreated PSII membranes.

to the O_2-evolution amplitude of the third flash in untreated PS II membranes. In the samples treated with high-salt washing, increasing concentrations of Dy^{3+} causes a decrease of O_2-amplitudes and a damping of oscillations. Dysprosium cations almost completely inactivate the system washed with $CaCl_2$. Even extra calcium ions cannot reverse this. PS II membranes washed with $MgCl_2$ are inactivated if 1.6 μM of Dy^{3+} is added to the medium and were not reactivated by Ca^{+2} (at least not during the 2 min of incubation, in darkness, on ice). The oscillatory pattern of oxygen evolution in PS II membranes washed with NaCl in the presence of increasing concentrations of dysprosium cations resembled that of preparations washed with $MgCl_2$ (Fig. 3B). The same is true for the pattern in untreated preparations after introduction of Dy^{3+} into the medium. The remarkable observation is that addition of 0.2 μM $DyCl_3$ causes an increase of the signal amplitude

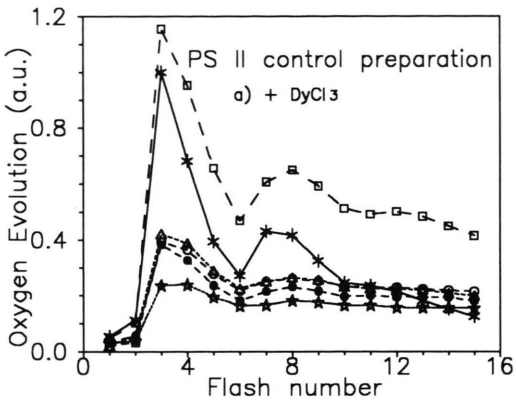

Fig. 3A

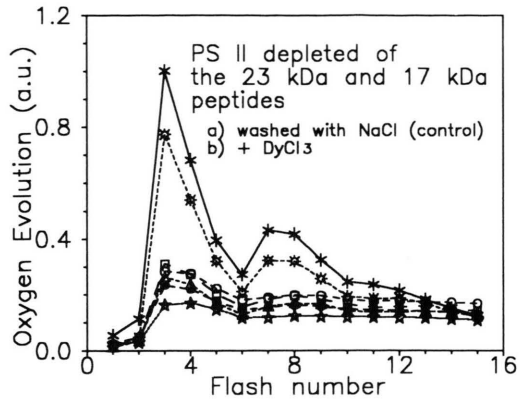

Fig. 3B

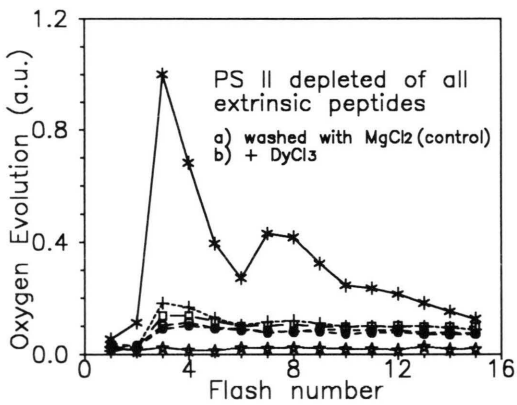

Fig. 3C

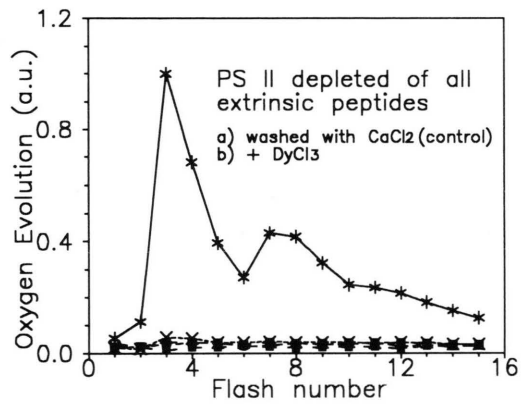

Fig. 3D

Fig. 3 Dependence of oxygen evolution as consequence of short saturating flashes in: **A,** intact photosystem (PS) II membranes (*), data shown in Fig. 3A, 3B, 3C and 3D; **B,** membranes washed with 1.5 M NaCl (✳), data shown in Fig. 3B; **C,** membranes washed with 1.5 M $MgCl_2$ (+), data shown in Fig. 3C; **D,** membranes washed with 1.5 M $CaCl_2$ (×), data shown in Fig. 3D. Conditions **A, B, C** and **D** were treated with the following $DyCl_3$ concentrations: 0.2 μM $DyCl_3$ (□), data shown in Fig. 3A, 3B, 3C and 3D; 0.4 μM $DyCl_3$ (△), data shown in Fig. 3A and 3B; 0.8 μM $DyCl_3$ (○), data shown in Fig. 3A, 3B and 3C; 1.6 μM $DyCl_3$ (☆), data shown in Fig. 3A, 3B and 3C. In addition 1.0 μM $CaCl_2$ were added after 2 min to the samples treated with 0.8 μM $DyCl_3$ (●), data shown in Fig.3A, 3B, 3C and 3D or 4.8 μM $CaCl_2$ were added after 2 min to the samples treated with 0.8 μM $DyCl_3$ (■), data shown in Fig. 3D. All amplitudes are normalized to the amplitude of O_2 -evolution after the third flash of untreated PSII membranes.

of oxygen evolution and changes the pattern in the sense that the ratio of the third amplitude to the fourth is decreased.

Treatment with Eu^{3+} ions

As shown in Fig. 4, incubation of intact PS II membranes with 0.2 μM $EuCl_3$ leads to a roughly two-fold increase of oxygen evolution amplitudes and an improvement of the oscillatory behaviour when compared to the corresponding treatment with $DyCl_3$ or $CaCl_3$. The oscillatory pattern itself remains apparently unchanged. The same concentration of europium cations inactivates preparations depleted of the extrinsic polypeptides. In the particle preparations washed with $CaCl_2$ and $MgCl_2$ the inactivation is irreversible, whereas the preparation which still contains the 33 kDa sub-

