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The function of 33-kDa protein in the photosynthetic oxygen-evolution system studied by reconstitution experiments

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Treatment of Photosystem II particles with 1.2 M $CaCl_2$ released three proteins of 33, 24 and 18 kDa of the photosynthetic oxygen evolution system, but left Mn bound to the particles as demonstrated by Ono and Inoue (Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 252–260). Oxygen-evolution activity of the $CaCl_2$-treated particles was very low in a medium containing 10 mM NaCl as a salt, but could be restored by the 33-kDa protein. When the particles were incubated in 10 mM NaCl at 0°C, two of the four Mn atoms per oxygen-evolution system were released with concomitant loss of oxygen-evolution activity. The 33-kDa protein suppressed the release of Mn and the inactivation during the incubation. These findings from reconstitution experiments suggest that the 33-kDa protein acts to preserve Mn atoms in the oxygen-evolution system. The 33-kDa protein could be partially substituted by 100 or 150 mM Cl^- for the preservation of the Mn and oxygen-evolution activity. The Mn in Photosystem II particles enhanced rebinding of the 33-kDa protein to the particles.

Introduction

The molecular organization of the oxygen-evolution system of photosynthesis remains one of the main themes of bioenergetics which has not been satisfactorily elucidated. Biochemical studies on oxygen-evolving PS II particles [1,2] and inside-out thylakoid vesicles [3] have revealed that three water-soluble membrane proteins, 33, 24 and 18 kDa, participate in the oxygen-evolution system. The PS II particles contain one molecule each of the three proteins, four Mn atoms and one reaction center II per 220 Chl molecules [4,5]. The 18-kDa and 24-kDa proteins lower the requirement of Cl^- [6,7], an essential anion for photosynthetic oxygen evolution [8]. The 24-kDa protein seems to provide the oxygen-evolution system with a high affinity site for Ca^{2+} [9] which is necessary for the development of full oxygen-evolution activity [9–11].

The 33-kDa protein is the most essential to oxygen evolution among the three proteins [4]. It interacts with two Mn atoms in the oxygen-evolution system [12,13]. We recently prepared PS II particles containing about four Mn atoms, but lacking all the 33-, 24- and 18-kDa proteins by treating PS II particles with urea in the presence of 200 mM NaCl [14]; the resultant particles were designated as (urea + NaCl)-treated PS II particles. By comparing these particles with NaCl-treated PS II particles, which contained four Mn

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Abbreviations: Chl, chlorophyll; PS, Photosystem; Mes, 4-morpholineethanesulphonic acid.

atoms and the 33-kDa protein but lacked the 24-kDa and 18-kDa proteins, we suggested that the 33-kDa protein is necessary for preserving the Mn atoms and full oxygen-evolution activity. Ono and Inoue [15], using $CaCl_2$-treated PS II particles which are similar to the (urea + NaCl)-treated PS II particles in their Mn content and polypeptide composition [14,16], suggested that the role of the 33-kDa protein was to maintain the conformation of the Mn atoms.

The reconstitution experiments of the present study confirmed that the 33-kDa protein plays a role in preserving oxygen-evolution activity and the Mn binding in the oxygen-evolution system.

Materials and Methods

PS II particles were prepared from spinach chloroplasts as described previously [1]. They were suspended in 300 mM sucrose/10 mM NaCl/25 mM Mes-NaOH (pH 6.5) at the Chl concentration of 1.5 mg ml^{-1}. To one volume of the particle suspension, four volumes of 1.5 M $CaCl_2$/25 mM Mes-NaOH (pH 6.5) were added, making the final concentrations of $CaCl_2$ and Chl 1.2 M and 0.3 mg ml^{-1}, respectively. After being left for 20 min at 0°C under room light, the suspension was centrifuged at $35000 \times g$ for 10 min. The pellet was suspended in 300 mM sucrose/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) and centrifuged as above. The resultant particles were designated as $CaCl_2$-treated PS II particles.

In a study on the effect of salts and the 33-kDa protein on oxygen-evolution activity, $CaCl_2$-treated PS II particles suspended in 300 mM sucrose/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) were diluted with an assay medium (described below) supplemented with salt(s) and/or the 33-kDa protein just before use in the experiments.

