Ca^{2+}-dependent restoration of O_2-evolving activity in $CaCl_2$-washed PS II particles depleted of 33, 24 and 16 kDa proteins

Taka-aki Ono and Yorinao Inoue*

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351, Japan

Received 26 January 1984

$CaCl_2$-washing of O_2-evolving PS II particles liberated 33, 24 and 16 kDa proteins concomitant with inactivation of O_2 evolution, whereas almost all Mn remained associated with membranes [FEBS Lett. 164 (1983) 252–260], and the lost O_2 evolution was significantly restored when 33 kDa protein rebound to the washed membranes [FEBS Lett. 166 (1984) 381–384]. Half of the Mn atoms retained in $CaCl_2$-washed particles were unstably associated with the membrane, being gradually released during incubation in the absence of Ca^{2+}, whereas in the presence of Ca^{2+} the release of Mn was suppressed concomitant with partial reactivation of O_2 evolution. These results were interpreted as indicating that Ca^2 as well as 33 kDa protein maintains the conformation around the Mn-binding sites as required for O_2 evolution.

O_2-evolution *$CaCl_2$-washing* *PS II particle* *Manganese* *33 kDa protein* *Ca^{2+}-specific restoration*

1. INTRODUCTION

Data accumulated during recent years have revealed that 3 peripheral proteins with molecular masses of 33, 24 and 16 kDa located on the inner surface of thylakoid membranes are involved in photosynthetic O_2 evolution [1–6]. Of these proteins, those of 24 and 16 kDa are specifically liberated from PS II particles by washing with concentrated NaCl solution to result in partial inactivation of O_2-evolving activity, while preserving Mn and 33 kDa protein associated with the particles [2,5]. On Tris- and/or alkaline-washing, all 3 proteins are liberated from the particles concomitant with release of Mn to cause total inactivation of O_2 evolution [1,3–5]. Besides these treatments, we have recently developed a new method of $CaCl_2$-washing, which liberates the 3 proteins concomitant with total inactivation of O_2 evolution, while leaving Mn unaffected and still associated with the membrane [7].

Reconstitution experiments with the use of these particles have been attempted by several investigators. Authors in [2] and [6] showed that purified 24 kDa protein rebinds to NaCl-washed particles and partially restores the inactivated O_2-evolving activity. The rebinding occurs at a specific site on the membrane with a molar ratio of one 24 kDa protein per PS II reaction center [6]. Since the NaCl-washed particles used in these experiments retained appreciable O_2-evolving activity (~20% of the original activity), the 24 kDa protein may not always be essential for O_2 evolution. In another experiment, authors in [8] applied urea treatment to NaCl-washed particles to remove 33 kDa protein, and showed that the protein partially rebinds to the treated particles concomitant with a slight reactivation of O_2 evolution.

* To whom correspondence should be addressed

Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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00145793/84/$3.00

However, the extent of reactivation was very low, i.e., less than 15% of the activity of NaCl-washed particles, so that it remains ambiguous whether the urea-treated particles preserved a sufficient amount of Mn. A more clear functional reconstitution of O_2 evolution has been shown with $CaCl_2$-washed particles. In [9] we showed that on rebinding of 33 kDa protein to $CaCl_2$-washed particles, the O_2-evolving activity almost completely lost after washing recovers to a level higher than that of NaCl-washed particles. This result suggests that the Mn atoms preserved in $CaCl_2$-washed particles are not capable of O_2 evolution due to a very slight conformational modulation brought about around the Mn-binding site by the loss of 33 kDa protein, which can be successfully reversed by rebinding of 33 kDa protein to the membrane.

The suggestion that the inactivation of O_2 evolution in $CaCl_2$-washed particles is caused by such slight conformational modulation raised the question as to whether the modulation could be reversed by some artificial factor other than the 33 kDa protein. We report here that Ca^{2+} is capable of partially restoring the O_2-evolving function of the Mn atoms in the complete absence of 33 kDa protein. Based on this result a possible role of 33 kDa protein in O_2 evolution is discussed.

