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Reconstitution of water-oxidizing complex in manganese-depleted photosystem 2 preparations with synthetic manganese complexes

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Abstract

Four synthetic manganese complexes in which Mn atoms have different coordination environments and valence states were used to reconstitute water-oxidizing complex (WOC) in Mn-depleted photosystem 2 preparations. Three Mn-complexes restored a significant rate of electron transfer and oxygen evolution except one complex in which Mn atom ligated to the O-atoms within the ligands by covalent linkage. The effect of coordination environment of the Mn-atom within the Mn-complexes on their efficiencies in reconstituting the electron transport and oxygen evolution was analysed.

Additional key words: manganese complexes; reconstitution; water-oxidizing complex.

Introduction

Elucidation of the structure of water-oxidizing complex (WOC) has been one of the most challenging researches in the photosynthesis field due to its unique role in photosynthetic water oxidation. Up to now, the structural information about WOC has been mainly obtained by EXAFS and EPR spectroscopy. These studies have well established that WOC is comprised of $Mn_4Ca_1Cl_x$ and a redox-active tyrosine, Yz (D1-Y161) (Debus 1992, Britt 1996, Yachandra *et al.* 1996). Recent study on the X-ray crystal structure of photosystem 2 (PS2) at 0.35 nm resolution revealed that WOC contains a cubane-like Mn_3CaO_4 cluster linked to a fourth Mn by a mono-μ-oxo-bridge and detailed information of surrounding coordination of Mn cluster for the first time was provided (Ferreira *et al.* 2004). Unfortunately, protein ligands ligated with Ca^{2+} were not detected at current resolution and Cl^- ligand was not observed either, even though Cl^- was suggested as a possible ligand of Mn cluster (Debus 1992). Moreover, the structure of Mn_4Ca cluster provided by X-ray crystal structure of PS2 at 0.3 nm resolution (Loll *et al.* 2005) differs considerably from the above cubane-like model. So, great challenges will still remain until higher resolution of WOC structure is obtained.

Bioinorganic model chemistry has provided important insights into the structure of WOC. In recent years, various Mn-complexes have been synthesized as models to mimic the Mn cluster of the WOC. Synthetic Mn-complexes could provide a powerful system to analyze the assembly of the WOC and thus may help in understanding the structure and function of the WOC. Photoassembly of WOC with synthetic Mn-complexes (Klimov *et al.* 1990, Allakhverdiev *et al.* 1994a,b, 1999, Li *et al.*

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Abbreviations: ADRY – acceleration of the deactivation reactions of the water-splitting enzyme system Y; apo-WOC – cofactor-depleted enzyme; Chl – chlorophyll; CP43 – PS2 chlorophyll binding proteins; DCBQ – 2,5-dichloro-*p*-benzoquinone; DCIP – 2, 6-dichlorophenol indophenol; EPR – electron paramagnetic resonance; EXAFS – extended X-ray absorption fine structure; Mes – 4-morpholine ethanesulfonic acid; PPBQ – phenyl-p-benzoquinone; pic – pyridinecarboxylate; PS – photosystem; pyz – pyrazine-2-carboxylate; RC – reaction centre; TEMED – *N′,N′,N′,N′*-tetramethylethylenediamine; Y_z – redox-active tyrosine of polypeptide D1; WOC – water-oxidizing complex.

2000, Karacan and Somer 2004, 2005, Han *et al.* 2005, Liu *et al.* 2006) in Mn-depleted PS2 membranes by a process called photoactivation is more effective in comparison with photoassembly with $MnCl_2$. Factors that influence the reconstruction of WOC are complicated and have not been elucidated clearly due to limited investigations on synthetic Mn-complexes. As a continuation of our former research (Han *et al.* 2005, Li *et al.* 2006), four synthetic Mn-complexes with different coordination environment were used to reconstitute the WOC in Mn-depleted PS2 preparations and some interesting hints between the chemical structure of Mn-complexes and their efficiencies in reconstituting the electron transport and oxygen evolution were found.

Materials and methods

Preparation of PS2 particles: PS2-enriched membrane fragments were prepared from market spinach by the method of Berthold *et al.* (1981). Chlorophyll (Chl) contents and Chl *a/b* ratios were assayed in 80 % acetone according to Arnon (1949). These preparations exhibited 100–110 mmol kg^{-1}(Chl) s^{-1} oxygen evolution rates under saturating irradiance in the presence of 0.1 mM 2,5-dichloro-*p*-benzoquinone (DCBQ) plus 1 mM $K_3Fe(CN)_6$ as electron acceptors. Samples were stored in liquid nitrogen until use.