The effects of various conditions on time-dependent losses of oxygen-evolution activity and Mn content were studied by incubating $CaCl_2$-treated PS II particles in appropriate media at 0°C under room light with stirring. After a designated period, a portion of the suspension was withdrawn and diluted with 15 volumes of assay medium (described below) supplemented with salts and/or the 33-kDa protein, and then the oxygen-evolution activity was measured. Another portion was diluted 7-fold with 300 mM sucrose/60 mM $CaCl_2$/25 mM Mes-NaOH (pH 6.5) and centrifuged at $35000 \times g$ for 10 min. The pellet was washed with 300 mM sucrose/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) by resuspension and recentrifugation. The resultant pellet was suspended in 300 mM sucrose/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) for the analyses of Mn content and polypeptide composition.

Oxygen-evolution activity was assayed using a Clark-type oxygen electrode unit (Hansatech, DW 1) at 25°C at a Chl concentration of about 12 $\mu g \cdot ml^{-1}$. The assay medium contained 0.3 mM phenyl-*p*-benzoquinone/300 mM sucrose/0.05% bovine serum albumin/25 mM Mes-NaOH (pH 6.5). Salt(s) and/or the 33-kDa protein were added to the medium as needed. The Chl concentration was photometrically determined in 80% acetone [17].

The 33-kDa protein was extracted from NaCl-treated PS II particles [18] with 1.2 M $CaCl_2$ and 25 mM Mes-NaOH (pH 6.5), which was performed as described above, and purified by column chromatography with DEAE-Sepharose CL-6B [19]. The purified protein was dialyzed against 300 mM sucrose/10 mM sodium phosphate buffer (pH 6.5), and stored at 77 K until use. The concentration of 33-kDa protein was determined from the absorbance at 276 nm using an absorption coefficient of 20 $mM^{-1} \cdot cm^{-1}$ [20].

Polyacrylamide gel electrophoresis was performed in the presence of sodium laurylsulfate and 6 M urea as described previously [18]. The relative amount of 33-kDa protein rebound to the particles was determined from the peak height of the stained band in the densitogram of the gel plate. The Mn content in the particles was determined using a flameless atomic absorption spectrometer (Jarell Ash, AA-835 equipped with FLA-100) as described previously [18].

Results

Effects of Cl^-, Ca^{2+} and 33-kDa protein on oxygen-evolution activity

Treatment of PS II particles with 1.2 M $CaCl_2$ released three proteins having molecular masses of 33 kDa, 24 kDa and 18 kDa, but left more than 90% of the Mn bound to the particles, as first

