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Ca^{2+} binding to the oxygen evolving enzyme varies with the redox state of the Mn cluster

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Oxygen evolution by the mangano-enzyme of photosystem II is inhibited by Ca^{2+} depletion induced by NaCl washing and restored by Ca^{2+} addition. The effectiveness of NaCl treatment in inhibiting oxygen evolution in photosystem II was studied after a series of preilluminating flashes. The susceptibility of the enzyme to NaCl treatment varied with the number of preilluminating flashes and this variation showed an oscillation pattern with a period of four. This pattern is characteristic of cycling through the four long-lived intermediate states in the enzyme cycle (i.e. the states, S_0, S_1, S_2, S_3). The relative extent of inhibition corresponding to each of the S states was as follows: $S_3 > S_0 \approx S_2 > S_1$. From these results it is concluded that Ca^{2+} binding is dependent on the S states and that Ca^{2+} probably plays a fundamental role in the mechanism of water splitting. The results also help to explain the conflicting reports of the extent of inhibition induced by NaCl washing and the controversy over which electron transfer step is inhibited by Ca^{2+} depletion.

Photosynthesis; Photosystem II; Oxygen evolution; Ca^{2+}; Water oxidase

1. INTRODUCTION

Oxygen evolution by plants is catalyzed by photosystem II which drives the four electrons from the oxidation of two H_2O molecules to the plastoquinone electron acceptors (see [1] for review). This reaction requires the storage of four positive charge equivalents involving a cluster of 4 atoms of Mn linked to the reaction center and 3 extrinsic membrane-bound proteins with molecular masses of 18, 24 and 33 kDa. The oxidizing side of PS II goes through a cycle of five different redox states which are denoted S_n, n varying from 0 to 4 indicating the number of the charge equivalents stored, according to the model of Kok et al. [2]. This process requires two cofactors, Cl^- and Ca^{2+} (see [3] for a review). The data pertaining to the electron transfer step(s) requiring Ca^{2+} have been extensively discussed [4], however, the molecular mechanism by which Ca^{2+} acts is unknown.

A treatment known to release Ca^{2+} is the removal of the 24 and 18 kDa proteins. In the absence of these proteins the affinity of the binding site for Ca^{2+} decreases [5-7]. The requirement of light for Ca^{2+} release has been under debate (see [4] for discussion). In some reports it was found that after NaCl washing, Ca^{2+} release was a light-dependent process [8,9]. In this regard we recently suggested that the Ca^{2+}-binding affinity is modulated by the distribution of the S states [4]. This suggestion was also based on the consideration of the following points. Firstly, although two binding constants had been measured for the Ca^{2+} required to reconstitute activity in the absence of the 18 and 24 kDa proteins [7,10], it was shown that a single Ca^{2+} per spinach reaction center was responsible for the Ca^{2+} effect on oxygen evolution [10]. Secondly, two different steps in the enzyme cycle have been shown to be inhibited in Ca^{2+}-depleted membranes (reviewed in [4]) and it was suggested that these two kinds of inhibition might correlate with the two different bin-

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Abbreviations: PS II, Photosystem II; PPBQ, phenyl *para*-benzoquinone; Chl, chlorophyll

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ding constants [4]. Approx. 30% and 70% of PS II centers possess dissociation constants of 1–2 mM and 50–100 μM, respectively [7,10]. Both of these considerations indicated the presence of heterogeneity in the enzyme. The most satisfactory explanation for heterogeneity on the donor side of PS II is that due to dark distribution of the S states.

In this report we have investigated the requirement for light in Ca^{2+} release by performing NaCl treatment of PS II membranes in each of the long-lived S states. Indeed an S-state dependence for the inhibition of oxygen evolution was found.

2. MATERIALS AND METHODS

PS II membranes from spinach chloroplasts were prepared according to Berthold et al. [11] with the modifications of Ford and Evans [12] and were stored at −80°C in 0.3 M sucrose, 30 mM NaCl, 25 mM Mes (2-(*N*-morpholino)ethane sulfonic acid), pH 6.5, 30% (v/v) ethylene glycol at approx. 4 mg Chl/ml. NaCl treatment was done as follows: 200 μl of PS II particles were put in 4 mm external diameter quartz tubes and dark adapted for 30 min on ice. Then the samples were given 1 saturating preflash and dark adapted for 9 min at room temperature to synchronize the PS II centers in the S_1 state [13]. After this treatment 2 mM PPBQ was added in the dark as an artificial electron acceptor. The samples were then given the desired number of flashes and quickly (< 10 s) transferred in the dark into 1.2 ml of a high-salt medium consisting of 1.7 M NaCl, 25 mM Mes, pH 6.5, 50 μM EGTA. After dark incubation on ice for 10 min, 40 μl of the diluted sample were further diluted into 1 ml (final volume) of a medium containing 25 mM Mes, pH 6.5, 50 μM EGTA, 5 mM $MgCl_2$, 0.6 mM PPBQ, ± 10 mM $CaCl_2$. The initial rate of oxygen evolution was measured under continuous saturating light, 30–60 s after the dilution, with a Clark-type electrode (time response < 2 s) at 22°C. The $MgCl_2$ was present to avoid any non-specific effects of divalent cations on oxygen evolution. When NaCl treatment was done under room light the same protocol was used except that no flashes were given and illumination by room light for 20 min was alloed in the presence of the high-salt medium. The samples were then dark adapted 15 min before the measurement. Flashes at room temperature were provided from a Nd-YAG laser (15 ns, 300 mJ, 530 nm) with a frequency of 1 Hz.