Depletion of manganese: The extractions of manganese and three extrinsic polypeptides from PS2 preparations were carried out using 20 mM *N,N,N′,N′*-tetramethylethylenediamine (TEMED) plus 0.5 M $MgCl_2$ treatments, which give a 97 % removal of Mn from WOC (Ananyev *et al.* 1992, Allakhverdiev *et al.* 1994b). The Mn content was assayed with flameless atomic absorption spectrophotometer (*Perkin-Elmer Zeeman 500* spectrometer) and the polypeptide composition was verified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Purification of 33 kDa protein: The 33 kDa protein was extracted from the PS2 preparations by 1.0 M $CaCl_2$ treatment and purified by column chromatography on a DEAE-*Sepharose CL-6B* column as described by Weng *et al.* (2004). The purified protein concentration was calculated from UV absorbance at 276 nm according to the method of Eaton-Rye and Murata (1989).

***In vitro* photoactivation of Mn-depleted (TEMED-treated) PS2 particles** was carried out as described by Li *et al.* (2006). Mn-depleted PS2 membranes, suspended in buffer containing 300 mM sucrose, 100 mM NaCl, 50 mM Mes-NaOH (pH 6.5), 20 mM $CaCl_2$, and 0.25 mM $K_3Fe(CN)_6$ at a chlorophyll (Chl) concentration of 200 g m^{-3} were pre-incubated with an indicated amount of $MnCl_2$ or Mn-complex in the dark at 4 °C for 10 min before photoactivation. Photoactivation was done at room temperature with five cycles of continuous irradiation (λ>600 nm, I = 55 W m^{-2} irradiation of 30–40 s periods separated by 30–40 s of dark). Suspensions were stirred during incubation and photoactivation. The photoactivated samples were centrifuged at 25 000×*g* for 10 min to remove unbound Mn-complexes, and then the pellets were washed two times before measurement of oxygen evolution activity.

O_2 evolution activity was measured with a Clark-type electrode fitted with a circulating water jacket at 25 °C. The assay mediums comprised 300 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 25 mM Mes-NaOH (pH 6.5) with 100 μM phenyl-*p*-benzoquinone (PPBQ) as electron acceptor.

Variable fluorescence: The kinetics of the photoinduced change of Chl fluorescence yield was measured in 1-cm cuvette at 20 °C using a phosphoroscopic set-up (Ananyev *et al.* 1992). The suspension for fluorescence measurements contained samples at 10 g m^{-3} Chl, 300 mM sucrose, 35 mM NaCl, and 50 mM Mes-NaOH (pH 6.5).

Photoinhibition and thermoinactivation: Photoinhibition of photoactivated samples was carried out by continuous heat-filtered irradiation (3 000 μmol m^{-2} s^{-1}) at a Chl concentration of 100 g m^{-3} at 20 °C. Thermoinactivation was carried out at certain temperature in the dark. Samples were incubated for 10 min in indicative temperature at a Chl concentration of 100 g m^{-3}. All the photoinhibition and thermoinactivation experiments were carried out in the medium containing 300 mM sucrose, 170 mM NaCl, 50 mM Mes-NaOH (pH 6.5), and 20 mM $CaCl_2$.

Synthesis of manganese complexes: Synthesis, structural characterization, and magnetic properties of manganese compounds $[Mn(II)Cl(pic)]_n$, $[Mn(II)(pic)_2]_n$, $[Mn(IV)_2O_2(pic)_4]$ (Huang *et al.* 2004) and $[Mn(III)(pic)_3]$ (Li *et al.* 2006) [(pic) = pyridinecarboxylate] are described in references. The coordination spheres of the Mn ion in the four Mn-complexes are shown in Fig. 1.

Results

Fluorescence measurements: Typical traces of photoinduced changes of Chl fluorescence yield in different PS2 preparations are shown in Fig. 2. Increase of fluorescence yield in untreated PS2 samples was observed due to light-induced reduction of Q_A, the primary electron acceptor of PS2. However, Mn-depleted

PS2 preparations showed markedly different pattern due to the loss of manganese. In this case, ΔF was largely reduced because of very limited electron donor capacity. Addition of $MnCl_2$ corresponding to four Mn per PS2 reaction centre (RC) caused a significant restoration of ΔF. This indicates that $MnCl_2$ can reconstitute the electron transport capacity of Mn-depleted PS2 preparations. This role of $MnCl_2$ had already been shown (Allakhverdiev *et al.* 1994b, 1999, Karacan and Somer 2004, 2005, Han *et al.* 2005).

