Mn-preserving extraction of 33-, 24- and 16-kDa proteins from O_2-evolving PS II particles by divalent salt-washing

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Divalent salt-washing of O_2-evolving PS II particles caused total liberation of 33-, 24- and 16-kDa proteins, but the resulting PS II particles retained almost all amounts of Mn present in initial particles. The retained Mn was EPR-silent when the particles were kept in high concentrations of divalent salt. By divalent salt-washing, the activity of diphenylcarbazide (DPC) photooxidation was not affected at all, neither suppressed nor enhanced, while O_2 evolution was totally inactivated. These results indicate that Mn can be kept associated with PS II particles even after liberation of the 33-kDa protein, and suggest that the 33-kDa protein is probably not responsible for binding Mn onto membranes, but is possibly responsible for maintaining the function of Mn atoms in the O_2-evolving center.

O_2-evolution *Divalent salt-washing* *PS II particle* *EPR* *Mn-binding* *33-kDa protein*

1. INTRODUCTION

Three proteins with approximate molecular masses of 33, 24 and 16 kDa have been revealed to be closely related to the function of photosynthetic O_2 evolution. These proteins are located on the inner surface of thylakoid membranes [1] and are liberated by various treatments from inside-out thylakoids [1,3], O_2-evolving PS II particles [2,4] or from broken thylakoids [5].

Monovalent salt-washing of PS II particles [4,6] or inside-out thylakoids [3] liberates 24- and 16-kDa proteins without release of Mn and the 33-kDa protein, and causes partial inactivation of O_2 evolution. These liberated proteins are capable of rebinding to the washed membranes concomitant with partial reconstitution of O_2-evolution [3,7,8]. One of these two proteins, the 24-kDa protein, has been suggested to play either a regulatory [8] or an essential [9] role in O_2 evolution.

Tris- and/or alkaline-washing of PS II particles [2,4] or inside-out thylakoids [1] liberates all of 33-, 24- and 16-kDa proteins concomitant with release of Mn [6], and causes total inactivation of O_2 evolution. Up to now, no successful reconstitution of O_2 evolution has been reported with Tris- and/or alkaline-washed PS II particles. The concomitant release of Mn and the 33-kDa protein seems to be the major cause for the difficulty in reconstituting experiments as well as in understanding the role of the 33-kDa protein in O_2 evolution. Obviously, the direct cause for inactivation of O_2 evolution is the release of Mn, but two (or more than two) interpretations may be possible regarding the role of the 33-kDa protein; it either provides binding sites of Mn on membranes in the vicinity of PS II reaction center, or functions as a proteinous factor for maintaining the conformation of the O_2-evolving center including Mn [8,10].

From the parallel relationship found for release

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Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; DMQ, 2,5-dimethylbenzoquinone; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Chl, chlorophyll

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courses of Mn and the 33-kDa protein during Tris- and/or alkaline-washing, the 33-kDa protein has been considered to be closely involved in maintaining Mn on membranes [6,13]; e.g., by providing Mn binding sites. Such a view may be supported by the report in [11] on algal mutants that loss of O_2 evolution is accompanied by a decrease in Mn abundance and a modification of the 33-kDa protein. However, this view does not always seem to be consistent with the fact that the purified 33-kDa protein bears no Mn [6,8,12,13].

Under these circumstances, a technique for discrete liberation of the 33-kDa protein leaving Mn unaffected is desirable. Here, we report a new method to liberate the 33-, 24- and 16-kDa protein from the O_2-evolving PS II particles without releasing Mn. The results provide evidence that none of these 3 proteins are directly responsible for binding Mn to PS II membranes, and in turn suggest that the protein is required in maintaining the function of Mn atoms in the O_2-evolving center.

2. MATERIALS AND METHODS

O_2-evolving PS II particles were prepared from spinach as in [14], with modifications. Thylakoid membranes were suspended (2 mg Chl/ml) in 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 40 mM Mes–NaOH (pH 6.5) and incubated with Triton X-100 (25 mg/ml Chl) at 4°C for 5 min. The precipitate sedimented by centrifugation (35000 × *g*, 20 min) was resuspended in the same buffer without Triton X-100 and centrifuged (7000 × *g*, 15 min) and the pelleted PS II particles were suspended in the same medium and stored at −80°C until use. The electron transport activity of this preparation (300–600 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$ and 350–700 μmol DCIP $\cdot$ mg $Chl^{-1} \cdot h^{-1}$ at pH 6.5 with DMQ and DCIP, respectively, as electron acceptor) did not change for more than 6 months at −80°C. The preparation, when used, was defrosted and washed at least 3-times with 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5) in order to completely remove non-specifically releasing material.

