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Studies on calcium depletion of PS II by pH 8.3 treatment

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We have investigated by EPR the hypothesis that the S_1 state of the oxygen-evolving complex (OEC) becomes unstable at pH 8.3 and is converted to the S_0 state in the dark (Plitjer et al. (1986) FEBS Lett. 195, 313–318). Treatment at pH 8.3 removed the ability to generate the S_2 multiline EPR signal, but this was restored by returning the sample to pH 6.3. The depletion of calcium by an NaCl-wash procedure at pH 8.3 in the dark inhibited oxygen evolution and the formation of the multiline EPR signal, even on returning to pH 6.3. The characteristic 25% reduction of the electron donor, D^+, over 4 h in untreated samples was absent in pH 8.3 NaCl-washed samples. Calcium and vanadyl ions, VO^{2+}, restore the normal dark stable S_1 state, as measured by the large decrease in D^+ and ability to form the S_2 state multiline EPR signal after 200 K illumination. Calcium, strontium and vanadyl ions also partially restore oxygen evolution in calcium-depleted PS II. These results are interpreted as indicating the loss of the S_1 state at pH 8.3 in the dark and that there is a calcium requirement for S_0 to S_1 at pH 6.3.

Introduction

Oxygenic photosynthesis requires the storage of four positive charges to enable two water molecules to be oxidised to molecular oxygen. The charges are stored by a cluster of two or four manganese ions in an oxygen-evolving complex (OEC) associated with PS II [1]. The OEC was suggested to have five different successive oxidation states, S_0 to S_4, which are interconverted in a cyclic system [2]. The S_2 state is EPR-detectable, showing a characteristic 'multiline' EPR spectrum [3,4] around $g = 2$ and under certain conditions a signal near $g = 4.1$ [5,6].

Calcium and chloride cofactors have been shown to be needed for maximum rates of oxygen evolution to occur [7,8]. It is uncertain how many calcium ions are required per reaction centre, either 1 or 2 in high-affinity or high- and low-affinity binding sites [9,10]. The removal of calcium ions from PS II has been linked to the loss of 17 kDa and 23 kDa extrinsic polypeptides from the PS II complex. These polypeptides have been suggested to protect a high-affinity calcium binding site [11]. A high-concentration salt wash is used to remove the 17 kDa and 23 kDa polypeptides. In this treatment, light is required to assist in calcium depletion and this has been linked to variable affinities for calcium binding in different S states [12]. S_3 has the lowest affinity for calcium, whilst S_1 has the highest affinity [13]. It has been suggested that when calcium ions are removed in the S_0 state, the advance of S states is blocked until depleted PS II is reconstituted with calcium [12,13]. A low-pH citrate treatment has also been used to remove one calcium per PS II without loss of the 17 kDa and 23 kDa polypeptides [9].

After 4 h in the dark the OEC of untreated PS II is completely in the S_1 state [14]. The higher S states are rapidly deactivated to S_1 in the dark. S_0 is oxidised to S_1 more slowly in the dark, with oxidation complete after 4 h [14]. The electron acceptor linked to this dark oxidation is a component known as D^+. During dark-adaptation of PS II there is a decrease in the size of the D^+ EPR spectrum, Signal II [15–17]. This is due to oxidation of the S_0 state to the S_1 state by D^+ [17]. D can also function as an electron donor involved in the deactivation of the higher S states [15,16,18]. D^+ has recently been identified as a tyrosine radical on the 32 kDa D2 reaction centre polypeptide [19,20].

Abbreviations: PS II, Photosystem II; OEC, oxygen-evolving complex; EPR, electron paramagnetic resonance spectrometry; Mes, *N*-morpholinoethanesulfonic acid; PPBQ, *p*-phenylbenzoquinone; OGP, *n*-octyl β-D-glucopyranoside.

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From measurements of ultraviolet absorbance changes in PS II membranes it was concluded that the S_1 state becomes unstable in the dark at pH 8.3 and is reduced to S_0 [21]. However, recent work on the flash number dependence of oxygen evolution and Chl a^+ nanosecond reduction kinetics suggests that the S_0 state was not formed and that the characteristics arise from an increased number of misses [22].

In the experiments described, EPR signals from D^+ and the S_2 state were studied in PS II preparations both before and after treatments to remove calcium. In particular, the S state distribution in pH-8.3-treated samples was investigated. The results suggest that pH 8.3 treatment perturbs the normal S_1 state, but that this does not involve the S_1D/S_0D^+ couple. Removal of calcium at pH 8.3 blocks the return to S_1 at pH 6.3. Calcium, strontium and vanadyl ions restore the normal dark stable S_1 state, as measured by the large decrease in D^+ and ability to form the S_2 state multiline EPR signal after 200 K illumination.

