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Different binding affinity sites of Ca^{2+} for reactivation of oxygen evolution in NaCl-washed Photosystem II membranes represent differently modified states of a single binding site

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Abstract

Oxygen evolution in Photosystem II (PS II) membranes which had been inactivated by treatment with a high concentration of NaCl in the light was restored by addition of a wide concentration of Ca^{2+} and kinetic analysis showed the occurrence of two binding sites with different affinity for Ca^{2+}; a low- (K_m = 1–2 mM) and a high-affinity (K_m = 50–100 μM) site. Additionally, removal of contaminating Ca^{2+} by treatment with Chelex 100 revealed a very-high-affinity binding site for Ca^{2+} (K_m = 4–10 μM). A substantial portion of the activity restored by Ca^{2+} survived treatment with a chelating reagent, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate, which removed Ca^{2+} bound to all the three affinity sites. Thus, there are three reversible binding sites and one irreversible binding site for Ca^{2+} in NaCl-washed membranes. The binding affinities of the reversible binding sites for Ca^{2+} were estimated after correcting for contribution from the irreversible binding site. When PS II membranes had been treated with increasing concentrations of NaCl, the irreversible binding site was partly converted into the reversible binding site and a low-affinity interaction of Ca^{2+} increased at the cost of high-affinity one. Addition of a low concentration of Triton X-100 to the washing medium also increased the high- and low-affinity sites and decreased the very-high-affinity site. Conversely, prolonged incubation of the salt-washed membranes with Ca^{2+} resulted in conversion of the high- and low-affinity sites to the very-high-affinity site. Thus, the four binding sites are at least partly interconvertible. The results show that the heterogeneity of Ca^{2+}-binding in terms of both binding affinity and reversibility derives from differently modified states of a single binding site.

Keywords: Calcium ion; Binding affinity; Oxygen evolution; Salt wash; Photosystem II membrane; (Spinach)

1. Introduction

Oxygen-evolving PS II membranes isolated from higher plants contain two [1–3] or three Ca^{2+} per PS II [1]. The oxygen-evolving activity is strongly reduced by wash of PS II membranes with a high concentration of NaCl in the light and the lost activity is restored by addition of Ca^{2+} [4–6]. Investigations on the Ca^{2+}-dependent reactivation of oxygen evolution and related reactions led to a proposal that more than one Ca^{2+} function in PS II electron transport (for reviews see [7–9]). There are, however, several lines of evidence indicating that only one Ca^{2+} functions in oxygen evolution. Oxygen-evolving PS II reaction center complexes prepared from the thermophilic cyanobacterium *Synechococcus elongatus* that are able to evolve oxygen at an extremely high rate contain one Ca^{2+} per PS II [10,11]. Highly active oxygen-evolving PS II complexes isolated from a chlorophyll-*b*-less mutant of rice [12] and normal spinach [13] also carry only one Ca^{2+} per PS II. A possibility that two Ca^{2+} present in spinach PS II membranes play different roles in electron transport from water to P680 was ruled out by our finding that the two Ca^{2+} are located in different functional domains of the membranes; one Ca^{2+} binds to the PS II reaction center core complex, while another is present in the peripheral LHC II assembly that functions in the light-harvesting [14]. Evidence was

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; LHC II, light-harvesting chlorophyll *a*/*b* proteins of PS II; P680, the primary electron donor chlorophyll of PS II.

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obtained indicating that the Ca^{2+} that is affected by the NaCl wash is the one bound to the core complex. Although none of the two Ca^{2+} present in PS II membranes is solubilized into the outer aqueous phase by treatment with NaCl alone [2], we showed that one Ca^{2+} does no longer bind to its functional site in the core complex and is readily solubilized in the presence of a detergent, leaving another Ca^{2+} bound to LHC II ([15], also see [1]). We concluded, therefore, that one and only one Ca^{2+} bound to the PS II core complex functions in oxygen evolution [14,15].