For divalent salt-washing, the particles were suspended (1 mg Chl/ml) in 1 M $CaCl_2$ (or 1 M $MgCl_2$), 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5), incubated at 0°C for 30 min in darkness, pelleted by centrifugation (35000 × *g*, 15 min) and then suspended in the respective buffer medium indicated for each experiment. For Tris-washing, the particles were suspended (1 mg Chl/ml) in 0.8 M Tris–HCl (pH 8.3), incubated at 0°C for 30 min, and then pelleted by centrifugation (35000 × *g*, 15 min).

The O_2 evolution was measured with a Clark-type oxygen electrode at 25°C in 300 mM sorbitol, 10 mM NaCl, 1 mM DMQ and 40 mM Mes–NaOH (pH 6.5). DCIP photoreduction was measured spectrophotometrically at 600 nm in 300 mM sorbitol, 10 mM NaCl, 0.07 mM DCIP, 40 mM Mes–NaOH (pH 6.5) and 0.5 mM DPC (when used) using the extinction coefficient of DCIP at pH 6.5, 18.0 $mM^{-1} \cdot cm^{-1}$. Saturating actinic light was provided by a 500 W tungsten lamp passing through a red filter (Toshiba, VR-65) and a heat absorbing filter (Nihon Shinku, Cold filter-B). The Chl concentration was determined as in [15].

Protein composition was analyzed by SDS–PAGE in the buffer system of [16] containing 6 M urea using a slab gel containing 6% (stacking gel) and 13.5% (resolving gel) acrylamide. Samples were dialyzed overnight against 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5) to minimize the concentration of divalent salts, and then dissolved in 1% SDS, 5% mercaptoethanol, 15% (w/v) sucrose and 10 mM Tris–HCl (pH 7.6). The gel was stained in 0.1% Coomassie brilliant blue R-250. The densitogram of stained gel was obtained with a Shimadzu dual-wavelength chromatoscanner (CS-900).

The abundance of Mn was determined with a Shimadzu atomic absorption spectrophotometer (AA-640-13) equipped with a graphite furnace atomizer (GFA-3). Samples (10 μl) were dried at 150°C for 40 s, ashed at 650°C for 30 s and atomized at 2400°C for 6 s.

The EPR spectrum of Mn was measured at room temperature with a JEOL EPR spectrometer, model JES-PE-3X at 9.45 GHz. The suspending medium is indicated in the legends of fig.2.

3. RESULTS AND DISCUSSION

Fig.1 shows the results of SDS–PAGE analysis of the protein composition of O_2-evolving PS II particles before and after washing with 1 M $CaCl_2$. Before washing, about 15 protein bands were

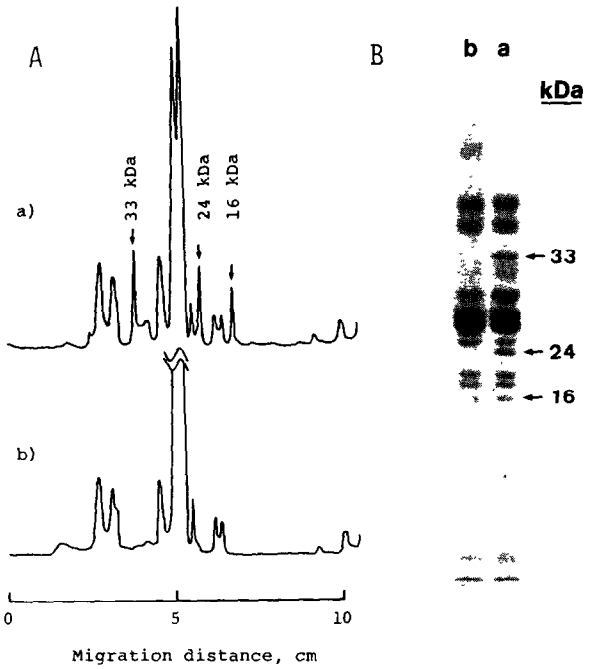

Fig.1. SDS–PAGE densitogram (A) and electrophoretogram (B) of PS II particles: (a) control particles before washing; (b) $CaCl_2$-washed particles.

observed (Aa, Ba) in agreement with the results of other workers [2,4]. After $CaCl_2$-washing, the proteins with apparent molecular masses of 33, 24 and 16 kDa are absent in the particles, as clearly seen on both the electrophoretogram (Bb) and the densitogram (Ab). The results indicate that the 3 proteins were mostly liberated from the PS II particles by 1 M $CaCl_2$-washing. The 3 proteins were comigrated with those liberated by washing the same particles with 0.8 M Tris–HCl (pH 8.3) and were confirmed to be identical with the proteins reported in [1], [2] and [4]. The same results were obtained by washing with 1 M $MgCl_2$ (not shown).