An effective restoration was also achieved with synthetic Mn-complexes (Fig. 2, traces *4–7*). But the efficiency of four Mn-complexes was obviously lower than that of $MnCl_2$. This may be related to coordination environment of Mn atoms in these complexes. Different numbers of chelate rings exist in these Mn-complexes, which may affect the capability of Mn atom to donate electron to RC. As shown in Fig. 2, the efficiency of Mn-complexes was decreased gradually in an order $[Mn(II)Cl(pic)]_n > [Mn(III)(pic)_3] > [Mn(IV)_2O_2(pic)_4]$ with the increasing number of the chelate rings. But the behaviour of Mn-complex $[Mn(II)(pic)_2]_n$ was very abnormal. Structurally, except the existence of two chelate rings, the only difference between this complex and other three complexes was that Mn atom within the complex ligated two carboxylic oxygen atoms by covalent bond. The existence of two O atoms in this Mn-complex may be the reason leading to severe decrease of its electron donor capacity. Detailed analysis will be carried out in Discussion.

Moreover, the valence state of Mn atom in the Mn-complexes may have some relationship with the efficiency of Mn-complexes in restoring electron transfer. As shown in Fig. 2 (traces *4*, *6*, and *7*), three Mn-complexes in which Mn atom is in +2, +3, and +4 valence states exhibited the variable fluorescence in an order $[Mn(II)Cl(pic)]_n > [Mn(III)(pic)_3] > [Mn(IV)_2O_2(pic)_4]$. Mn-complexes in which Mn atom is at lower valence state show stronger donation electron capacity than Mn-complex, in which Mn atom is at higher valence state.

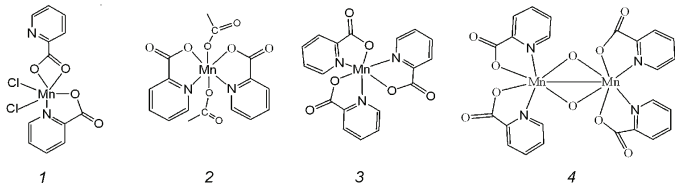

Fig. 1. Coordination spheres of the four Mn complexes, of which complexes *1* and *2* are coordination polymers of MnCl(pic) and $Mn(pic)_2$ moieties, respectively. *1*: $[Mn(II)Cl(pic)]_n$. *2*: $[Mn(II)(pic)_2]_n$. *3*: $[Mn(III)(pic)_3]$. *4*: $[Mn(IV)_2O_2(pic)_4]$.

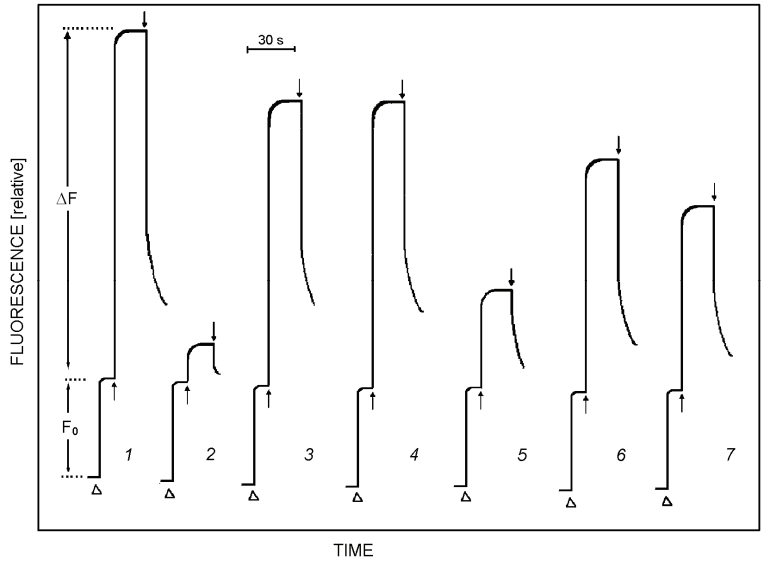

Fig. 2. Kinetics of the photoinduced changes of the PS2 chlorophyll fluorescence yield, related to photoreduction of Q_A in the sub-chloroplast membrane fragments enriched in PS2 before (*1*) and after (*2–7*) removal of Mn in the absence of other additions (*2*) and after the addition of 0.2 μM $MnCl_2$ (*3*); 0.2 μM $[Mn(II)Cl(pic)]_n$ (*4*); 0.2 μM $[Mn(II)(pic)_2]_n$ (*5*); 0.2 μM $[Mn(III)(pic)_3]$ (*6*); 0.1 μM $[Mn(IV)_2O_2(pic)_4]$ (*7*). Δ, switching on the measuring radiation (λ = 480 nm, 0.15 W m^{-2}), or ↑ and ↓ "actinic light" (λ>600 nm, 100 W m^{-2}) on and off, respectively.