The mode of rebinding of Ca^{2+} for reactivating of oxygen evolution in NaCl-washed PS II preparations is, however, not so simple as would be expected from functioning of a single Ca^{2+} in PS II electron transport. Oxygen evolution is reactivated by addition of Ca^{2+} over a wide range of concentrations from several micromolar to 10–20 mM [1,4,16–18]. Kinetic analyses provided evidence for the occurrence of three sites of different affinity for Ca^{2+}, i.e., a low-affinity site with K_m of 1–5 mM, a high-affinity site with K_m of 50–100 μM [1,16–18], and a very-high-affinity site with K_m of 1–4 μM [19]. This has been taken as evidence for the presence of multiple binding sites of Ca^{2+} [8,19]. In particular, the very-high-affinity site has so far been reported only for highly purified oxygen-evolving PS II reaction center complexes with a Ca^{2+} to PS II ratio of less than one [19], but not in NaCl-washed PS II membranes, which have one or two Ca^{2+} per PS II depending upon the presence of a detergent during NaCl-wash [1,15]. It was suggested, therefore, that Ca^{2+} bound to this site has a function different from that of Ca^{2+} bound to other affinity sites [19].

The binding of Ca^{2+} to NaCl-washed PS II membranes is also heterogeneous in terms of reversibility. The oxygen-evolving activity restored by Ca^{2+} is strongly but incompletely suppressed by addition of a chelating reagent, indicating that the binding of Ca^{2+} is partly irreversible [1,16]. The irreversible binding of Ca^{2+} was related to a high-affinity site [1].

The present study dealt with the heterogeneity of Ca^{2+} binding in terms of affinity and reversibility. We addressed the following two questions. The first question we asked was whether the very-high-affinity site is indeed unique to the highly purified PS II complexes and absent from NaCl-washed PS II membranes? Our conclusion that a single Ca^{2+} bound to the core complex functions in oxygen evolution predicts that there should be no difference in the mode of Ca^{2+} binding between the two PS II preparations. It is difficult, however, to evaluate the effect of very low concentrations of Ca^{2+} in the presence of either a chelating reagent or contaminating Ca^{2+}. Reactivation of oxygen evolution by different concentrations of Ca^{2+} was, therefore, investigated with NaCl-washed PS II membranes after removal of extraneous Ca^{2+} with Chelex 100. We also separately analyzed binding affinities of the reversible and irreversible binding sites for Ca^{2+}. The second question we addressed was why there are different binding sites for Ca^{2+} in spite of the functioning of only one Ca^{2+} in oxygen evolution? There is evidence suggesting that high- and low-affinity sites are mutually convertible [17,18]. Thus, a possibility is that different binding sites for Ca^{2+} derive from a single binding site through a series of structural modifications. We examined this possibility by investigating binding of Ca^{2+} in PS II membranes which had been washed with NaCl in the presence and absence of Triton X-100 or with different concentrations of NaCl. Reactivation of oxygen evolution in the salt-washed membranes which had been incubated with Ca^{2+} for different periods of time was also studied. The results show that the heterogeneity of Ca^{2+} binding does not contradict the notion that only one Ca^{2+} functions in oxygen evolution.

2. Materials and methods

Oxygen-evolving PS II membranes were prepared from spinach with Triton X-100 according to the method of Berthold et al. [20] with slight modifications [2]. The membranes were once washed with and suspended in 0.4 M sucrose, 40 mM Mes-NaOH (pH 6.5), 10 mM NaCl and 0.5 mM EGTA, and stored at 77 K.

For the NaCl wash, PS II membranes were incubated with 0.4 M sucrose and 40 mM Mes-NaOH (pH 6.5) and indicated concentrations of NaCl for 30 min under room light (7 μmol photons $m^{-2}s^{-1}$) at 0°C, collected by centrifugation at $35000 \times g$ for 10 min and washed with and suspended in 0.4 M sucrose, 40 mM Mes-NaOH (pH 6.5) and 10 mM NaCl. NaCl wash was also performed in the presence of 0.03% Triton X-100 [15]. Oxygen evolution was determined with a Clark-type oxygen electrode at 25°C as described previously [2]. The electron acceptor added was 0.4 mM phenyl-*p*-benzoquinone. Ca^{2+} contaminating the suspending media and membrane suspensions was removed by treatment with Chelex 100 [10,14].