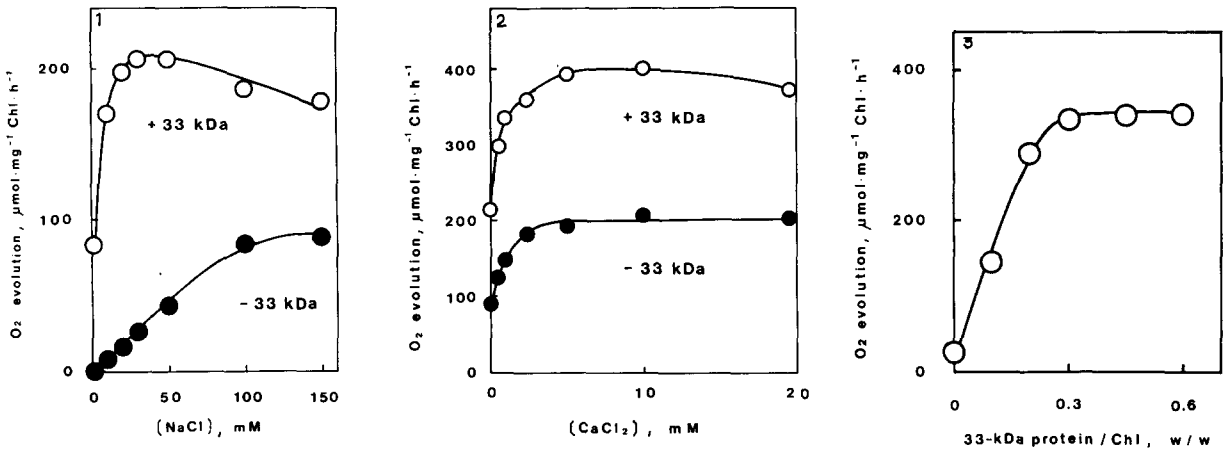

Fig. 1. Effect of NaCl on oxygen-evolution activity of $CaCl_2$-treated PS II particles. Activity was measured in the presence of the designated NaCl concentration in the assay medium. O——O, Activity in the presence of the 33-kDa protein (protein/Chl = 0.6, w/w); ●——●, activity in the absence of the 33-kDa protein. Activity of untreated PS II particles measured in the presence of 10 mM NaCl was 500 μmol O_2/mg Chl per h.

Fig. 2. Effect of $CaCl_2$ on oxygen-evolution activity of $CaCl_2$-treated PS II particles. Activity was measured in the presence of 100 mM NaCl and the designated $CaCl_2$ concentration in the assay medium. O——O, Activity in the presence of the 33-kDa protein (protein/Chl = 0.6, w/w); ●——●, activity in the absence of the 33-kDa protein. Activity of untreated PS II particles measured in the presence of 100 mM NaCl/5 mM $CaCl_2$ was 530 μmol O_2/mg Chl per h.

Fig. 3. Effect of 33-kDa protein on oxygen-evolution activity of $CaCl_2$-treated PS II particles. Activity was measured in the presence of 5 mM $CaCl_2$ and the designated amount of the 33-kDa protein in the assay medium. Activity of untreated PS II particles measured in the presence of 5 mM $CaCl_2$ was 550 μmol O_2/mg Chl per h.

observed by Ono and Inoue [16]. The Mn content of the $CaCl_2$-treated particles was 3.6 ± 0.3 atoms per 220 Chl molecules, or per reaction center II, whereas that in the untreated particles was 3.8 ± 0.3 atoms per 220 Chl molecules [4,5].

The $CaCl_2$-treated particles showed very little oxygen evolution in 10 mM NaCl (Fig. 1, lower trace). In 100 mM NaCl, the activity was restored to about 20% of the original level of untreated PS II particles. This effect was not observed with 100 mM $NaCH_3COO$ (data not shown), suggesting that the manifestation of the activity can be ascribed to Cl^- but not to Na^+. When $CaCl_2$-treated particles were supplemented with the 33-kDa protein, much of the activity was restored with an optimum NaCl concentration of 30 mM (Fig. 1, upper trace). The maximal activity was 2.5-fold higher than that in 100 mM NaCl without the protein.

The oxygen-evolution activity of $CaCl_2$-treated particles was enhanced by $CaCl_2$ either in the presence or absence of the 33-kDa protein. Fig. 2 shows the concentration dependence of the activity with a half-maximum effect at 1 mM and saturation at 5 mM $CaCl_2$ where the activity was doubled in both cases. Oxygen evolution was similarly enhanced by 5 mM $Ca(CH_3COO)_2$, but not by 5 mM $MgCl_2$ or $MnCl_2$ (data not shown). This suggests that the enhancement was induced by only Ca^{2+} [10,11]. Activity restored by the 33-kDa protein and 5 mM $CaCl_2$ reached 75% of the original level of untreated PS II particles.

Fig. 3 shows the effect of the 33-kDa protein on oxygen-evolution activity of $CaCl_2$-treated PS II particles. The activity was saturated at a protein to Chl ratio of 0.3 : 1 (w/w).

Effect of Cl^-, Ca^{2+} and 33-kDa protein on preservation of oxygen-evolution activity and Mn content

The stability of oxygen-evolution activity was studied by incubating $CaCl_2$-treated particles in media containing various salts (Fig. 4). The activity was rather stable in 50 mM $CaCl_2$ or 100 mM