Oxygen evolution activity of these preparations is shown in Table 1. In Mn-depleted PS2 preparations, this activity was almost completely lost, but it was partially recovered after photoactivation with $MnCl_2$. A more pronounced effect was obtained with synthetic Mn-complexes. Efficiency of photoactivation was further enhanced with the addition of $CaCl_2$ or 33 kDa protein in the case of both Mn-complexes and $MnCl_2$. But the maximal recovery of O_2 evolution activity was required in the presence of $CaCl_2$ and 33 kDa protein together. The effect of $CaCl_2$ and 33 kDa protein on photoactivation was more pronounced in Mn-complexes than in $MnCl_2$. In the presence of 20 mM $CaCl_2$ and 40 g m^{-3} of 33 kDa polypeptide, this activity could be restored up to 30.0, 28.5, and 28.0 % of the original level in PS2 membranes when apo-PS2 was photoactived with $Mn(II)Cl(pic)]_n$, $[Mn(III)(pic)_3]$, and $[Mn(IV)_2O_2(pic)_4]$, respectively, while $MnCl_2$ was only 22 %. Obviously, three Mn-

complexes are more efficient than $MnCl_2$ in reactivation of O_2 evolution activity. Likewise, the Mn-complex $[Mn(II)(pic)_2]_n$ showed special behaviour in reactivation of O_2 evolution activity (Fig. 2).

Detailed description of the reactivation of oxygen evolution activity is shown in Figs. 3 and 4. Fig. 3*A* shows the effect of the concentration of synthetic Mn-complexes on photoactivation. Restoration of O_2 evolution activity photoactivated with Mn-complexes was rapidly enhanced with the addition of exogenous Mn and almost saturated at a concentration corresponding to 4 Mn per RC. More additions of complexes did not induce more pronounced increase. Such traces showed that these Mn-complexes except $[Mn(II)(pic)_2]_n$ participated in a more effective way than $MnCl_2$ during the whole concentration range. Complexes $[Mn(III)(pic)_3]$ and $[Mn(IV)_2O_2(pic)_4]$ exerted almost the same capability in reactivation of O_2 evolution activity in Mn-depleted PS2 particles. Complex $Mn(II)Cl(pic)]_n$ was a little more efficient than the above two Mn-complexes.

Table 1. Reactivation of O_2 evolution in Mn-depleted PS2 preparations with Mn-complexes [mmol(O_2) kg^{-1}(Chl) s^{-1}]. $MnCl_2$ or Mn complexes were added at a concentration corresponding to 10 Mn per RC, the 33 kDa polypeptide at 40 g m^{-3}, and $CaCl_2$ at 20 mM. O_2 evolution activity of native PS2 preparations was about 105 mmol(O_2) kg^{-1}(Chl) s^{-1}. For other conditions see Materials and methods.

Addition during photoactivation	Rate of O_2 evolution				
	$MnCl_2$	$Mn(II)Cl(pic)]_n$	$[Mn(II)(pic)_2]_n$	$[Mn(III)(pic)_3]$	$[Mn(IV)_2O_2(pic)_4]$
None	0	0	0	0	0
10 Mn/RC	13.9	13.6	10.6	12.8	13.1
10 Mn/RC+$CaCl_2$	19.4	21.1	15.8	21.1	19.7
10 Mn/RC+33 kDa	21.7	18.6	15.0	22.8	21.9
10 Mn/RC+$CaCl_2$+33 kDa	23.3	31.7	16.7	30.0	29.7