3. Results

3.1. Is the very-high-affinity site absent from NaCl-washed PS II membranes?

The very-high-affinity site for Ca^{2+} with a K_m of several micromolar has so far been reported for the salt-treated highly purified oxygen-evolving PS II complexes [19] but not for NaCl-washed PS II membranes. We first investigated whether the site is indeed absent from NaCl-washed PS II membranes. PS II membranes inactivated by treatment with 1.5 M NaCl in room light were incubated with different concentrations of Ca^{2+} for 10 min and the oxygen-evolving activity restored was determined (Fig. 1). Although the maximum reactivation required 20 mM Ca^{2+}, the activity was substantially restored on addition of Ca^{2+}

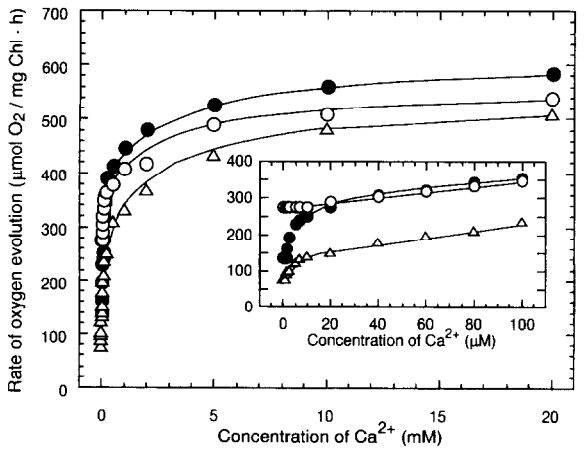

Fig. 1. Reactivation of oxygen evolution by different concentrations of Ca^{2+}. PS II membranes washed with 1.5 M NaCl in the presence or absence of 0.03% Triton X-100 under room light for 30 min were incubated with indicated concentrations of Ca^{2+} for 10 min at 0°C. ○, NaCl-washed membranes not treated with Chelex 100; ● and △, NaCl- and NaCl/Triton X-100-washed membranes that were treated with Chelex 100 prior to incubation with Ca^{2+}, respectively.

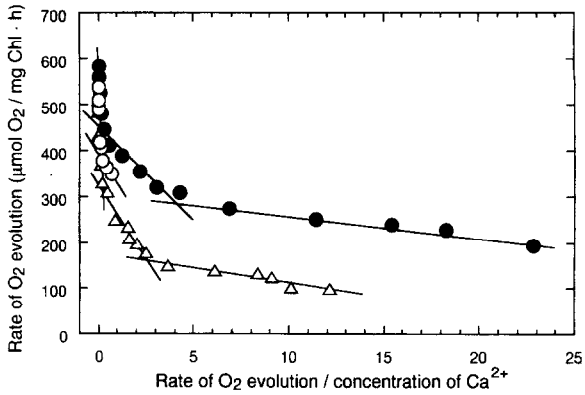

Fig. 2. Woolf-Hofstee plot of rates of oxygen evolution versus concentration of Ca^{2+} added. Symbols are as in Fig. 1.

below 1 mM. The inset shows the reactivation of oxygen evolution by Ca^{2+} below 100 μM. A notable difference was found before and after treatment with a chelating resin, Chelex 100, an effective procedure to remove extraneous Ca^{2+} [10,14]. Before the treatment, the membranes showed a high residual activity and addition of Ca^{2+} below 10 μM had no effect on the activity (open circles). Removal of contaminating Ca^{2+} with Chelex 100 resulted in a large decline in the residual activity and a sharp rise of the activity was observed on addition of Ca^{2+} in the mM region (solid circles). A smaller magnitude of the activity rise at low concentrations of Ca^{2+} was also detected in PS II membranes that had been washed with NaCl in presence of 0.03% Triton X-100 (open triangles). The oxygen evolving activity restored by low concentrations of Ca^{2+} gradually decreased during illumination, reflecting an instability of the Ca^{2+} binding in PS II membranes depleted of the extrinsic 23 kDa protein in the light [15]. The activity was therefore determined from the initial slope of recorder tracings. Addition of 0.5 mM EGTA also diminished the residual activity but it was difficult to estimate effects of low concentrations of Ca^{2+} in the presence of the chelator (not shown).

Plots of oxygen evolving activity vs. activity divided by Ca^{2+} concentration (Woolf-Hofstee plot) are shown in Fig. 2 and the K_m values estimated are listed in Table 1. There are two sites with different binding affinities for Ca^{2+} in NaCl-washed membranes that had not been treated with Chelex 100 (Fig. 2). The K_m values of the two sites correspond to those of the high- and low-affinity sites [1,6,16,17]. When contaminating Ca^{2+} had been removed, an additional Ca^{2+}-binding site appeared, which had a K_m value of 4 μM. The result shows that the very-high-affinity binding site is present in NaCl-washed PS II membranes.