2. MATERIALS AND METHODS

O_2-evolving PS II particles were prepared from local market spinach as in [10] with modifications as described in [7], and washed 3 times with 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5) to remove nonspecifically releasing materials before use. For $CaCl_2$-washing, the particles were suspended in 1 M $CaCl_2$, 300 mM sorbitol, 10 mM NaCl, 40 mM Mes–NaOH (pH 6.5) as in [7], and centrifuged at 35000 × *g* for 10 min. The resulting pellet was washed once with the same medium containing 1 M $CaCl_2$, then twice with 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5), and subsequently suspended in the latter washing medium supplemented with various concentrations of divalent cations at a chlorophyll concentration of 500 μg Chl/ml. For NaCl-washing, the particles were suspended in 1 M NaCl, 300 mM sorbitol and 40 mM Mes–NaOH (pH 6.5) as in [7].

O_2 evolution was measured with a Clark type oxygen electrode with 2,5-dimethylquinone as electron acceptor at 25°C in 300 mM sorbitol, 10 mM NaCl and 1 mM 2,5-dimethylquinone and 40 mM Mes–NaOH (pH 6.5) with supplement of various concentrations of divalent cations. The fluorescence transient was measured at room temperature as in [11]. Samples were suspended in 300 mM sorbitol, 10 mM NaCl and 40 mM Mes–NaOH (pH 6.5) at a chlorophyll concentration of 5 μg Chl/ml. Protein composition was analyzed by SDS–PAGE in the buffer system of [12] containing 6 M urea as in [7]. The gel was stained with Coomassie brilliant blue R-250. The densitogram of stained gel was obtained with a Shimadzu dual-wavelength chromatoscanner (CS-900). The relative abundance of proteins was estimated from the peak area of densitograms. The abundance of Mn was determined with a Shimadzu atomic absorption spectrometer (AA-640-13) as in [7].

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 (a) and after (b) washing with 1 M $CaCl_2$. As reported in [7], 3 proteins of molecular masses of 33, 24 and 16 kDa were liberated from PS II particles by 1 M $CaCl_2$-washing. To estimate correctly the trace amount of the 3 proteins remaining in the washed particles, different amounts of the samples before and after washing were loaded on the gel: 5 and 40 μg Chl for the samples before and after washing, respectively. As clearly shown by trace (b), the bands corresponding to the 3 proteins are negligibly low, indicating that these proteins were almost completely liberated from the particles. The amount of 33 kDa protein remaining in washed particles was as low as 3% of that in original particles.

As reported in [7], the liberation by $CaCl_2$-washing of the 3 proteins was accompanied by specific inactivation of O_2 evolution, and the activity of the washed particles was less than 1.5% of that of the original particles. However, when the washed particles were suspended in a medium containing Ca^{2+}, appreciable activity of O_2 evolution was observed. As shown in table 1, such a restoration effect was pronounced with Ca^{2+}, appreciable with Mg^{2+} but negligible with Sr^{2+} and