Materials and Methods

Photosystem II preparations with high rates of oxygen evolution were prepared from *Spinacea oleracea* [23,24]. Untreated PS II was stored at 77 K in 25 mM Mes, 5 mM $MgCl_2$, 15 mM NaCl and 20% glycerol (pH 6.3).

Light-harvesting chlorophyll was removed from untreated PS II preparations using *n*-octyl β-D-glucopyranoside. This preparation is referred to as OGP PS II, and lacks the 17 and 23 kDa extrinsic polypeptides [25].

PS II membrane preparations were depleted of the 17 and 23 kDa extrinsic polypeptides and calcium ions using a high-concentration salt wash [26]. PS II membranes were washed with 25 mM Mes-NaOH/20 mM NaCl/5 mM $MgCl_2$ (pH 6.3), and centrifuged at 40000 $\times g$ for 25 min. The pellet was resuspended in a small volume of the above buffer, and then diluted to 1.0 mg Chl/ml with 2.0 M NaCl/25 mM Mes-NaOH/1 mM EGTA/50 μM PPBQ (pH 6.3). This was stirred on ice in room light for 30 min and then centrifuged as above. The pellet was washed and resuspended in the same buffer and then centrifuged as above. The PS II membranes were then resuspended in 25 mM Mes-NaOH/30 mM NaCl/5 mM $MgCl_2$/100 μM EGTA/20% glycerol (pH 6.3) and stored at 77 K. This preparation is termed pH 6.3 NaCl-washed PS II. All further treatments were carried out in room light with 50 μM PPBQ added. The artificial electron acceptor (PPBQ) was added to increase the S-state turnover under the room light illumination conditions.

Calcium ions were also removed without polypeptide depletion using a low-pH citrate wash [9]. After washing the PS II membranes with 400 mM sucrose/20 mM NaCl at pH 6.5 and centrifuging at 40000 $\times g$ for 20 min, they were resuspended in 400 mM sucrose/20 mM NaCl/10 mM citrate (pH 3.0) to 2.0 mg Chl/ml and stirred on ice in the dark for 5 min. The PS II membranes were then diluted 5-fold with 400 mM sucrose/40 mM Mes-NaOH (pH 6.5) and centrifuged as above. The pellet was resuspended in glycerol buffer as for NaCl-washed PS II. This preparation is termed pH 3.0 citrate-washed PS II.

To remove calcium at pH 8.3 in the dark, PS II membranes were washed with 20 mM Mes-NaOH/40 mM NaCl/100 μM EGTA (pH 6.3), centrifuged at 40000 $\times g$ for 20 min then resuspended in 30 mM NaCl/5 mM $MgCl_2$/5 mM Tricine-NaOH (pH 8.3). Following centrifugation as above they were resuspended in a small volume of 10 mM Tricine/40 mM NaCl (pH 8.3) and then placed in the dark for 15 min to equilibrate. The PS II suspension was then diluted to 1 mg Chl/ml in the dark with 2.2 M NaCl/25 mM Tricine-NaOH/1 mM EGTA (pH 8.3) to give a final concentration of 2 M NaCl. This was stirred on ice for 20 min in the dark, the pH was then adjusted to pH 6.3 in the dark followed by centrifugation as above. The membranes were then washed with 25 mM Mes-NaOH/40 mM NaCl/100 μM EGTA (pH 6.3), centrifuged and resuspended as for pH 6.3 NaCl-washed PS II. This preparation is termed pH 8.3 NaCl-washed PS II.

20–40 mM Cl^- was maintained during all procedures to prevent chloride depletion.

Oxygen evolution measurements were made with a Clark type oxygen electrode, using an assay buffer of 25 mM Mes-NaOH/40 mM NaCl (pH 6.3) at 18°C with additions as described in the text. At least three measurements were used for each assay.