Fig. 1 and Table 1 indicate that the three sites of differing affinity are also present in NaCl/Triton X-100-washed PS II membranes. We showed previously that NaCl-wash in the presence of a low concentration of Triton X-100 liberates one Ca^{2+} into the outer aqueous phase, leaving another Ca^{2+} bound to the membranes [15]. Thus, the appearance of all the three affinity sites is related to release of one Ca^{2+} per PS II. This argues against the notion that one of the two Ca^{2+} present in PS II membranes binds to the very-high-affinity site and another to a lower-affinity site. Note that the magnitude of the very-high-affinity reactivation was appreciably reduced, accompanied by increases in those of the high- and the low-affinity reactivations, in the presence of the detergent during the NaCl wash. Because the total activities restored by Ca^{2+} were comparable, the result suggests that the very-high-affinity site is partly converted into lower-affinity sites under influence of the detergent.

Table 1
K_m values of Ca^{2+} binding in NaCl- and NaCl/Triton X-100-treated PS II membranes

Treated with	Chelex 100 treatment	Activity restored (μmol O_2/mg Chl per h)	K_m (μM)		
			very high	high	low
NaCl	–	262	–	55 (45%)	2200 (55%)
NaCl	+	432	4 (37%)	68 (23%)	2000 (40%)
NaCl + Triton	+	446	7 (23%)	86 (28%)	2200 (49%)

Numbers in parenthese denote relative magnitudes of the activity restored by Ca^{2+} bound to each site.

3.2. Irreversible binding of Ca^{2+}

The oxygen-evolving activity of NaCl-washed membranes which is restored by Ca^{2+} is strongly but incompletely suppressed by addition of EDTA [3] or EGTA [1,18]. This indicates that the binding of Ca^{2+} that is effective in the reactivation of oxygen evolution is partly irreversible. The K_m values were estimated above without distinguishing between the reversible and irreversible bindings. The following experiments were carried out to separately determine affinities of the reversible and irreversible binding sites for Ca^{2+}. PS II membranes washed with 1.5 M NaCl were incubated with various concentrations of Ca^{2+} for 10 min and, after 50-fold dilution with a Ca^{2+}-free medium, 0.5 mM EGTA was added to the membrane suspension to sequester free and reversibly bound Ca^{2+}. Without dilution, addition of EGTA resulted in acidification of media containing high concentrations of Ca^{2+}. Rates of oxygen evolution were determined before and after addition of EGTA. The EGTA-resistant activity was largely restored by addition of 100 μM Ca^{2+}, while about 5 mM of the metal cations was required for the full reactivation of the EGTA-sensitive activity (Fig. 3A). Kinetic analysis of the EGTA-sensitive activity showed that there are three reversible interactions of Ca^{2+} with different binding affinities (Fig. 3B). The K_m values estimated are shown in Table 2. It is concluded from these results that the very-high-affinity site is present in NaCl-washed PS II membranes.

The restoration of the EGTA-resistant activity depended upon concentration of Ca^{2+} as if the activity was restored by Ca^{2+} bound to the high-affinity site ('K_m', 148 μM) and to the very-high-affinity site ('K_m', 4 μM) (Fig. 3B). Cammarata and Cheniae [1] also showed previously that Ca^{2+} irreversibly binds to a site with a 'K_m' of about 65 μM in NaCl-washed membranes. It is to be stressed, however, that the kinetic analysis is not valid for an irreversible binding and the agreement of the 'K_m' values with the K_m values of the high- and the very-high-affinity sites is superficial. Nonetheless, the data show that the irreversible binding site has a high-affinity for Ca^{2+}: the concentration of Ca^{2+} that is needed to occupy 50% of the irreversible binding sites during incubation time of 10 min is about 100 μM.