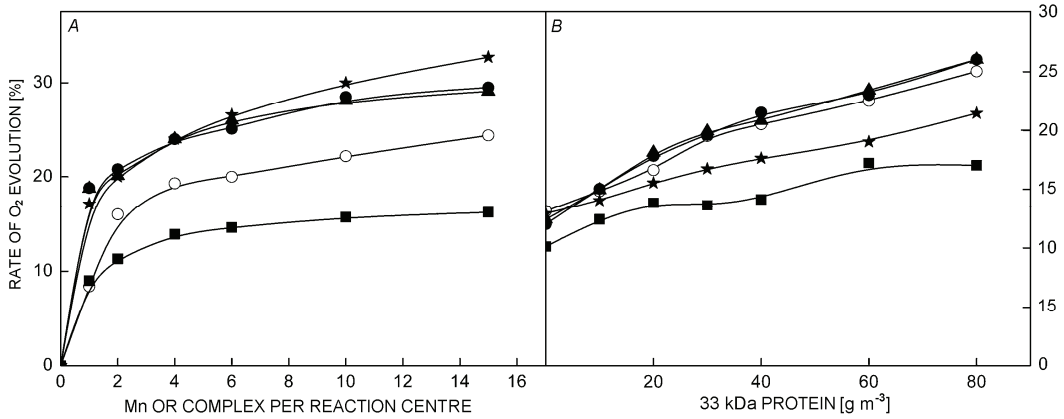

Fig. 3. Reactivation of O_2 evolution in Mn-depleted PS2 preparations reconstituted (*A*) with various concentrations of Mn or (*B*) with various concentrations of 33 kDa protein in the absence of Ca^{2+}. $MnCl_2$ (○); $[Mn(II)Cl(pic)]_n$ (★); $[Mn(II)(pic)_2]_n$ (■); $[Mn(III)(pic)_3]$ (●); $[Mn(IV)_2O_2(pic)_4]$ (▲). (*A*): The reactivation medium contained 20 mM $CaCl_2$ and 40 g m^{-3} 33 kDa protein. The PS2 enriched membranes were diluted to 20 g(Chl) m^{-3}. (*B*): The reaction medium contained 20 g(Chl) m^{-3}, 10 Mn per PS2 RC. The rates are given as percent of the rates obtained in intact PS2 preparations. For other details see Materials and methods.

The actions of Mn-complexes on 33 kDa protein in the absence of 20 mM $CaCl_2$ are described in Fig. 4. The restoration of O_2 evolution activity is quickly enhanced with increase in concentration of the 33 kDa protein. This role of 33 kDa protein in stimulating the photoactivation has been mentioned earlier (Berthold *et al.* 1981, Bricker and Frankel 1998). In the present photoactivation experiment, the presence of 80 g m^{-3} of the 33 kDa protein raised O_2 evolution activity by 90, 66, 68, 117, and 110 % when photoactivated with $MnCl_2$, $Mn(II)Cl(pic)]_n$, $[Mn(II)(pic)_2]_n$, $[Mn(III)(pic)_3]$, and $[Mn(IV)_2O_2(pic)_4]$, respectively. Moreover, the activation of O_2 evolution activity with $Mn(II)Cl(pic)]_n$ was largely decreased in the absence of 33 kDa protein compared to other Mn-complexes.

The requirement for $CaCl_2$ in the presence of 33 kDa protein during photoactivation is shown in Fig. 5. $CaCl_2$ was also required for photoactivation even though 40 g m^{-3} of 33 kDa protein was already presented in the medium. The addition of 2 mM $CaCl_2$ increased the oxygen evolution more than found in its absence. The presence of 5 mM $CaCl_2$ increased the oxygen evolution to almost its maximum and then saturated at this concentration. The actions of three different synthetic

Mn-complexes except $[Mn(II)(pic)_2]_n$ to $CaCl_2$ were more evident than those of $MnCl_2$. But the rate of O_2 evolution with Mn-complex $[Mn(II)(pic)_2]_n$ was decreased when concentration of $CaCl_2$ was over 10 mM. It seems there was a competition between Mn and Ca^{2+} at higher concentrations during photoactivation.

Photoinhibition and thermoinactivation: In order to understand the function of reconstituted WOC, we compared the capability of photoactivated samples with $MnCl_2$ and Mn-complexes, respectively, in preventing photoinhibition and thermoinactivation. Fig. 5*A* shows the rate of photoinhibition of photoactivated samples with the increasing time of irradiation. Three photoactivated WOC with Mn-complexes showed stronger capacity against photoinhibition than $MnCl_2$. Fig. 5*B* shows the rate of thermoinactivation of photoactivated samples with the increasing temperature. With the increase in temperature, photoactivated WOC with Mn-complexes also showed stronger ability against thermoinactivation than $MnCl_2$. Moreover, all samples lost O_2-evolving activity at 47.5 ºC.