EPR spectrometry was performed at cryogenic temperatures using a JEOL X-band spectrometer with 100 kHz field modulation and an Oxford instruments liquid helium cryostat. Samples of 0.3 ml in 3 mm diameter calibrated quartz tubes were used. Care was take to ensure that changes in signal amplitude were not due to changes in the microwave power saturation characteristics of signals. EPR samples were prepared in room light in a solution of 20 mM Mes/5 mM $MgCl_2$/20 mM NaCl/20% glycerol (pH 6.3), and then dark-adapted on ice. Chlorophyll concentrations of 5–6 mg/ml, measured as in Ref. 27, were used. The samples were then either frozen in the dark and then illuminated at 200 K with a 1000 W lamp for 8 min using an ethanol/solid CO_2 bath, or illuminated at 4°C with a 1000 W lamp for 1 min (with 100 μM DCMU added) and frozen at 77 K under illumination. Samples were reconstituted with 10 mM $CaCl_2$, $SrCl_2$ or micromolar concentrations of vanadyl ions as discussed in the text. A fresh stock solution of 0.3 M vanadyl sulphate hydrate at approx. pH 3.0 was prepared for each reconstitution. The properties of the paramagnetic oxovana-

dium(IV) make it a particularly useful probe. The cation has extremely flexible coordination properties and is therefore able to form strong complexes with a variety of ligands and binds to numerous metalloproteins [28–30]. Vanadyl ions, however, tend to be air-oxidised to V(V) at physiological pH. It is therefore important to keep all samples under oxygen-free nitrogen.

Results

Removal of calcium from PS II membranes

Illumination of PS II at 200 K allows only the transition of S_1 to S_2, giving the S_2 multiline EPR signal; no higher S states are formed [31,32]. At pH 6.3 a normal multiline EPR signal was induced by 200 K illumination (Fig. 1a). This signal or the signal due to the alternative form of the the S_2 state, the $g = 4.1$ signal [5,6,33] (not shown), were not formed when the pH was raised to pH 8.3 (Fig. 1b). When the pH of the sample was returned to pH 6.3, the ability to form the S_2 multiline EPR signal on 200 K illumination was rapidly restored (Fig. 1c). If a large fraction of the OEC were converted to the S_0 state at pH 8.3 in the dark, when the sample was returned to pH 6.3, the S_0 state would have to be oxidised to the S_1 state before the S_2 state could be formed by 200 K illumination.

Changes in the S_0/S_1 distribution might be expected to be associated with the D/D^+ redox couple. No changes in D^+ were observed upon pH 8.3 treatment, nor upon returning the sample to pH 6.3. This indicates that D was not involved in S_0/S_1 state changes at pH 8.3 and after return to pH 6.3.

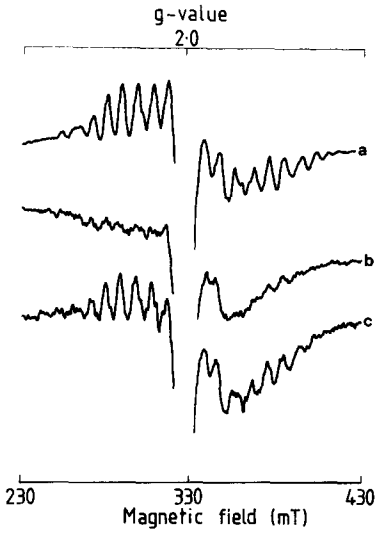

Fig. 1. EPR spectra of the S_2-state multiline region following 200 K illumination after 4 h dark adaptation on ice. PS II membranes (a) at pH 6.3, (b) at pH 8.3 and (c) returned to pH 6.3 from pH 8.3. Chl concentration, 5 mg/ml. EPR conditions; power, 10 mW; temperature, 8.5 K; modulation width, 1 mT. The spectra in (a) and (b) are an average of three scans.

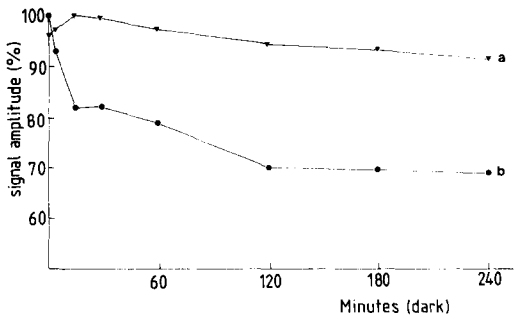

Fig. 2. Time-course of D^+ reduction at pH 6.3 during dark-adaptation after salt washing at pH 8.3 in the dark. (a) No additions; (b) 20 mM $CaCl_2$ added. Chl and EPR conditions: Microwave power, 1 μW; temperature, 15 K; modulation width, 0.2 mT.