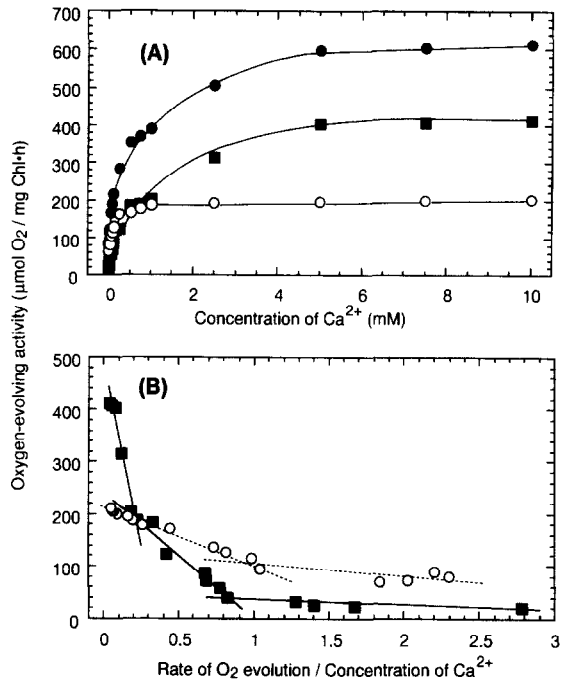

Fig. 3. (A) Effects of EGTA on the oxygen-evolving activity restored by different concentrations of Ca^{2+}. PS II membranes washed with 1.5 M NaCl were incubated with various concentrations of Ca^{2+} for 10 min and rates of oxygen evolution were determined before and after 50-fold dilution with a Chelex 100-treated solution containing 0.4 M sucrose and 40 mM Mes-NaOH (pH 6.5) and addition of 0.5 mM EGTA. ●, total activity restored; ○, EGTA-resistant activity; ■, EGTA-sensitive (total-EGTA-resistant) activity. (B) Woolf-Hofstee plot of EGTA-sensitive and -resistant activities versus concentration of Ca^{2+} added.

Table 2
Reversible and irreversible bindings of Ca^{2+} in PS II membranes washed with three different concentrations of NaCl

Binding sites	NaCl concentration								
	0.5 M			1.5 M			3.0 M		
	K_m (μM)	activity restored (μmol O_2/mg Chl per h)		K_m (μM)	activity restored (μmol O_2/mg Chl per h)		K_m (μM)	activity restored (μmol O_2/mg Chl per h)	
Reversible									
Very high	9	17	8%	17	35	9%	6	32	9%
High	195	140	67%	375	264	63%	376	193	54%
Low	460	52	25%	946	109	28%	953	132	37%
Total		209			390			357	
Irreversible									
	(3)	75	30%	(4)	14	9%	(8)	11	8%
	(37)	78	31%	(148)	141	91%	(110)	126	92%
	(299)	98	39%						
Total		250			155			137	

The residual activities determined in the presence of 0.5 mM EGTA were 200, 75 and 59 μmol O_2 mg^{-1} Chl h^{-1} for membranes washed with 0.5, 1.5 and 3.0 M NaCl, respectively.

3.3. Are the four different binding sites interconvertible?

Experiments described above show that, in total, there are four different binding sites for Ca^{2+} for the restoration of oxygen evolution in NaCl-washed membranes; one irreversible and three reversible binding sites. In view of involvement of a single Ca^{2+} in oxygen evolution [14,15], an explanation would be that the different binding sites for Ca^{2+} derive from a single binding site through a series of structural modifications. The observation that addition of Triton X-100 increased the high- and the low-affinity sites at the cost of the very-high-affinity site (Table 1) is consistent with this explanation. In the following, we investigated interconvertibility of the four binding sites by comparing the binding of Ca^{2+} in PS II membranes washed with three concentrations of NaCl (Table 2). More than 50% of the activity restored by Ca^{2+} was irreversible in PS II membranes washed with 0.5 M NaCl. Rates of oxygen evolution that remained after addition of EGTA decreased from 250 to 137 μmol O_2/mg Chl per h as the concentration of NaCl was raised from 0.5 M to 3.0 M. This indicates conversion of the irreversible to the reversible binding sites because the total activities restored by Ca^{2+} were comparable at the three NaCl concentrations. Conversion from the high- to the low-affinity site was also indicated by changes in the relative contribution of the two affinity sites: with increasing concentration of NaCl, the percent contribution of the low-affinity interaction increased and that of the high-affinity interaction decreased. PS II membranes washed with 0.5 M NaCl showed smaller K_m values for the high- and low-affinity sites than did the membranes treated with higher concentrations of NaCl. Thus, the binding of Ca^{2+} is progressively weakened by treatment of PS II membranes with increasing concentrations of NaCl. Table 2 also shows that the Ca^{2+} concentration required for a half-maximum irreversible reactivation during incubation time of 10 min was significantly smaller in PS II membranes washed with 0.5 M NaCl than in the membranes washed with 1.5 or 3.0 M NaCl.