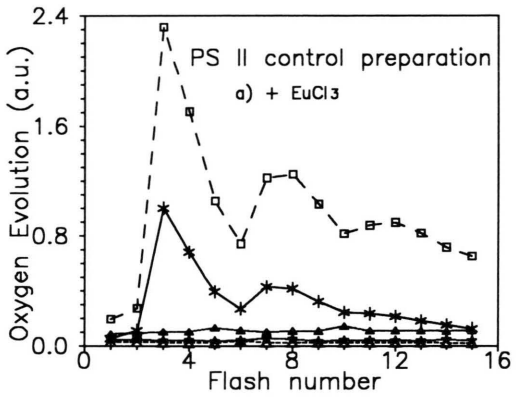

Fig. 4A

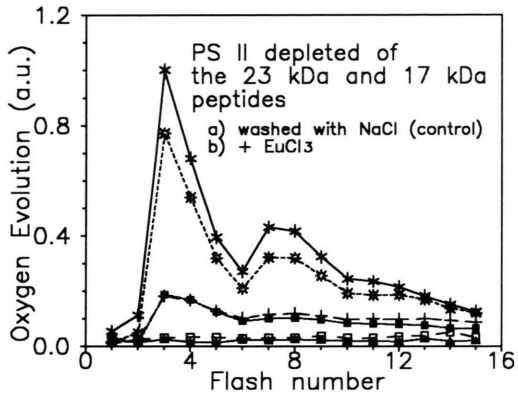

Fig. 4B

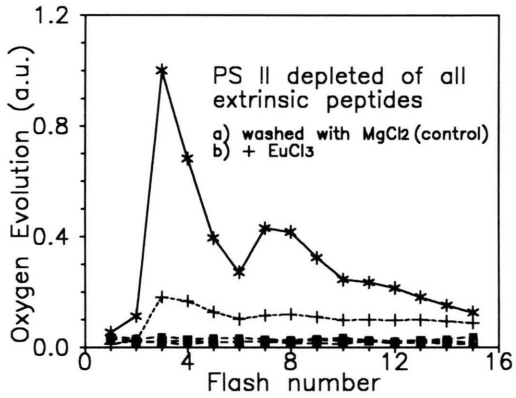

Fig. 4C

Fig. 4. Dependence of oxygen evolution as consequence of short saturating flashes in: **A,** intact photosystem (PS) II membranes (*) , data shown in Fig. 4A, 4B, 4C and 4D; **B,** membranes washed with 1.5 M NaCl (✳), data shown in Fig. 4B; **C,** membranes washed with 1.5 M $MgCl_2$ (+), data shown in Fig. 4C. Conditions **A, B** and **C** were treated with the following $EuCl_3$ concentrations: 0.2 μM $EuCl_3$ (□), data shown in Fig. 4A, 4B and 4C, 0.4 μM $EuCl_3$ (△), data shown in Fig. 4A. Then 1.0 μM $CaCl_2$ were added after 2 min to the samples treated with 0.2 μM $EuCl_3$ (▲) – data respectively shown in Fig. 4B and 4C or 4.8 μM $CaCl_2$ were added after 2 min to the sample treated with 0.2 μM $EuCl_3$ (■), data shown in Fig. 4B and 4C or 1.0 μM $CaCl_2$ were added after 2 min to the samples treated with 0.4 μM $EuCl_3$ (▲), data shown in Fig. 4A or 4.8 μM $CaCl_2$ were added after 2 min to the sample treated with 0.4 μM $EuCl_3$ (★), data shown in Fig. 4A. All amplitudes are normalized to the amplitude of O_2 -evolution after the third flash of untreated PSII membranes.

unit is reactivated after addition of 4.8 μM $CaCl_2$. The pattern appears changed and corresponds to that observed with PS II membranes depleted of all their extrinsic peptides (Fig. 4B).

Discussion

Studies considering the role of Ca^{2+} in PS II provide evidence that calcium is a necessary cofactor for optimal oxygen evolution activity. The requirement for calcium cations as well as that for chloride anions is increasing in high-salt treated PS II membranes. It confirms the predictions that the binding of the inorganic cofactors depends on the extrinsic 17 kDa, 23 kDa and 33 kDa polypeptides (Miyao and Murata, 1984; Åkerlund and Jansson, 1981; Kuwabara and Murata, 1983).

The present measurements (Fig. 1) clearly show that the oxygen evolution rate depends on the composition and condition of the PS II membranes. The depletion of the 17 kDa and 23 kDa subunits lowers the oxygen yield by 25% in comparison to intact membrane preparations. The presence of an insufficient amount of chloride ions and calcium ions, which are supposed to be released during NaCl washing, is most probably responsible for the phenomenon (Homann, 1985) Introducing different concentrations of $CaCl_2$ shows that 0.4 μM of $CaCl_2$ gives the highest rate of O_2 evolution in PS II membranes when the 17 kDa and 23 kDa proteins are absent whereas twice the amount of calcium chloride is required with untreated membranes (Fig. 2A and B). Higher concentrations do not enhance the signal, lower it rather somewhat, but without changing the