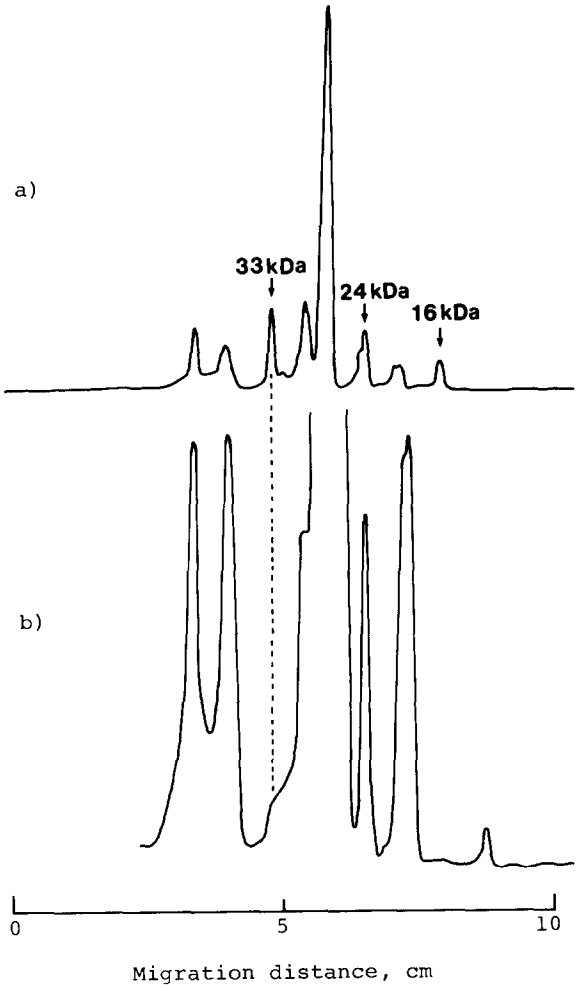

Fig.1. SDS–PAGE densitograms of original PS II particles (a) and 1 M $CaCl_2$-washed PS II particles (b). The amount of particles loaded on the gel was 5 and 40 μg Chl for (a) and (b), respectively.

Ba^{2+}. This result suggests that Ca^{2+} is capable of partially restoring the O_2-evolving activity in $CaCl_2$-washed particles even if 33 kDa protein is totally absent.

Fig.2 shows the dependency of Ca^{2+}-induced restoration on Ca^{2+} concentration. The restoration effect became appreciable at 5 mM Ca^{2+}, increased with increasing Ca^{2+} concentration to reach a plateau at about 50 mM, and then decreased with further increase in Ca^{2+} concentration. As reported by us in [7,9], $CaCl_2$-washing specifically liberates the 3 proteins (33, 24 and 16 kDa proteins) but not Mn atoms, so that the present results suggest that the Mn atoms preserved in the particles partially recover their function of O_2 evolution if a suitable concentration of Ca^{2+} is present in the suspending medium. It is noteworthy that this recovery in activity involves none of the 3 proteins but is solely dependent on Ca^{2+}. The contribution of residual 33 kDa protein may be precluded, since the amount of this protein remaining in the washed particles was less than 3% of the original amount (fig.1), being too low to account for the present activity recovery. The extent of activity recovery by Ca^{2+} was only 19% of the

Table 1

Effect of various divalent cations on Ca^{2+}-induced restoration of O_2-evolving activity

Type of PS II particles	Addition	O_2 evolution (μmol O_2/mg Chl per h)	Mn abundance (atoms/400 Chl)
Control	none	270	11
1 M $CaCl_2$-washed	none	7	8
	+ $MgCl_2$	11	8
	+ $CaCl_2$	38	8
	+ $SrCl_2$	9	8
	+ $BaCl_2$	7	8
	+ 33 kDa protein	76[b]	9[b]
1 M NaCl-washed	none	54[a]	10[a]

[a] Data from a separate experiment
[b] Data from [9]

The concentration of divalent cations was 50 mM

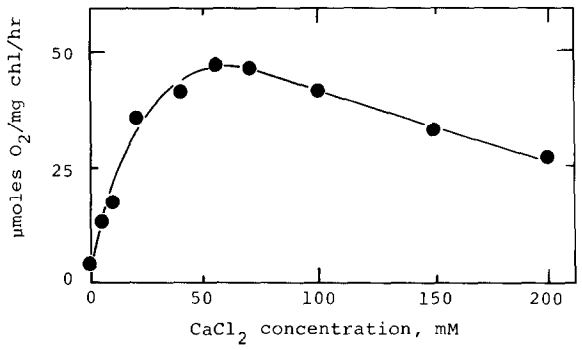

Fig.2. Dependence on $CaCl_2$ concentration of Ca^{2+}-induced restoration of O_2-evolving activity in $CaCl_2$-washed PS II particles. The activity of control PS II particles was 250 μmol O_2/mg Chl per h, and that of 1 M NaCl-washed particles was 54 μmol O_2/mg Chl per h.

original activity in PS II particles, but this value is significant if we refer to previous data that the activity of NaCl-washed particles (depleted of 24 and 16 kDa proteins) and that of reconstituted particles (reconstitution between $CaCl_2$-washed particles and 33 kDa protein) are about 20 and 28%, respectively, of the original activity [7,9].