Fig. 4 shows changes in the three affinity interactions of Ca^{2+} during a prolonged incubation of NaCl-washed PS II membranes with different concentrations of Ca^{2+}. The magnitude of the activity restored by Ca^{2+} bound to the very-high-affinity site slowly increased during incubation with Ca^{2+}, accompanied by a decrease in that of the low-affinity reactivation. Because the magnitude of the total activity that was reversibly restored by Ca^{2+} was constant after 10 min of the incubation, the result indicates that the low-affinity site is gradually converted into the very-high-affinity site during incubation with Ca^{2+}. Changes in the magnitude of the high-affinity reactivation were small, perhaps reflecting a balanced conversion of the low to the high- and the high to the very-high-affinity site. There were gradual decreases in the K_m values of the very-high- and low-affinity sites during incubation with

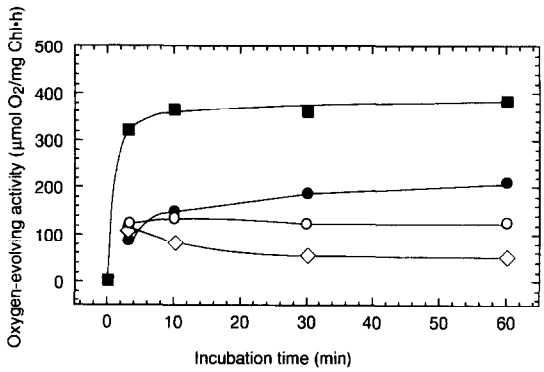

Fig. 4. Changes in the magnitude of reactivation of oxygen evolution by Ca^{2+} bound to the three affinity sites during incubation of NaCl-washed PS II membranes with Ca^{2+}. PS II membranes washed with 1.5 M NaCl were incubated with different concentrations of Ca^{2+} for the indicated periods of time. ■, total activity that was reversibly restored by Ca^{2+}. ●, ○ and ◇, activities restored by Ca^{2+} bound to the very-high-, the high- and the low-affinity sites, respectively.

Ca^{2+} (data not shown). The result show that NaCl-induced changes in the reversible binding sites are reversible.

4. Discussion

The present study shows that the heterogeneity of Ca^{2+} binding for reactivation of oxygen evolution in NaCl-washed PS II membranes in terms of affinity and reversibility does not contradict our proposal that only one Ca^{2+} functions in oxygen evolution. The high- and low-affinity interactions of Ca^{2+} have repeatedly been found in NaCl-washed PS II membranes that contain one or two Ca^{2+} per PS II [1,16–19], whereas the very-high-affinity one has been reported only for salt-washed purified PS II reaction center complexes that have less than one Ca^{2+} per PS II [19]. This was taken as evidence that one Ca^{2+} that remains bound to the very-high-affinity site in PS II membranes but not in the purified complexes after salt-wash has a function different from that of another Ca^{2+} bound to other affinity sites [19]. The present study excludes this possibility by showing that the very-high-affinity site is present in NaCl-washed PS II membranes. Furthermore, the very-high-affinity interaction of Ca^{2+} was observed in NaCl/Triton X-100-washed membranes that lost one of the two Ca^{2+} present per PS II [15]. This shows that the binding site of the Ca^{2+} remaining is not the very-high-affinity site. The results are consistent with our previous observation that, out of the two Ca^{2+} present in PS II membranes, one Ca^{2+} binds to a LHC II assembly and another Ca^{2+} that is located in the PS II core complex and susceptible to NaCl-wash functions in oxygen evolution [14,15]. The low abundance of Ca^{2+} in the salt-washed purified PS II complexes is therefore ascribed to the absence of LHC II from the complexes and depletion of the core Ca^{2+}. Thus, irrespective of preparations, the

appearance of all the three affinity sites is related to dislodging of one Ca^{2+} bound to the PS II core complex.