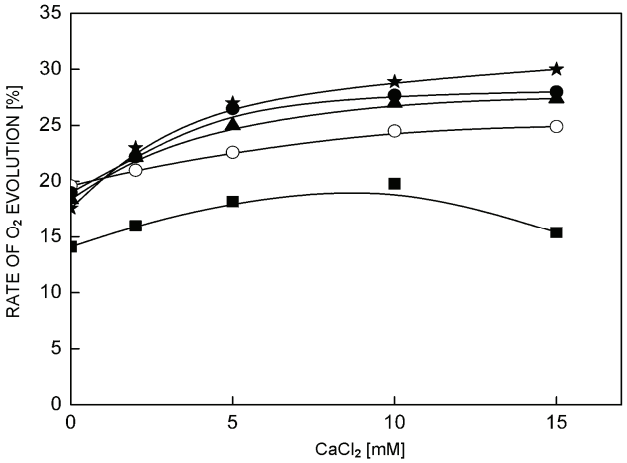

Fig. 4. Reactivation of O_2 evolution in Mn-depleted PS2 preparations reconstituted with Mn complexes in the presence of various concentrations of $CaCl_2$. $MnCl_2$ (○);, $Mn(II)Cl(pic)]_n$ (★); $[Mn(II)(pic)_2]_n$ (■); $[Mn(III)(pic)_3]$ (●); $[Mn(IV)_2O_2(pic)_4]$ (▲). The reaction medium contained 20 g(Chl) m^{-3}, 10 Mn per PS2 RC, and 40 g m^{-3} of 33 kDa protein.

Discussion

The above results demonstrate that synthetic Mn-complexes can restore significant rates of electron transport and oxygen evolution and reconstitute the WOC in Mn-depleted PS2 preparations. Moreover, the reconstituted WOC show higher capacity against photoinhibition and thermoinactivation compared to $MnCl_2$. However, their efficiencies in restoration of electron transport and oxygen evolution of WOC vary between Mn-complexes. There are some relationships between the chemical structure and their functions in restoring the electron transport and O_2 evolution.

In our previous investigations (Han *et al.* 2005, Li *et al.* 2006), we proposed that existence of ligation between Mn atom and N atom within the ligands determine whether Mn-complexes are able to restore electron transport and O_2 evolution. Several secondary amines with bulky aromatic substituents and acidic –NH group are strong catalysts of the ADRY effect in an intact WOC (Hanssum *et al.* 1985). Based on the above finding, Allakhverdiev *et al.* (1994b) suggested that aromatic ligands containing Mn complexes can facilitate interaction of Mn atoms with exposed donor side of PS2 during photoactivation. Our previous results showed that ligation of Mn atom with N atom within the aromatic ring facilitated its interaction with exposed donor side of PS2.

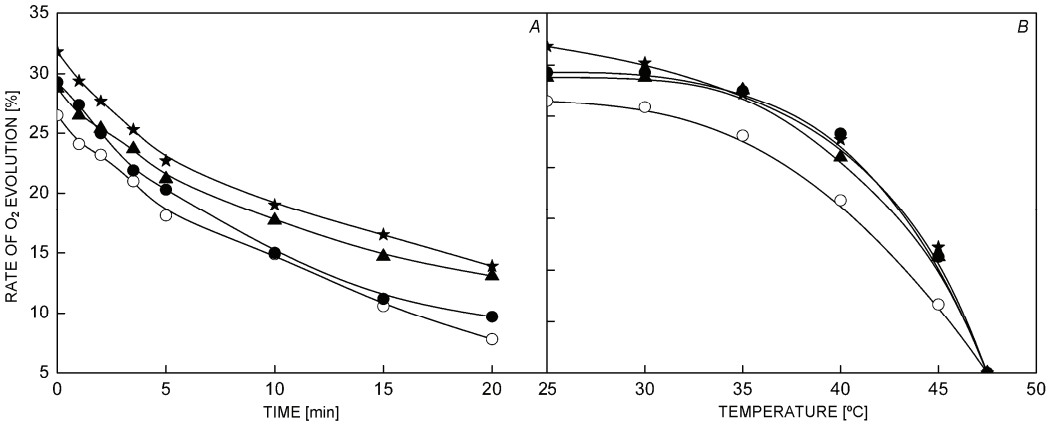

Fig. 5. Effect of photoinhibition (A) or thermodeactivation (*B*) on oxygen-evolving activity of reconstituted PS2 with $MnCl_2$ or Mn complexes at 10 Mn/RC. For symbols see Fig. 4. The medium contained 100 g(Chl) m^{-3}, 20 mM $CaCl_2$, and 40 g m^{-3} of 33 kDa protein. For details see Materials and methods.