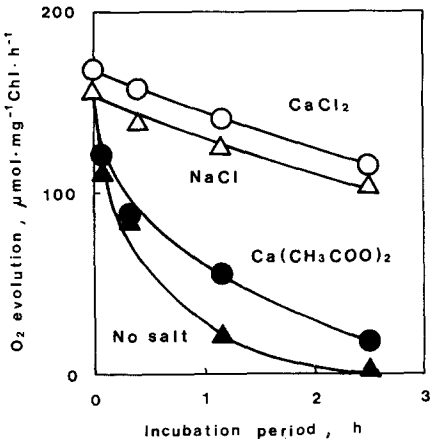

Fig. 4. Loss of the oxygen-evolution activity of $CaCl_2$-treated PS II particles during incubation in the presence of various salts. $CaCl_2$-treated PS II particles were incubated at 0.2 mg Chl per ml in 300 mM sucrose, 25 mM Mes-NaOH (pH 6.5) and a designated salt. Activity was measured in the presence of 100 mM NaCl/5 mM $CaCl_2$ in the assay medium. ○——○, 50 mM $CaCl_2$; △——△, 100 mM NaCl; ●——●, 50 mM $Ca(CH_3COO)_2$; ▲——▲, no salt added.

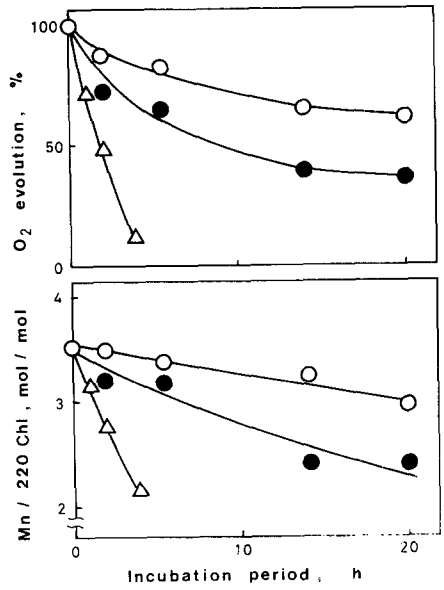

Fig. 5. Effect of the 33-kDa protein and Cl^- on the losses of oxygen-evolution activity and Mn during incubation. $CaCl_2$-treated particles were incubated at 0.4 mg Chl per ml in 300 mM sucrose and 25 mM Mes-NaOH (pH 6.5) together with 10 mM NaCl, 150 mM NaCl, or 10 mM NaCl plus the 33-kDa protein (protein/Chl = 0.4, w/w). Activity was measured in 100 mM NaCl/5 mM $CaCl_2$ in the assay medium. ○——○, 10 mM NaCl plus 33-kDa protein; ●——●, 150 mM NaCl; △——△, 10 mM NaCl.

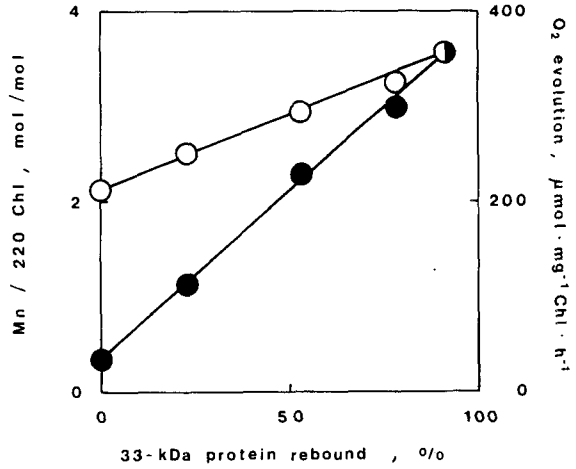

Fig. 6. Effect of rebound 33-kDa protein on preserving Mn and oxygen-evolution activity during incubation. $CaCl_2$-treated PS II particles were incubated at 0.2 mg Chl per ml in 300 mM sucrose/10 mM NaCl/25 mM Mes-NaOH (pH 6.5) for 3 h. The amount of rebound 33-kDa protein was changed by incubating $CaCl_2$-treated particles with the protein to Chl ratio of 0, 0.05, 0.10, 0.15 or 0.20 (w/w). The amount of 33-kDa protein rebound was shown relative to the level of untreated PS II particles. Activity was measured in the presence of 30 mM NaCl, 5 mM $CaCl_2$ and the 33-kDa protein (protein/Chl = 0.6, w/w) in the assay medium. ○——○, Mn content; ●——●, oxygen-evolution activity.