In most of the previous reports (6,8,12,13], liberation of the 33-kDa protein has resulted in concomitant release of Mn from PS II particles. In contrast to these, however, washing with divalent salts did not cause any marked release of Mn. As shown in table 1, the Mn abundance in particles after $CaCl_2$- or $MgCl_2$-washing was 7–8 Mn/400 Chl, being not much changed from that of the control, while the abundance after Tris-washing was markedly decreased to a level as low as 0.1 Mn/400 Chl. The above Mn determination for divalent salt-washed samples is not very accurate because of inevitable strong interference in atomic absorption measurements due to divalent salts used for washing. However, the results indicate clearly that the effect of $CaCl_2$-washing on Mn in PS II particles is very different from that of Tris-washing, although both treatments similarly liberate the 3 proteins.

In order to confirm the presence of Mn in the particles after extraction of the 3 proteins, EPR signals of Mn were investigated (fig.2). Firstly, the $CaCl_2$-treated sample was subjected to EPR measurement without removing the liberated material. As shown by curve b, no EPR signal due

Table 1

Effect of various washings on Mn abundance and O_2-evolving activity of PS II particles

Treatment	O_2 evolution (μmol $O_2 \cdot$ mg Chl$^{-1} \cdot h^{-1}$)	Mn abundance (gatoms/400 Chl)	Protein released (kDa)
Control	380	7.0	None
1 M $CaCl_2$, pH 6.5	15	7.4	33, 24, 16
1 M $MgCl_2$, pH 6.5	30	7.0	33, 24, 16
0.8 M Tris, pH 8.3	20	0.1	33, 24, 16 [2,4]
1 M NaCl, pH 6.5[a]	95	6.5	24, 16 [3,6]
2 mM NH_2OH, pH 6.5[b]	20	1.5	[27 > 34 ⩾ 17][c] [10]

[a] Washed in 1 M NaCl, 300 mM sorbitol, 40 mM Mes–NaOH, pH 6.5
[b] Washed in 2 mM NH_2OH, 300 mM sorbitol, 10 mM NaCl, 40 mM Mes–NaOH, pH 6.5
[c] Data from [10]

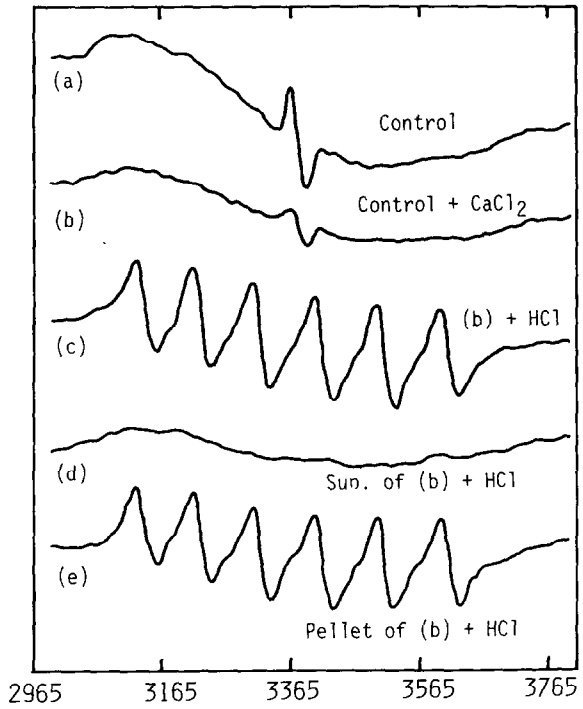

Fig.2. Effect of $CaCl_2$-washing on EPR signal of Mn in PS II particles: (a) Control PS II particles in 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5); (b) PS II particles during washing with $CaCl_2$ (buffer for (a) plus 1 M $CaCl_2$); (c) (b) plus 0.5 N HCl; (d) 33-, 24- and 16-kDa proteins (supernatant fraction obtained by centrifugation of the sample for (b)) in the presence of 0.5 N HCl; (e) $CaCl_2$-washed PS II particles (pellet fraction obtained by centrifugation of the sample for (b)) suspended in $CaCl_2$ containing medium in the presence of 0.5 N HCl. Chl concentration, 1 mg/ml. Instrumental conditions (microwave power, 30 mW; modulation amplitude, 20 G; time constant, 1 s; receiver gain 7.9×10^3) were the same for all spectra.