oscillation pattern. The difference in $CaCl_2$ requirement for the improvement of oxygen evolution can easily be explained by the protective role against external factors (mechanistic as well as electrostatic) of the 17 kDa and 23 kDa extrinsic polypeptides of the OEC. This function of the subunits is also evident when a treatment with lanthanides is carried out. If the studied system is depleted of all extrinsic polypeptides (17 kDa, 23 kDa and 33 kDa) inhibition of the O_2 yield is about 75% in the case of $MgCl_2$ washing. Surprisingly, oxygen evolution is more inhibited after $CaCl_2$ washing, namely to about 92% (Fig. 1). These differences are considerably beyond any experimental error. It looks as if calcium cations themselves were responsible for the observed effect. $MgCl_2$ washing (the same concentration as that used in $CaCl_2$ washing, see Materials and Methods) was even more efficient in the removal of the 33 kDa polypeptide (Fig. 5). Thus, it seems possible that in the intrinsic polypeptides low affinity binding sites for calcium cations exist. Depletion of the 33 kDa subunit might influence the OEC surrounding by creating Ca^{2+} attracting regions, thus leading to inactivation of the PS II particles. Due to the lower coordination number, the same sites cannot be occupied by magnesium. Even if Mg^{2+} cations reached the binding site, they can not bind tightly. It looks as if, due to the much smaller ionic radius of 0.65 Å of Mg^{2+} comparing to 0.94 Å for the Ca^{2+} ion, the protein structure is not changed. Thus, it has been shown that magnesium cations do not influence oxygen evolution (Homann, 1985). The removal of the 33 kDa peptide destabilizes the structure of OEC which by this event seems to loose protection against external factors. The addition of low $CaCl_2$ concentrations to the PS II membranes which are depleted of all extrinsic proteins increases the signal of oxygen yield with, however, the maintenance of a changed oscillation pattern. Thus, the ratio of the fourth to the third flash amplitude is higher than in intact PS II particles. This means that the signal increase is to be considered as a direct consequence of the higher concentration of chloride anions (Homann, 1985). Only an amount of 1.6 μM $CaCl_2$ changes the oscillation pattern towards a pattern of a "normal sequence" and enhances the signal amplitude further (Fig. 2C). The modification of the pattern is influenced by calcium cations, as will be confirmed by the observations that follow. Thus, the highest concentration of calcium chloride reactivates the enzyme cycle in $MgCl_2$ washed systems up to 40%, and it has been demonstrated earlier (Burnap *et al.*, 1992) that the absence of the 33 kDa subunits inhibits O_2 evolution up to 40%.