In the presented photoactivation experiments, the above proposal was further testified. Mn-complexes that contain Mn-N ligation restored significant rate of electron transport and O_2 evolution (Figs. 3 and 4). In addition, the comparison of the efficiency of complexes in restoring the electron transport and the O_2 evolution activity and their chemical structures showed that the capability of Mn-complexes in reconstitution of WOC was also related to the valence state of Mn atom and other structural factors.

Mn(II) in complex $[Mn(II)Cl(pic)]_n$ displays a coordination environment consisting of two Cl^-, two carboxylic oxygen atom from the same pic ligand, and a chelate ring formed with one carboxylic oxygen atom and one nitrogen atom from same pic ligand. Besides two same chelate rings as above, $[Mn(II)(pic)_2]_n$ is ligated with two carboxylic oxygen atoms from two ligands. Mn(III) in $[Mn(III)(pic)_3]$ is coordinated by the three same chelate rings. $[Mn(IV)_2O_2(pic)_4]$ is a dinuclear μ-oxo Mn-complex, which is composed of four same chelate rings.

Fig. 2 shows that the capability of these Mn-complexes as an effective electron donor is gradually decreased with the increasing number of chelate ring. The existence of chelate ring, on the one hand, makes the electron flow onto the ring, which weakens donation electron capacity of the Mn atom. On the other hand, increasing chelate ring enhances the size of the Mn-complex to a great extent and leads to the augmentation of steric hindrance surrounding Mn atom. As shown in Fig. 2, complex $[Mn(II)Cl(pic)]_n$, which has the smallest chelate ring number, is most efficient in restoring the electron transport capacity while the complex $[Mn(IV)_2O_2(pic)_4]$ with most chelate rings, displays lowest efficiency. The order of Mn-complexes as effective electron donor is opposite to the number of chelate rings except the complex $[Mn(II)(pic)_2]_n$.

Moreover, the valence state of Mn atom in Mn-complexes also affects recovery of electron transport capacity. Mn-complex in which Mn atom is at lower valence state can easier donate electron to RC than the Mn-complex in which Mn atom exists at higher oxidation state. Based on the above analysis we suggest that restoration of electron transport capacity is affected by both valence states of Mn atom and structure factors such as chelate ring number and size of ligand.

However, the behaviour of complex $[Mn(II)(pic)_2]_n$ is very special and does not obey the above rule. Though only two chelate rings exist in the complex in which Mn atom is of +2 valence, this complex shows lower ΔF than other Mn-complexes. This complex also shows lower efficiency than other Mn-complexes in reactivating O_2 evolution (cf. Figs. 3–5). Excluding the difference in the number of chelate rings, the only difference between complex $[Mn(II)(pic)_2]_n$ and other complexes in chemical structure is that Mn atom in this complex is coordinated with two carboxylic oxygen atoms by covalent linkage. A similar phenomenon was also found in our previous work (Li *et al.* 2006). Complex $[Mn(II)(pyz)_2(H_2O)_4]$ in which Mn(II) atom is coordinated by four water molecules and two oxygen atoms from two pyz ligands can not restore the electron transport and O_2 evolution in a reconstituted PS2 sample. It is inferred from the above analysis that decreasing capability of this complex in the recovery of electron transport and O_2 evolution may be related to the covalent linkage between Mn atom and O atom within ligands.

The oxygen atom has a strong electronegativity. When the Mn atom in the complex is coordinated with the O atom by covalent bond, the donation electron capacity of the Mn atom is largely weakened due to stronger attraction electron capability of the O atom unless there is a delocalization structure formed between the Mn atom and the O atom within ligand. That may be why the native Mn cluster forms a cubane-like Mn_3CaO_4 cluster in which the Mn atom is linked with the O atom in a μ-oxo bridge form. Existence of delocalization structure in Mn_3CaO_4 cluster not only helps avoid the centralization of higher positive charge of Mn atom but also benefits the output of electron and stability of Mn cluster. Hence covalent locating linkage between Mn atom and O atoms within the ligands may be one of the reasons leading to the lower efficiency of this complex in the reconstitution of WOC.