3. RESULTS

Oxygen-evolution activities measured in the presence of 10 mM $CaCl_2$ in untreated PS II membranes or after NaCl treatment in the light were respectively 435 and 325 μM O_2/mg Chl × h. When NaCl treatment was performed in the dark the activity after addition of 10 mM Ca^{2+} was 400 μM O_2/mg Chl × h irrespective of the flash number (all these values are ± 10% depending on the PS II preparations). The fraction of centers irreversibly inhibited after NaCl treatment in room light is as expected from previous results [9] and is probably due to some weak-light photoinhibition [14,15].

Fig.1 shows a plot of the oxygen-evolution activity in the absence of Ca^{2+} divided by the activity in the presence of Ca^{2+}, in NaCl-washed samples in the dark, versus the flash number i.e. versus the S state. When NaCl treatment was carried out in the S_1 state (0 flash) the Ca^{2+} stimulation was similar to that observed in untreated PS II membranes. This level of activity is defined as 100% on the right scale of fig.1. This is in good agreement with the observation of Miyao and Murata [9] who showed that NaCl washing in the dark did not significantly inhibit oxygen evolution. The maximum inhibition, interpreted as being due to Ca^{2+} depletion, occurred when NaCl treatment was performed in the light. Such a treatment routinely gave 25% residual activity. This level of inhibition can be considered as the maximum inhibition which could be obtained with such a treatment in these particles (by definition, 0% on the right scale of fig.1). After a given flash number, it can be seen that the inhibited, Ca^{2+}-reactivatable, activity oscillated with a period of four, characteristic of the charge-storage mechanism in the oxygen-evolving system of PS II [16]. The maximum inhibition occurred in the S_3 state. The overall susceptibility to NaCl treatment in the presence of 50 μM EGTA appears to be as follows: $S_3 > S_0 \approx S_2 > S_1$ if we take into account the misses and the lifetimes of the S states. The release of the 24 and 18 kDa proteins from the membrane is a prerequisite for Ca^{2+} removal [5,6]. It has been reported that in 1.5 M NaCl the detachment of these proteins is largely complete in 5 min in the dark or in the light [18]. In our PS II particles we have found by gel electrophoresis that the depletion of the 18 and 24 kDa proteins after NaCl treatment for 6 min in the S_1 state or 30 min in room light is similar (not shown). This indicates that it is not light modulated polypeptide release which is responsible for the S state dependent inhibition.

At room temperature, in PS II membranes in the presence of PPBQ as an electron acceptor, the S_2 state and S_3 state decay monophasically with half-decay times of 3 and 4 min, respectively [13]. In-

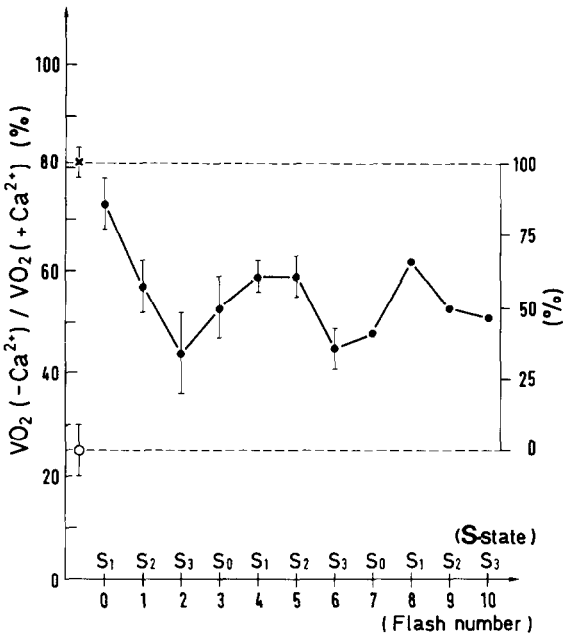

Fig.1. Initial rate of oxygen evolution in the absence of Ca^{2+} divided by the activity in the presence of 10 mM Ca^{2+}. (●) Samples NaCl washed in the dark after a given flash number. (×) Untreated PS II membranes. (○) A sample NaCl washed in the light. Other conditions are described in the text. Each point accompanied by an error bar (SD) is the average of 4–5 experiments. Each experiment is the average of 3 measurements. The righthand scale refers to the maximum inhibition obtained in the light (0%) and to the oxygen evolution in untreated sample in the absence of (Ca^{2+}) (100%).