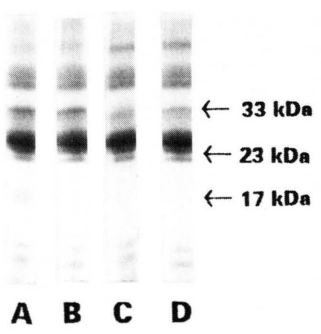

Fig. 5. Polypeptide composition of photosystem II- reaction center preparations in PAGE: **A,** untreated; **B,** washed with NaCl; **C,** washed with $MgCl_2$; **D,** washed with $CaCl_2$.

The observed effect of treatment of intact and modified PS II membrane particles with $DyCl_3$ allows to understand the mechanism of inhibition of oxygen evolution by lanthanide cations. Addition of 0.2 μM $DyCl_3$ to intact particles increases the measured amplitudes and changes the oscillation pattern (Fig. 3A). Thus, the ratio of the fourth to the third peak increases. The surprising

increase of the signal is apparently caused by two factors: (a) by the increase of cations in the medium which are acting as acceptors and (b) by the higher concentration of chloride anions. It is evident that the low amount of Dy^{3+} used does not inhibit O_2 evolution (at least not after 2 min of incubation in darkness on ice). But an increase of the $DyCl_3$ concentration clearly leads to a decrease of the amplitudes and a decrease of the ratio of the third to the fourth peak. However, the sequence could be restored by adding an amount of calcium ions comparable to the used dysprosium amount. After incubation with 1.6 μM of $DyCl_3$ the signal size and the sequence pattern are very similar compared to those observed with PS II membranes washed with $MgCl_2$ (Fig. 1 and 3A). Thus, it has been found (Ghanotakis *et al.*, 1985) that lanthanides cause the release of the extrinsing water soluble polypeptides (17 kDa, 23 kDa and 33 kDa). In NaCl washed preparations $DyCl_3$, applied in increasing amounts, inhibits the enzyme cycle (i.e. the Kok cycle) more and more (Fig. 3B). Thus, the treatment with 0.8 μM dysprosium chloride could not be compensated or repaired by two minutes incubation with calcium cations. Again the ratio of the third to the fourth peak is increased but the amplitudes remain unchanged at a very low level. In the case of PS II membranes washed with $MgCl_2$ (depleted of all extrinsic subunits) the addition of Ca^{2+} had no beneficial effect on the sequence pattern. 1.6 μM of Dy^{3+} completely inactivate the sample (Fig. 3C). When the preparation was washed with $CaCl_2$ no oxygen evolution was observed even under low dysprosium concentrations. The inhibition was obviously irreversible (Fig. 3D) and presents the necessary evidence for the serious modifications inflicted upon the structure of the OEC in PS II membranes treated with high $CaCl_2$ concentrations.