The restoration of O_2 evolution induced by Ca^{2+} was also confirmed by means of the fluorescence transient. As shown in fig.3, the fluorescence transient of $CaCl_2$-washed particles showed very small variable fluorescence, indicating inhibition on the donor side of PS II (B). When the same particles were measured in the presence of 50 mM Ca^{2+}, an appreciable variation in fluorescence took place (C), the extent of which was more or less the same as that of 1 M NaCl-washed particles (D) which retained a level of O_2 evolution activity of about 25% of the original activity.

Fig.4 shows the effect of Ca^{2+} on the stability of Mn atoms in $CaCl_2$-washed PS II particles. When $CaCl_2$-washed particles were suspended in a medium containing no Ca^{2+}, gradual release of Mn from the membranes took place, and the Mn abundance in the particles decreased to about one half of the initial level after incubation at 0°C in darkness for 7 h. However, the Mn abundance did not change any more during further incubation to 20 h (open circles). This suggests that the binding states of the 4 Mn atoms per reaction center in $CaCl_2$-washed particles (namely in the absence of

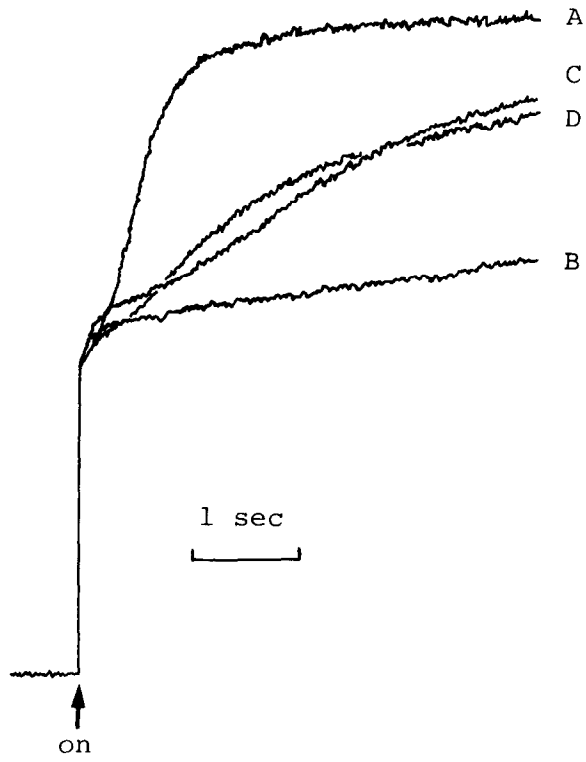

Fig.3. Ca^{2+}-induced recovery of fluorescence variation in $CaCl_2$-washed PS II particles. (A) Control PS II particles, (B) 1 M $CaCl_2$-washed particles, (C) 1 M $CaCl_2$-washed particles + 50 mM $CaCl_2$, (D) 1 M NaCl-washed particles.

33 kDa protein) are not the same: half become destabilized to be gradually liberated during incubation, whereas the other half are stable and remain associated with membranes even after long incubation (20 h). However, when the washed particles were incubated in the presence of 50 mM $CaCl_2$, the decrease in Mn abundance was markedly suppressed and only 10% of Mn was released from membranes after 20 h incubation (triangles). When the Ca^{2+} concentration was increased to 1 M, practically no Mn was released from membranes (squares). These results indicate that Ca^{2+} stabilizes the association with membranes of those Mn atoms which become destabilized on removal of 33 kDa protein.

The above results and considerations may be summarized as a scheme in fig.5. It is assumed that in normal PS II particles, 4 Mn atoms are held in a cavity which is probably located in the reaction