The possibility that the pH 8.3 effect may be due to changes in the affinity of binding of the calcium cofactor associated with the S_0 state was investigated. A 2.0 M salt treatment was incorporated into the pH 8.3 wash to remove calcium ions. Washing with buffer alone at pH 8.3 had not perturbed the S-state cycle at pH 6.3 (Fig. 1). After salt washing at pH 8.3 in the dark and returning to pH 6.3, the characteristic decay of D^+ on 4 h dark adaptation was not seen (Fig. 2a). The EPR spectrum of a 4 h dark-adapted sample of pH 8.3 NaCl-washed PS II is shown in Fig. 3a. This shows a weak dark stable 'multiline-like' EPR signal consisting of several faint but reproducible lines extending over 200 mT, suggesting that the manganese complex was not in the normal EPR-silent S_1 state. No S_2 multiline

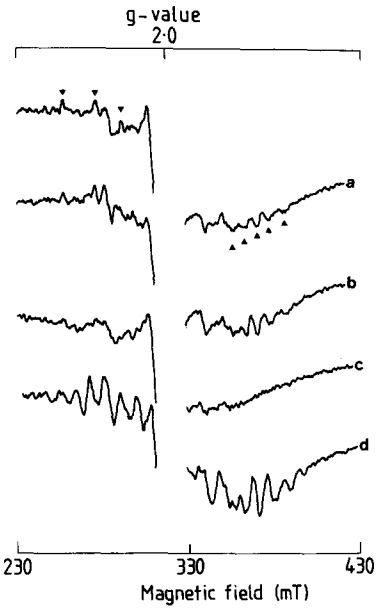

Fig. 3. The effect of calcium depletion at pH 8.3 on the ability to induce the S_2 state multiline EPR signal by 200 K illumination at pH 6.3. (a) 4 h dark, no additions; (b) 4 h dark then illuminated at 200 K; (c) as (a) but 20 mM $CaCl_2$ added in the dark; (d) as (c), illuminated at 200 K. Chl and EPR conditions as in Fig. 1. The most prominent features of the reproducible dark stable spectrum (a) are marked.