In comparison to $DyCl_3$, addition of $EuCl_3$ influences much stronger the oxygen yield. Already the lowest used amount of 0.2 μM Eu^{3+} cations added to washed PS II membranes caused their inactivation (Fig. 4B and C). In the assay system without all three extrinsic proteins the europium-caused inhibition cannot be overcome by calcium addition. Addition of calcium in a concentration 3-times higher than europium is of no effect. However, if the assay system contained the 33 kDa, the $EuCl_3$-caused inhibition was relieved by the addition of Ca^{2+} in high concentration. The obtained signals indicated that the PS II particles are depleted of the large 33 kDa extrinsic subunit (Fig. 4B). On the other hand, the intact membrane system treated with 0.2 μM Eu^{3+} gives enhanced O_2 evolution without modification of the sequence pattern. As has been shown already for dysprosium, the europium cations and chloride anions are responsible for the higher value of the measured amplitudes. The same concentration of Cl^- and europium cations as that used with $DyCl_3$ (Fig. 3) causes a much stronger effect. The observed high amplitudes and the unchanged oscillation pattern (in comparison to the untreated pattern) obtained for the lowest europium concentration, might indicate that calcium is substituted by europium to the same level observed with dysprosium.

The differences between Eu^{3+} and Dy^{3+}, observed with respect to their action on PS II membranes, can be explained by the different ionic radii ($r_{Dy^{3+}}$ = 0.92 Å, $r_{Ca^{2+}}$ = 0.94 Å and $r_{Eu^{3+}}$ = 0.98 Å) (Latscha *et al.*, 1990). It has been shown in earlier work (Bakou *et al.*, 1992; Ghanotakis *et al.*, 1985) that the similar ionic radii of lanthanides and calcium seems to be the most important factor in substitution experiments. Moreover, lanthanides and calcium have the same coordination numbers. Here, Eu^{3+} has a larger ionic radius than Ca^{2+} and therefore substitutes for calcium only with difficulties, and when it manages to reach the Ca-binding sites, it causes more damage in the surrounding of the binding sites than dysprosium. In intact PS II membranes, low concentration of Eu^{3+} and Dy^{3+} cations can enhance oxygen evolution as Ca^{+2} does. It looks as if lanthanides form less stable complexes in comparison to calcium, thus leading to the consequence that already smaller concentrations cause the release of the extrinsic polypeptides.

Conclusions

The data represented in this report are in agreement with other studies carried out in PS II membranes. Our investigation confirms that the ionic radius of lanthanides is the crucial factor in substitution experiments. Europium has a larger ionic radius than dysprosium with the consequence that

lower europium ion concentrations cause already an irreversible inactivation of oxygen evolution. The one unit charge difference between the divalent calcium (2+) and trivalent lanthanides (3+) also seems to be of great importance for the proper functioning of the OEC. It is obvious that Eu^{3+} and Dy^{3+} produce modified structural situations in the system. This in combination with the higher electronic states leads apparently to different redox conditions of the enzyme cycle (i. e. the Kok cycle).

Our experiments with dysprosium and europium provide the evidence that two different regions on the donor side of PS II exist where calcium is required. One of the sites, highly dependent on Ca^{2+}, as well as on Eu^{3+} and Dy^{3+}, is situated on the extrinsic polypeptides. The enhancement of O_2 evolution in intact PS II membranes after the addition of 0.2 μM Eu^{3+} with change and alteration of the sequence pattern in the case when the same amount of Dy^{+3} was in the reaction medium, suggests that a binding site exists which somehow affects the Mn complex. As an increase of the signal was not obtained in PS II membranes depleted of the 17 kDa and 23 kDa subunits, one site where the lanthanides can bind, must be on the two extracted peptides or in the connection area between the 33 kDa and 23 kDa peptides. It has been found by others that some segments of the 23 kDa polypeptide can complement the calcium binding site on the 33 kDa subunit. An extrinsic protein binding domain is predicted to be located in the same region as calcium (Beauregard, 1992). The effect of the 23 kDa subunit on the calcium requirement in higher plants has been shown earlier (Shen *et al.*, 1988). Thus, the absence of the 23 kDa and 17 kDa polypeptides in cyanobacteria represents an incomplete Ca^{+2}-binding site and could be the explanation for one less calcium cation found in this type of OEC.