The previous failure to observe the very-high-affinity reactivation of Ca^{2+} in the salt-treated membranes is ascribed to the presence of either contaminating Ca^{2+} or a chelating reagent in the reaction media [1,6,16–18]. Suspensions of PS II membranes usually contain extraneous Ca^{2+} at concentrations sufficient to mask the very-high-affinity site. EGTA or EDTA has been added to assay media to sequester contaminating Ca^{2+}, but this makes it difficult to determine reactivation of oxygen evolution by Ca^{2+} in the micromolar region. An important advantage of Chelex 100 treatment is that the chelating resin is not only effective in removal of Ca^{2+} bound to the very-high-affinity site but also easily separated from membrane suspensions by precipitation [10]. The successful demonstration of the very-high-affinity interaction of Ca^{2+} in the present study is, therefore, ascribed to Chelex 100 treatment of the membrane suspensions which allowed us to measure oxygen evolution in the absence of both contaminating Ca^{2+} and a chelating reagent.

Evidence was obtained to indicate that the three reversible binding sites and one irreversible binding site of Ca^{2+} are, at least partly, interconvertible. Treatment of PS II membranes with increasing concentrations of NaCl resulted in increases in the reversible binding sites at the cost of the irreversible binding site. Among the reversible binding site, contribution of the low-affinity site increased and that of the high-affinity site decreased as concentrations of NaCl was raised. Addition of a low concentration of Triton X-100 to the washing medium also increased the high- and the low-affinity sites at the cost of the very-high-affinity site. Conversely, a prolonged incubation of the salt-washed membranes with Ca^{2+} resulted in an increase in the very-high-affinity site that is associated with decreases of the high- and low-affinity sites. It is concluded, therefore, that the different binding sites for Ca^{2+} represent differently modified states of a single Ca^{2+}-binding site.

The site that irreversibly binds Ca^{2+} may be or close to the native state of the binding site. The extraction of the extrinsic 23 kDa is incomplete at 0.5 M NaCl, but the protein is totally solubilized at 1.5 M NaCl [21]. The irreversible binding of Ca^{2+} cannot be related to the 23 kDa protein remaining bound to PS II membranes because a substantial portion of the activity restored by Ca^{2+} is irreversible in PS II membranes washed with 1.5 or 3.0 M NaCl. The irreversible binding site has a relatively high affinity for Ca^{2+}. The concentration of Ca^{2+} that is needed for 50% occupancy of the irreversible binding sites during 10 min of incubation was about 100 μM in PS II membranes washed with 1.5 M and 3.0 M NaCl. It is stressed, however, that this Ca^{2+} concentration is a function of incubation time and will decrease with increasing time in contact with Ca^{2+}. The membranes washed with 0.5 M NaCl required a lower concentration of Ca^{2+} for a half-maximum irreversible reactivation. The difference may be ascribed to a subtle change in the conformation of the binding site, or the 23 kDa protein that remained partly unextracted at 0.5 M NaCl [21]. Loss of the irreversibility of the Ca^{2+} binding may be related to a structural change in the Ca^{2+}-binding site. Further modifications of the binding site may result in successive reduction of the binding affinity for Ca^{2+} from the very-high to the high, then to the low-affinity site. The small K_m values found in PS II membranes washed with 0.5 M NaCl may be related to a smaller extent of structural modification or an incomplete extraction of the 23 kDa protein.

Our results show that there are two different functions of Ca^{2+} in the reactivation of oxygen evolution in NaCl-washed PS II membranes. First, Ca^{2+} reactivates PS II electron transport by binding to its functional site. This effect of Ca^{2+} is independent of the affinity and reversibility of the binding and seems to appear immediately after rebinding of Ca^{2+}. Presumably, optimal operation of the water-oxidation machinery requires the presence of Ca^{2+} near the Mn cluster. The second effect of Ca^{2+} is a reversal of NaCl-induced changes in the binding affinity for Ca^{2+}. This Ca^{2+} effect may involve conformational changes of the Ca^{2+}-binding site and hence develop more slowly than the first Ca^{2+} effect. It is known that inactivation of oxygen evolution by NaCl wash is strongly diminished when Ca^{2+} is present in the washing medium [16]. The protecting effect of Ca^{2+} may be, at least partly, ascribed to the second effect of Ca^{2+}.

In conclusion, the two questions we asked in the Introduction were answered in the present study. All the three different affinity sites and the one irreversible binding site are present in NaCl-washed PS II membranes. The heterogeneity of Ca^{2+} binding in terms of both affinity and reversibility are related to differently modified states of the Ca^{2+} binding site in the PS II core complex. Thus, the complex mode of Ca^{2+}-binding for reactivation of oxygen evolution does not contradict the notion that only one Ca^{2+} functions in PS II electron transport.

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