to aqueous Mn^{2+} could be found. Secondly, the $CaCl_2$-treated sample was centrifuged, and the resulting pellet and supernatant were separately subjected to EPR measurement in the presence of 0.5 N HCl. The supernatant fraction which contained almost all amount of the 3 proteins did not show any signal (curve d), whereas the pellet fraction which did not contain any of the 3 proteins showed a marked signal of aqueous Mn^{2+} (curve e) at a signal amplitude comparable to that of the control sample (curve c). These results clearly indicate that the Mn atoms in PS II particles are not liberated by $CaCl_2$-washing but are safely preserved, probably at the original binding site, even after complete extraction of the 3 proteins. Quite a similar result was obtained by washing with 1 M $MgCl_2$ (not shown).

The failure in detecting aqueous Mn^{2+} in the first experiment (curve b) may imply that most of the Mn atoms present in the initial PS II particles are not those loosely bound to the particles to be replaced by externally added divalent salts as in [17,18], but are those closely associated with the particles as functional constituents for oxygen evolution. This interpretation seems reasonable if we take into account that the Mn abundance in the presence PS II particles was 7–8 Mn/400 Chl, close to the proposed number of Mn atoms per functional O_2-evolving center [6,10].

Table 2 shows the effect of $CaCl_2$-washing on the electron transport activity of PS II particles. By $CaCl_2$-washing, the activity from H_2O to DCIP or DMQ was completely inhibited. Since $CaCl_2$-washed particles retain almost all Mn, the total inhibition of water-oxidation activity indicates that the Mn atoms remaining in the washed-PS II complex are not capable of oxidizing water. Probably, the 33-kDa protein is necessary as a counterpart for the Mn atoms to be functional. In most of the previous works reporting the involvement of this protein in O_2 evolution [2,4,8,10], the liberation of the 33-kDa protein was accompanied by the release of Mn, so that there remained an ambiguity regarding the function of this protein: whether the protein simply provides the binding site for Mn on the PS II complex or the protein is required for the function of the Mn atoms in the PS II complex. Here, the 33-kDa protein was released independently, leaving Mn unaffected. The total inhibition of water-oxidation activity by $CaCl_2$-washing, therefore, seems to support the latter view that the protein is required not for binding Mn to the PS II complex but for the function of the Mn atoms even if they are attached to PS II complex.

In contrast to water oxidation activity, the electron transport activity from DPC to DCIP was not affected at all by $CaCl_2$-washing, neither suppressed nor stimulated (table 2). This absolute indifference of $CaCl_2$-washing to DPC photooxidation sharply contrasts with the effect of Tris-washing which usually stimulates DPC photooxidation ac-

Table 2

Effect of $CaCl_2$-washing on electron transport reactions in O_2-evolving PS II particles

Conditions	Electron transport activity (e^- μequiv. · mg $Chl^{-1} \cdot h^{-1}$)		
	$H_2O \rightarrow DMQ$	$H_2O \rightarrow DCIP$	$DPC \rightarrow DCIP$
Control	1520	600	630
1 M $CaCl_2$-washed	10	10	650

tivity by a factor of 2 as in [4]. This contrast can reasonably be ascribed to the difference in Mn abundance between the two samples: most Mn is preserved safe in $CaCl_2$-washed particles, while mostly removed in Tris-washed particles. In Mn-depleted PS II particles, DPC will directly donate electron to Z^+, the secondary donor of PS II, which may result in a stimulated rate of DPC photooxidation. Whereas in Mn-retaining PS II paticles the direct electron donation from DPC to Z^+ may be restricted by some structural hindrance due to the presence of Mn atoms or by redox mediation by Mn between DPC and Z^+. The fact that DPC photooxidation activity was not affected at all by $CaCl_2$-washing, therefore, seems to support the view that the state of Mn atoms remaining in the PS II complex after $CaCl_2$-washing is not much modified, but preserves the original binding state, although this view has to await further confirmation by some other means, such as reconstitution experiments. Quite similar results have been obtained by washing with $MgCl_2$ (not shown).

This study revealed that $CaCl_2$-washing as well as $MgCl_2$-washing liberates 33-, 24- and 16-kDa proteins without any release of Mn from O_2-evolving PS II particles. The results indicate that Mn atoms are kept associated with the PS II reaction center complex even after liberation of the 33-kDa protein, if the concentration of divalent salts in the medium is high. The Mn atoms in such a reaction center complex are inactive in O_2 evolution, but seem to preserve the original binding state. Chemical and functional characterization of these Mn atoms are underway including reconstitution with the 33-kDa protein and two other proteins.

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