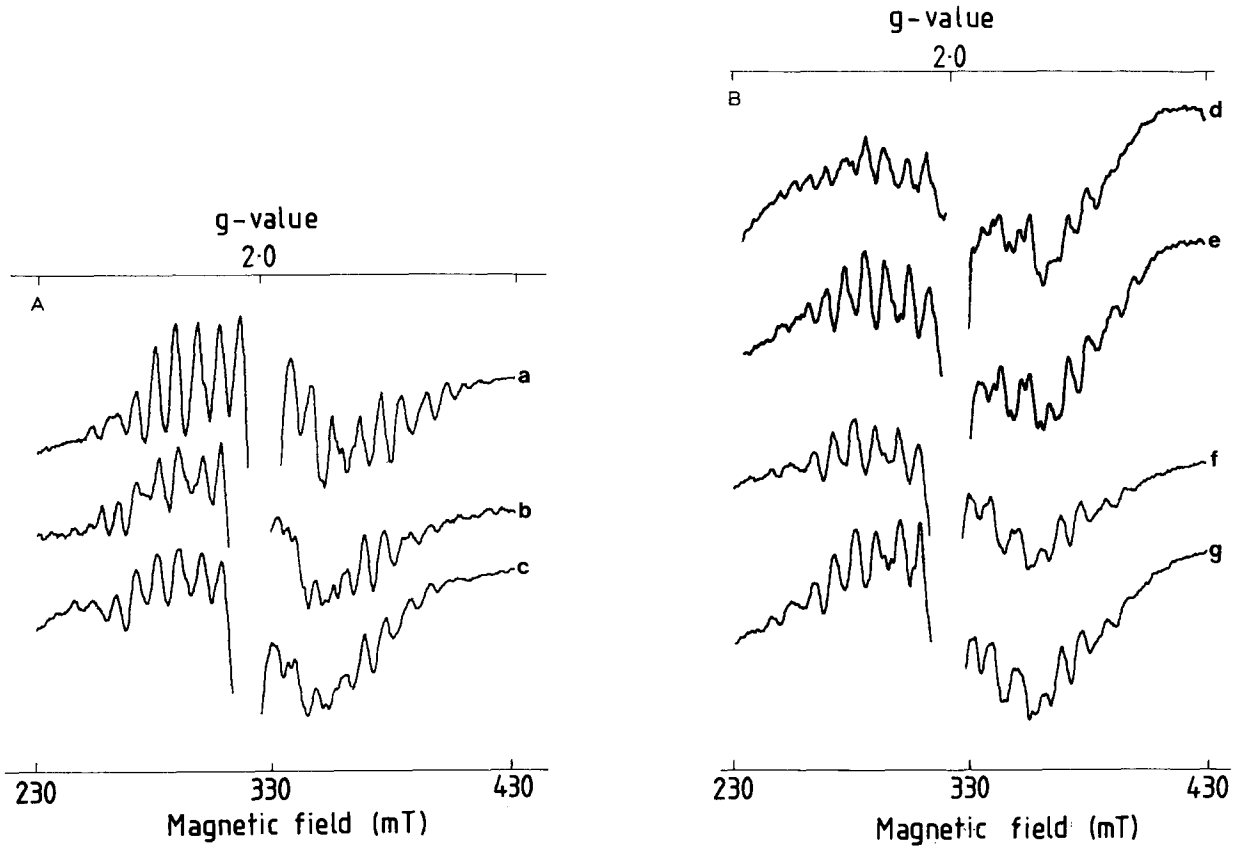

Fig. 4. Comparison of the conventional methods of calcium removal on the amplitude of the S_2 state multiline EPR signal. S_2 induced by 200 K illumination. (a) Untreated PS II membranes; (b) pH 6.3 NaCl-washed PS II membranes; (c) as in (b) with 20 mM $CaCl_2$ added before freezing; (d) pH 3.0 citrate-washed PS II membranes; (e) as (d) with 20 mM $CaCl_2$ added before freezing; (f) OGP-treated PS II membranes; (g) as (f) with 20 mM $CaCl_2$ added. Chl and EPR conditions as in Fig. 1.

EPR signal or $g = 4.1$ signal was formed on 200 K illumination (Fig. 3b). When calcium ions were reconstituted into the depleted samples there was an immediate loss of the unusual dark spectrum (Fig. 3c). During dark-adaptation of the reconstituted sample, a fast decrease in D^+ was followed by slow reduction of D^+ over 4 h (Fig. 2b). After 4 h dark-adaptation a normal S_2 state multiline EPR signal was produced by 200 K illumination (Fig. 3d).

This suggests that when calcium was depleted during the pH 8.3 salt wash in the dark, the S state cycle became trapped in one state, i.e., no multiline EPR

TABLE I

Rates of oxygen evolution by pH 8.3 NaCl-washed PS II and pH 3.0 citrate-washed PS II

Other details of assay in Materials and Methods

Assay conditions	Rate (μmol O_2/mg Chl per h)	
A. Untreated PS II		
No addition	583	
B. pH 8.3 NaCl-washed PS II		(% Ca^{2+} rate)
(1) no addition	35	(13.0)
(2) 20 mM $CaCl_2$	265	(100.0)
(3) 20 mM $SrCl_2$	176	(66.5)
(4) 20 mM VO^{2+}	102	(38.5)
C. pH 3.0 citrate-washed PS II		
(1) no addition	65	(12.0)
(2) 10 mM $CaCl_2$	516	(100.0)
(3) 20 mM $SrCl_2$	310	(60.0)
(4) 20 mM VO^{2+}	260	(50.5)

TABLE II

Rates of oxygen evolution by OGP PS II after reconstitution with calcium and vanadyl ions

Other details of assay in Materials and Methods

Assay conditions	Rate (μmol O_2/ mg Chl per h)	
		% Ca^{2+} rate
(1) 5 mM $CaCl^2$	530	(100)
(2) No addition	148	(28)
(3) 3 mM VO^{2+} (dark)	223	(42)
(4) 3 mM VO^{2+} (illuminated) then 5 mM $CaCl_2$	270	(51)
(5) 3 mM VO^{2+} (illuminated then dark 3 min) then 5 mM $CaCl_2$	281	(53)
(6) 3 mM VO^{2+} + 5 mM $CaCl_2$ (dark)	413	(78)

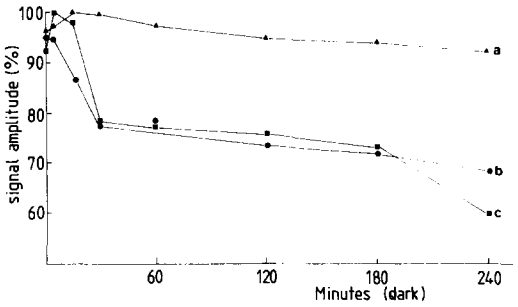

Fig. 5. The effect of calcium analogues on the time-course of D^+ reduction at pH 6.3 after salt washing at pH 8.3 in the dark. (a) No additions; (b) 20 mM $SrCl_2$ added; (c) 50 μM VO^{2+} added. Chl and EPR conditions as in Fig. 2.

signal could be formed and no oxygen evolution occurred. When calcium ions were reconstituted, the OEC assumed a functional S_1 state, restoring the ability to induce the S_2 multiline signal, and to evolve oxygen.