evitably in our experiment the S_2 state and S_3 state would have deactivated in some of the centers before the release of the 18 and 24 kDa proteins was completed. This could explain the less than 100% inhibition. It is of note that the 1 flash and 2 flash samples are expected to have been largely in the S_1 state after the dark incubation in the high-salt medium before the measurements of oxygen evolution were made. The observation of diminished activity in these samples compared with samples in the S_1 state after 0 and 4 flashes indicates that the effect of NaCl treatment in the higher S states is not reversed by deactivation.

It has been previously shown that after dilution of the sample some rebinding of the 24 and 18 kDa proteins occurred [9]. Nevertheless under such conditions the reactivation of oxygen evolution requires exogenous Ca^{2+} with the same two dissociation constants as found in the absence of both polypeptides [7]. This is due to a time lag required for reappearance of the ultrahigh-affinity Ca^{2+}-binding site [5,7]. Thus the rapid polypeptide rebinding in the diluted salt does not affect Ca^{2+}-binding behaviour under the conditions of our experiment.

After NaCl treatment, irrespective of the treatment (0 to 10 flashes or continuous light), the rate of residual oxygen evolution in the absence of Ca^{2+} was linear for only 10 to 15 s after which time it considerably decreased whatever the flash number. In contrast, in the presence of Ca^{2+}, linearity was observed for at least 1 min (not shown). This suggests that in NaCl-treated PS II although Ca^{2+} depletion seems to occur effectively after flash illumination, the release of the residual Ca^{2+} under continuous light requires several turnovers. Similar effects have been reported earlier [8,17]. This is probably due to the shorter lifetime of the susceptible S states when being turned over by strong light compared to the long deactivation time after flash illumination.

4. DISCUSSION

The results presented in this paper show that the susceptibility of PS II centers to NaCl treatment in the presence of 50 μM EGTA is modified by the S states. Maximum inhibition occurs in the S_3 state and no inhibition in the S_1 state. The ability to observe these effects relies on the rapid depletion of the 18 and 24 kDa proteins (less than 5 min [18]) and rapid loss of Ca^{2+} during the lifetime of the S state. It is shown, for S_3, that inhibition due to Ca^{2+} release occurs to more than 50% in 5 min. However it has been reported that NaCl washing under weak continuous light inhibits oxygen evolution with a half time of approx. 20 min [9]. This discrepancy may be due to the 300 mM sucrose in the experiments in [9]. We have previously shown that sucrose greatly decreases the rate of Ca^{2+} rebinding [18]. It is possible that sucrose may also slow down Ca^{2+} release. Alternatively, the long half time for inhibition under continuous light could be due to a decrease of the steady-state concentration of PS II centers in the S_3 state and probably to an electron transfer limitation imposed by the limited capacity of the plastoquinone pool to accept electrons. Under these circumstances, in a proportion of the centers, the formation of the

susceptible S states could be limited by air oxidation of the plastoquinone pool. This could also explain the residual activity in NaCl-washed PS II membranes in the light, which has been found to vary from 10% to 40% depending on the preparation [7] and on the length of time they are exposed to the light [9].