Higher concentration of lanthanides, calcium and sodium (to a lesser degree) destroy the organization of the extrinsic peptides, leading to a full exposition of the otherwise well protected part of the OEC. In addition to this site, another calcium binding site seems to exist, which can be influenced by lanthanides. However, this time the effect of lanthanides consists in an inactivation of oxygen evolution. When PS II membranes contain the 33 kDa subunit, an inhibition caused by $DyCl_3$ or $EuCl_3$ can be overcome by a short period of incubation (2 min.) with calcium cations. However, when the system is completely depleted of the extrinsic polypeptides, the inactivation seems to be irreversible or at least much more severe. It is well known that the lanthanides are bound much stronger than calcium. Thus, it looks as if the 33 kDa peptide, which is supposed to bind in a close relationship to the Mn complex and probably also to the Tyr/Z apparently slows down or prevents irreversible changes in the region of the second Ca-binding site.

The inactivation of the O_2 yield induced by lanthanides supports the idea that the binding sites of Mn and Ca are in a close relationship and interact with each other (Ono and Inoue, 1983). For example, a Concanavalin-A-type organization (Becker *et al.*, 1973) proposed already in Ghanotakis *et al.* (1985) is thinkable. The direct influence of calcium on oxygen evolution can alternatively also be explained by a Ca^{2+}-binding domain of a Calmodulin-type protein (Goodman *et al.*, 1979) between Asp_{59} and Glu_{65} in the luminal region of D_1, what has been postulated in Ono and Inoue (1989). There is evidence that lanthanides block electron transport from the Mn complex to Tyr^+/Z^+ and that they affect electron transport from Tyr/Z to $P680^+$ (Bakou and Ghanotakis, 1993). Moreover, it has been demonstrated that the Ca^{2+} lanthanide binding site has an influence on th S-state formation and their stabilization (Ghanotakis *et al.*, 1985). Our measurements show that depletion of the 33 kDa protein as well as the substitution of Ca^{2+} by Eu^{3+} and Dy^{3+} can serve as an explanation for the increase of the so-called miss transition probability used in quantitative descriptions of the O_2-yielding process (Kok *et al.*, 1970; Delrieu, 1974, 1983; Lavorel, 1978). Our results demonstrate that lanthanides subsequently influence the redox features of the OEC and lower the number of transitions between S states (Burda and Schmid, in preparation).

In conclusion, the obtained data give evidence for two Ca-binding regions. One of the sites is situated in/on the contact area surface of the 23 kDa and 33 kDa proteins and easily accessible by external cations (Fig. 6). This site can be influenced by trivalent or monovalent cations. Cations with a different ionic radius than calcium apparently re-

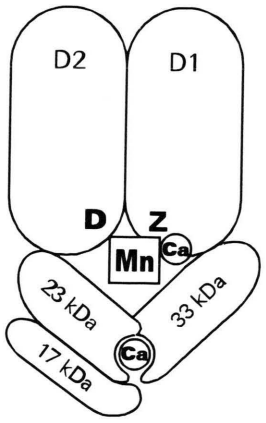

Fig. 6. Scheme of the reaction center of photosystem II with indication of calcium binding sites.

arrange the 33 kDa subunit in such a way that oxygen evolution comes out less efficient. Thus, the extra calcium ion in higher plants (not appearing in cyanobacteria: Ichimura *et al.*, 1992; Kashino *et al.*, 1986), due the the presence of the 23 kDa protein keeps the OEC in more "tense" form which is apparently good for effective electron transport. In more sensitive organisms like cyanobacteria, the oxygen evolution system is not supported by extra protection of this sensitive apparatus. During evolution the extrinsic 23 kDa and 17 kDa polypeptides have been developed in higher plants. The second, tightly bound calcium ion is responsible for the activity of the enzyme cycle, i.e. the Kok cycle. Any influence on this binding site inhibits oxygen yield. This Ca^{2+} is located close to the Mn cluster and Tyrosine-161.

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