When calcium was depleted from PS II membranes by the established methods using (1) a salt wash (in the light or dark) at pH 6.3 [8,13], (2) a citrate wash at pH 3.0 [9] or (3) treatment with OGP [25], the S_2 multiline EPR signal was formed at pH 6.3 on 200 K illumination (Fig. 4a, b, d, f). These results agree with those of Boussac and Rutherford [12]. The signal amplitude increased slightly with calcium reconstitution (Fig. 4c, e, g). There are some differences in amplitude and lineshape of the S_2 multiline signal between treatments, but there is a clear difference between these methods and the pH 8.3 NaCl-wash which removed the ability to generate the multiline EPR signal. This indicates that PS II depleted of calcium at pH 8.3 was trapped in a state which may be equivalent to the S_0 state, whilst PS II depleted at pH 6.3 and/or pH 3.0 remained in the S_1 state in the dark.

Calcium analogues

Strontium and vanadyl ions but not magnesium ions partially replace calcium activity by stimulating oxygen evolution after calcium depletion in pH 8.3 salt-washed PS II (Table I), pH 3.0 citrate-washed PS II (Table I), and in OGP PS II (Table II). Table II shows that slow cycling of the S states by illumination allows vanadyl ions to be tightly bound to OGP PS II. The rate of oxygen evolution following this treatment could not be stimulated to the higher rate associated with calcium addition (Table II-4). When both calcium and vanadyl ions were added in the dark, an intermediate rate was observed (Table II-6). These results suggest that vanadyl ions are competing for one or more calcium binding site/s involved in water oxidation. Both strontium and vanadyl ions restore the normal S_1 state after salt washing at pH 8.3 in the dark, illustrated by the 25% loss of D^+ over 4 h in the dark (Fig. 5).

Discussion

Plijter et al. [21] suggested that at pH 8.3 in the dark the oxygen-evolving complex is set to the S_0 state. This change was reversed at pH 6.0 [21]. In our study, oxygen evolution was inhibited at pH 8.3 and no S_2 multiline EPR signal could be formed. On returning to pH 6.3, oxygen evolution and the ability to induce the S_2 multiline EPR spectrum was restored.

We have established that calcium depletion at pH 8.3 in the dark removed the reversibility of the pH 8.3 treatment by transfer to pH 6.3. At pH 6.3 both the S-state cycle leading to oxygen evolution and the formation of the S_2 multiline EPR signal were inhibited in depleted samples. It has been suggested earlier that removal of calcium from the S_0 state might result in the loss of S-state turnover [12]. The dark stable EPR signal detected in the depleted sample suggests that the OEC was not in the EPR-silent S_1 state in the dark. After calcium depletion at lower pH, the formation of the S_2 multiline EPR signal was not inhibited, suggesting that S_1 was still present in the dark in these samples. Our results suggest that the S_0 state or an equivalent oxidation state, S_0^*, with different structural or protonation characteristics was formed at pH 8.3. The S_1 state was rapidly reformed on returning to pH 6.3. We suggest that the S_0 or S_0^* state was trapped by the calcium depletion at pH 8.3. When pH 8.3 NaCl-washed PS II was reconstituted with calcium ions, the normal S-state cycle occurred, and the normal function of D in the dark was restored.

An alternative explanation could be that calcium depletion at the higher pH removes calcium from a higher-affinity site than at lower pH, thereby blocking S_1 to S_2 advancement. However, the rapid reduction of D^+ observed upon calcium reconstitution strongly suggests that this is involved in the restoration of normal S_1. The reduction of D was more rapid than seen in the oxidation of S_0 in untreated samples, again suggesting that the manganese cluster was not in a normal S_0 state in depleted preparations, i.e., it was in the S_0^* state.

These experiments therefore provide evidence that the pH 8.3 treatment does perturb the normal S_1 state of the OEC in the dark. They suggest that calcium ions are essential for the oxidation of S_0 to S_1 using D^+ as the oxidant and show that calcium binding influences the advance of the S-state cycle. The reconstitution of activity was also achieved by the calcium analogues, strontium and vanadyl ion. The paramagnetic vanadyl ion may provide a useful probe to investigate the role of calcium ions, the manganese cluster and the EPR-silent states.

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