The inhibition of oxygen-evolution activity in NaCl-treated PS II membranes is directly related to the release of Ca^{2+} since this activity can be restored by Ca^{2+} addition. The point at which electron transfer is blocked when Ca^{2+} is released has been demonstrated to be largely at the S_3 to S_0 transition [4,19]. In addition, a second mode of inhibition at the level of electron donation to the tyrosine Z^+ has been detected in dark-adapted materials (e.g. [20], see [4] for a review). This was attributed to a block of the S_1 to S_2 transition by some workers (e.g. [21]) however we have suggested that it could be a block of the S_0 to S_1 transition [4]. In addition, we suggested earlier [4] that these two modes of inhibition correlate with the two different binding constants measured for Ca^{2+} reconstitution of oxygen-evolution activity in the absence of the 18 and 24 kDa proteins and that this heterogeneity might relate to the dark distribution of the S states. More specifically, the PS II centers inhibited by Ca^{2+} depletion in the dark are blocked at the S_0 to S_1 transition and these correspond to the PS II centers in the S_0 state at the start of the treatment. The PS II centers inhibited at the S_3 to S_0 transition correspond to centers in the S_1 state at the start of NaCl washing. From the current work it seems likely that Ca^{2+} depletion occurs mainly in the S_3 state which means that light is required to form the susceptible S states. Thus, on a percentage basis, the association of the low-affinity binding site for Ca^{2+} ($k_d = 1$–2 mM) with S_0 finds some support. The high-affinity binding constant may reflect Ca^{2+} binding to S_1 or S_2 state. Clearly, since S_3 is more susceptible to Ca^{2+} release than S_0, it is unlikely to have a higher affinity for Ca^{2+}. However, extra complexity must be introduced to explain some other relevant observations in the literature. Miyao and Murata [9] provided two lines of evidence indicating that, in the dark (i.e. in the S_1 state) bound Ca^{2+} is not in rapid equillibrium with free Ca^{2+}. Firstly, no inhibition was observed when long dark-adapted material was incubated 1 h with 20 mM EGTA. Secondly, Ca^{2+} readdition to Ca^{2+}-depleted PS II resulted in only a slow and partial reconstitution of this dark-occluded Ca^{2+} behaviour. A model was put forward involving accessible and inaccessible Ca^{2+} sites [9]. In addition, using the Sr^{2+}-modified S_2-state multiline signal as a probe [4], we have preliminary evidence (unpublished) that Sr^{2+} reconstitution occurs much more rapidly in S_3 or S_3 and S_0 than in S_1, indicating a kinetically occluded Ca^{2+}-binding site in S_1. The exact relationship between these phenomena and the effects reported here remains to be clarified by experimentation but there seems little doubt that a final model will involve the S-state dependence not only of binding equilibria but also of Ca^{2+} accessibility.

The observation that PS II centers in the S_0 state are inhibited by NaCl washing while those in the S_1 state are unaffected agrees with our prediction [4] but also explains an earlier report of Miyao and Murata [22]. These authors found no inhibition of oxygen evolution by NaCl washing in long dark-adapted material incubated with EGTA while a small fraction (14%) of centers was inhibited under the same conditions when the sample was dark adapted for only a short time. It has been shown that long dark-adapted material consists of essentially 100% of centers in the S_1 state while short dark-adapted material consists of approximately 25% S_0 and 75% S_1 [23,24]. It seems likely from the present results that the inhibited centers in the experiment in [22] correspond to those in the S_0 state. The requirement for PS II centers to be in the S_3 or S_0 states before inhibition due to Ca^{2+} depletion could be observed explains the nature of the light requirement observed earlier for this kind of inhibition [4,8,9].

The different binding of Ca^{2+} depending on the S states allows some speculation on a possible role for Ca^{2+} in the oxygen-evolving process. Ono and Inoue [25] have recently reported that the incubation of PS II membranes at pH 3 in a citrate containing medium led to the release of one Ca^{2+} per PS II center resulting in total inhibition of oxygen evolution. This effect suggests the involvement of a carboxyl group, such as aspartic acid or glutamic acid with an apparent pK_a equal to 4, in the Ca^{2+} ligation [25]. The weak and tight binding of Ca^{2+}, which is demonstrated here as being dependent on the S states, may indicate the making and breaking, especially in the S_3 state, of ligands with car-

boxyl groups. This process could be associated with S-state-dependent protons release. Also relevant perhaps to this idea, is the argument that the most energetically favorable water-oxidation reaction is when the protons released along with O_2 are initially bound to nearby basic groups ($pK_a = 8$) on the surrounding protein [26]. Following this argument deprotonation of these putative basic groups was suggested to occur due to a lowering of their pK_a values triggered by a conformational change [26]. Alternatively, the proton release could occur via carboxyl groups on the protein from which Ca^{2+} binding and debinding could induce subsequent deprotonation or protonation, respectively. Carboxyl groups in close proximity may possess a pK_a close to 8 [27]. It is interesting to note that a large number of the characteristics of the Ca^{2+}-binding site in the oxygen-evolving enzyme resemble to that of the sarcoplasmic reticulum calcium pump [27] (e.g. modification of binding affinity and accessibility to the site depending on the step in the enzyme cycle, changes in pK_a of carboxyl groups, structural modification induced by Ca^{2+} release). Very recently, Homann [28] found an higher susceptibility of PS II centers to NaCl washing at low pH than at high pH, which can be accentuated by preillumination of the sample. Moreover, he reports that the high-affinity binding site for Ca^{2+} ($k_d = 50$–$100\ \mu M$) can be converted into the low-affinity binding site by decreasing the pH. These results agree with our earlier proposal [4] that the two Ca^{2+}-binding affinities represent different states of the same binding site. It was also shown [28] that the Ca^{2+} stimulation of oxygen evolution in NaCl-washed PS II is increased by lowering the pH whereas the activity in the presence and the absence of Ca^{2+} decreased as expected if Ca^{2+} binding modulates and is modulated by the protonation state of a particular group involved in water oxidation.

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