NaCl with 70% of the original activity remaining after 2.5 h. In contrast, when the particles were incubated without added salts, the activity completely diminished in 2.5 h. The medium containing 50 mM $Ca(CH_3COO)_2$) was almost ineffective. These results suggest that the activity can be preserved by Cl^-, but not by Na^+ or Ca^{2+}.

Fig. 5 shows the time-course of Mn release and loss of oxygen-evolution activity during the incubation of $CaCl_2$-treated particles in 10 mM NaCl, in 150 mM NaCl, or in 10 mM NaCl plus 33-kDa protein. The oxygen-evolution activity and the Mn content rapidly decreased in 10 mM NaCl, but very slowly in 150 mM NaCl. In the presence of the 33-kDa protein, more of the oxygen-evolution activity and Mn content were preserved even in 10 mM NaCl. This result indicates that the 33-kDa protein is more effective than 150 mM Cl^- for preserving the oxygen-evolution activity and Mn.

The effect of the 33-kDa protein on the losses of Mn and oxygen-evolution activity during in-

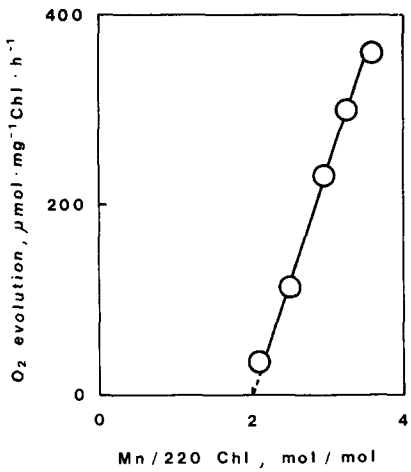

Fig. 7. Relationship between oxygen-evolution activity and Mn content in $CaCl_2$-treated PS II particles incubated under the conditions of Fig. 6. Activity and Mn content were changed by incubating the particles for 3 h in 300 mM sucrose/10 mM NaCl/25 mM Mes-NaOH (pH 6.5) supplemented with various amounts of the 33-kDa protein. Samples were the same as those of Fig. 6.

cubation in a low-salt medium was quantitatively studied with $CaCl_2$-treated PS II particles supplemented with various proportions of the 33-kDa protein. Fig. 6 shows that the Mn content and oxygen-evolution activity which remained after the incubation in 10 mM NaCl for 3 h were linearly correlated with the amount of rebound 33-kDa protein. Without 33-kDa protein rebound, the oxygen-evolution activity was almost completely lost and the Mn content dropped to about two atoms per 220 Chl molecules. This suggests that the 33-kDa protein bound to the $CaCl_2$-treated PS II particles suppresses the release of two of the four Mn atoms. These results essentially agree with our previous conclusion for the interaction of the 33-kDa protein and two Mn atoms in the oxygen-evolution complex [12,13].

Fig. 7 shows the relationship between the amount of Mn left bound and the oxygen-evolution activity remaining in the samples of Fig. 6. A linear relationship existed between the activity and bound Mn, as previously observed in the (urea + NaCl)-treated particles [14]. Extrapolation of the line crossed the abscissa at two Mn atoms per 220 Chl molecules indicating that the oxygen-evolution activity is completely lost when the Mn content approaches two atoms per reaction center II.

TABLE I

EFFECT OF BOUND Mn ON REBINDING OF THE 33-kDa PROTEIN TO $CaCl_2$-TREATED PS II PARTICLES

The designated types of PS II particles suspended in 300 mM sucrose/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) were mixed with the 33-kDa protein (protein/Chl = 0.6, w/w). The mixture was diluted with 300 mM sucrose/25 mM Mes-NaOH (pH 6.5) for a final NaCl concentration of 10 mM. After being left for 5 min at 0°C, the particles were collected by centrifugation at $35000 \times g$ for 10 min. Unbound 33-kDa protein was removed by resuspension in 25 mM Mes-NaOH/300 mM sucrose/10 mM NaCl (pH 6.5) and recentrifugation.