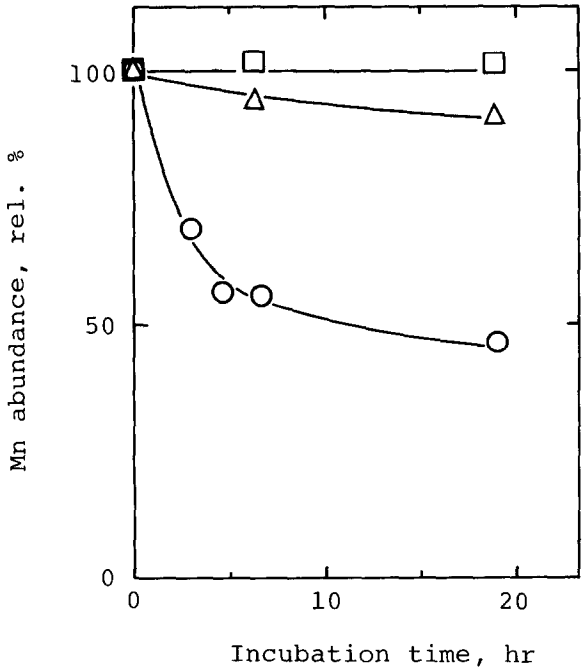

Fig.4. Effect of Ca^{2+} on the stability of association with membranes of the Mn atoms preserved in $CaCl_2$-washed PS II particles. $CaCl_2$-washed particles were suspended in a medium containing no $CaCl_2$ (○), 50 mM $CaCl_2$ (△) and 0.5 and 1 M $CaCl_2$ (□). Abundance of Mn in the initial $CaCl_2$-washed particles was 8.3 Mn atoms/400 Chl.

center complex consisting of 47, 43, 32, 30 and 10 kDa proteins [13], and the opening of the cavity is sealed with 33 kDa protein, by which the conformation of the 4 Mn atoms is maintained in a state functional for O_2 evolution (A). On washing with $CaCl_2$, 33 kDa protein is liberated from the membranes, and the opening of the cavity is unfastened to modulate the conformation of the two functional Mn atoms to become incapable of O_2 evolution (B), which may result in destabilization of the Mn atoms resulting in gradual release out of the membrane. It is of note that the two other nonfunctional Mn atoms are firmly bound to the membrane after removal of the destabilized Mn atoms (C, see also fig.4). In the presence of Ca^{2+}, however, the opening of the cavity is refastened by the action of Ca^{2+} to recover simultaneously the conformation of the Mn atoms to be capable of O_2 evolution (D).

This scheme involves much speculation in addition to the major assumption as to the role of

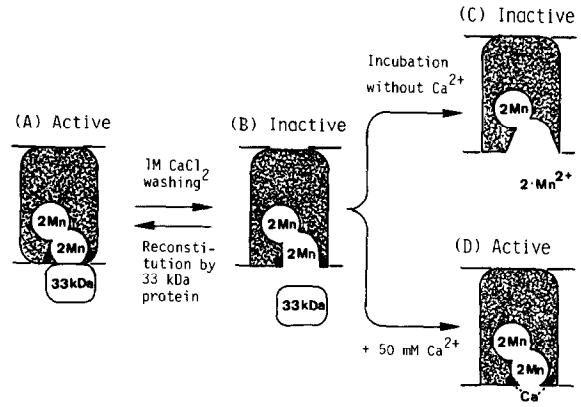

Fig.5. A scheme for the possible role of 33 kDa protein and Ca^{2+} in O_2 evolution. Four Mn atoms were assumed to be held in a cavity (guitar-shaped hole) in a PS II reaction center complex (shadowed area) with binding sites (solid circles) tentatively assumed to be common for both 33 kDa protein and Ca^{2+}. No account was taken of 24 and 16 kDa proteins in the present scheme.

33 kDa protein in O_2 evolution. The scheme, however, accounts for the liberation of 33 kDa protein by $CaCl_2$-washing not inducing any release of Mn [7], while similar liberation of the protein by Tris- and/or alkaline-treatment induces total release of Mn [5], and for the restoration of appreciable O_2-evolving activity in the complete absence of 33 kDa protein when Ca^{2+} is added to Mn-preserving particles. At present we have no direct evidence for interaction of Ca^{2+} with PS II reaction centers. Although we have observed that Ca^{2+} is required for photoactivation of the latent O_2-evolving system [14], the effective concentration of Ca^{2+} is different from that for reactivation in this study. Despite these ambiguities, however, our finding that Ca^{2+} partially plays the role of 33 kDa protein in O_2 evolution appears to support the view that the role of 33 kDa protein is to maintain the conformation of the Mn-binding sites as required for O_2 evolution probably in the PS II reaction center complex.

ACKNOWLEDGEMENTS

This study was supported by a research grant on Solar Energy Conversion by Means of Photosynthesis given by Science and Technology Agency of Japan (STA) to The Institute of Physical and Chemical Research (RIKEN).

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