In addition, we found that the efficiency of Mn-complexes in the recovery of electron transport is not in accord with that in O_2 evolution. For example, $[Mn(II)Cl(pic)]_n$, $[Mn(III)(pic)_3]$, and $[Mn(IV)_2O_2(pic)_4]$ showed higher efficiency than $MnCl_2$ in reactivation of O_2 evolution activity while the three Mn-complexes had a lower efficiency than $MnCl_2$ in restoration of electron transport. Similar results were also presented by Karacan and Somer (2004). Thus reconstitution of electron transport and O_2 evolution with synthetic Mn-complexes is influenced by different factors.

The properties of ligand, especially the structural factor, seem to have close relation with the reconstitution of electron transfer (Fig. 2). The size of ligand obviously affects the donation electron capacity of Mn-complex. All these Mn-complexes show lower efficiency than free Mn^{2+} in restoring electron transport. But the function of ligand during the photoactivatiom with Mn-complex may only help the coordination of Mn atom to the apo-WOC (cofactor-depleted enzyme). From comparison with the results of $MnCl_2$, it is inferred that the existence of ligands, especially those containing aromatic ring, is important for the ligation of Mn atom. The ligand itself may not participate in the formation of WOC and will be stripped after the Mn atom ligated to the apo-WOC. The size of ligand is not an essential factor affecting the efficiency of Mn-complex in the recovery of O_2 evolution. In the present photoactivation experiment, the difference in the number of chelate rings did not cause large difference in the O_2 evolution between Mn-complexes.

Moreover, to our knowledge, complex $[Mn(IV)_2O_2(pic)_4]$ in which Mn atom is at +4 valence state was used for the first time to reconstitute the WOC. This complex restores the O_2 evolution activity with higher efficiency. That means that Mn (IV) is photo-oxidized to Mn(V) or higher valence state. Brudvig's group has proposed a mechanism of water oxidation in which involvement of an electrophilic Mn(V) = O species in O_2 formation is postulated (Limburg *et al.* 1999). Our present photoactivation experiments showed that the existence of a Mn(V) intermediate was possible in the cycle of S states.

Ca^{2+} is an essential cofactor required for photo-activation. A Ca^{2+} ion was involved in the conversion of the first Mn^{2+} mononuclear intermediate to the Mn^{3+}–Mn^{2+} intermediate in photoactivation experiments using $MnCl_2$ (Ananyev and Dismukes 1996). $CaCl_2$ is also required for mononuclear Mn-complexes during photo-activation (Karacan and Somer 2004). But in the case of binuclear Mn-complexes, there was no special requirement for addition of exogenous Ca^{2+} during photo-activation (Han *et al.* 2005). The Ca^{2+} retained in the isolated PS2 preparations was sufficient or Ca^{2+} was not required for insertion of binuclear complexes.

The 33 kDa protein is referred as manganese stabilizing protein (MSP) due to its key role in the stabilization of the manganese cluster of the WOC. The presence of this polypeptide decreases Cl^- requirement and stimulates photoactivation (Miyao and Murata 1983).

In our photoactivation experiment with Mn-complexes, addition of the 33 kDa protein or 20 mM $CaCl_2$ alone markedly stimulated the oxygen-evolving activity. But the maximal recovery of O_2 evolution activity required the presence of both $CaCl_2$ and 33 kDa protein. The requirement for $CaCl_2$ and 33 kDa protein during photoactivation seems to be more important to Mn-complexes than to $MnCl_2$. The presence of 40 g m^{-3} 33 kDa protein and 20 mM $CaCl_2$ during photoactivation with Mn-complexes raised the O_2-evolution activity to a greater extent compared with $MnCl_2$ (Table 1). An interaction between $CaCl_2$ and 33 kDa protein may exist and this interaction provides favourable environment for the ligation of Mn atom with apo-WOC during photo-activation with Mn-complexes.

The 33 kDa protein is conformation flexible in solution (Hutchison *et al.* 1998, Lydakis-Simantiris *et al.* 1999, Shutova *et al.* 2000). It may play a role in the stabilization of optimal conformation of PS2 RC proteins engaged in Mn-Ca coordination (Seidler 1996). Moreover, the isolated 33 kDa protein of PS2 contains one calcium low-affinity binding site (Kruk *et al.* 2003). Binding of calcium to this site may influence the conformation of the 33 kDa protein itself and/or the neighbouring proteins (*e.g.* 23 kDa protein) and also indirectly affect the oxygen evolution activity. Hence we suggest, based on the above analysis, that presence of $CaCl_2$ during photoactivation possibly modulates the conformational change of 33 kDa protein engaged in reconstitution of WOC and therefore increases the O_2 evolution activity.

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