Type of PS II particles	Mn / 220 Chl	33-kDa protein rebound [a]	O_2 evolution [b] (μmol/mg Chl per h)
Treated with $CaCl_2$	3.7 [d]	95%	400
Treated with $CaCl_2$ and incubated [c]	2.1 [d]	60%	35

[a] Relative to the amount in untreated PS II particles.
[b] Measured in the assay medium supplemented with 5 mM $CaCl_2$/30 mM NaCl after rebinding of the 33-kDa protein.
[c] Prepared by incubating $CaCl_2$-treated PS II particles in 300 mM sucrose/10 mM NaCl/25 mM Mes-NaOH (pH 6.5) for 3 h.
[d] Mn atoms per 220 Chl molecules.

Effect of Mn release on rebinding of the 33-kDa protein

Previous studies [15,21] indicated that the 33-kDa protein can rebind to PS II particles which are depleted of the three water-soluble proteins. We further demonstrated [21] that there is a specific binding site for this protein on PS II particles. The possible participation of Mn in the binding of 33-kDa protein was studied using two types of PS II particles having different Mn contents; $CaCl_2$-treated particles which contained 3.7 Mn atoms per 220 Chl molecules and particles treated with $CaCl_2$ and then incubated, which contained 2.1 Mn atoms per 220 Chl molecules. Table I shows that the rebinding of 33-kDa protein to the former type reached 95% of the original level of untreated PS II particles, whereas rebinding to the latter particles reached only 60%. This observation suggests that both types of PS II particles can bind the 33-kDa protein, and that the binding of the 33-kDa protein to the oxygen-evolution complex is supported in part by the two Mn atoms, that are among the four Mn atoms in the oxygen-evolution complex and can be released by depletion of the 33-kDa protein.

Discussion

The present study indicates that $CaCl_2$-treated PS II particles are very similar to (urea + NaCl)-treated PS II particles of our previous study [14] in the modes of actions of Cl^-, Ca^{2+} and the 33-kDa protein on the oxygen-evolution system. In both types of particles, the Mn contents decreased from about 3.6 to 2.0 atoms per 220 Chl molecules during incubation in 10 mM NaCl.

$CaCl_2$-treated PS II particles could evolve oxygen in the presence of concentrated Cl^-. However, the maximal activity brought about by 100 mM Cl^- was only 40% of that in the particles supplemented with the 33-kDa protein. This finding suggests that the Cl^- cannot fully replace the 33-kDa protein, and that the protein is necessary for the achievement of full oxygen-evolution activity.

Ca^{2+} at 5 mM enhances oxygen-evolution activity in NaCl-treated PS II particles [10,11]. The present study shows that this cation accelerated the oxygen evolution of $CaCl_2$-treated PS II particles depleted of the 33-kDa protein. This suggests that Ca^{2+} acts on a membraneous component other than the 33-kDa protein in the acceleration. Ghanotakis et al. [9] recently revealed that the 24-kDa and/or 18-kDa protein(s) facilitates the rebinding of Ca^{2+} to (NaCl + EGTA)-treated PS II particles.

Our previous study [14] suggested that the 33-kDa protein plays an essential role in preserving Mn in the oxygen-evolution system and clarified its effect by comparing NaCl-treated PS II particles containing the 33-kDa protein and (urea + NaCl)-treated PS II particles depleted of the 33-kDa protein. The present study compared two types of $CaCl_2$-treated PS II particles, one supplemented with the 33-kDa protein and the other without it, and the results offer stronger evidence for the role of the 33-kDa protein in Mn preservation.

Ono and Inoue [22], observing that 50 mM $CaCl_2$ preserved Mn atoms and stimulated oxygen evolution in the $CaCl_2$-treated PS II particles, claimed that Ca^{2+} was essential for these effects. However, our present study clearly indicates that 100 mM Cl^-, but not 50 mM Ca^{2+}, is essential for the preservation of Mn and oxygen-evolution activity. As shown in Fig. 5, however, 150 mM Cl^- was less effective than the 33-kDa protein. This suggests that Cl^- can only partially substitute for the 33-kDa protein.

The 33-kDa protein seems to be bound to PS II particles by hydrogen or hydrophobic bonding, since it can be released from the particles with urea [12–14] and SCN^- [12]. A previous study [21] indicated that there may be a specific binding site for the 33-kDa protein on PS II particles. Our finding that the rebinding of 33-kDa protein is affected by the elimination of two of the four Mn atoms from PS II particles may be due to these two Mn atoms being constituents of such a binding site for the 33-kDa protein.

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