Photochemistry and Photobiology, 1999, 70(1): 57–63

Reconstruction of the Water-Oxidizing Complex in Manganese-Depleted Photosystem II Preparations Using Mononuclear Manganese Complexes

S. I. Allakhverdiev[1–3], U. Ozdemir[4], J. Harnois[1], N. Karacan[4], S. Hotchandani[1], V. V. Klimov[2], N. Murata[3] and R. Carpentier*[1]

[1]Groupe de Recherche en Énergie et Information Biomoléculaires, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada;
[2]Institute of Soil Science and Photosynthesis, Russian Academy of Science, Pushchino, Moscow Region, Russia;
[3]Department of Regulation Biology, National Institute for Basic Biology, Myodaiji, Okazaki, Japan and
[4]Department of Chemistry, University of Gazi, Ankara, Turkey

Received 26 January 1999; accepted 16 April 1999

ABSTRACT

The water-oxidizing complex of chloroplast photosystem II is composed of a cluster of four manganese atoms that can accumulate four oxidizing redox equivalents. Depletion of manganese from the water-oxidizing complex fully inhibits oxygen evolution. However, the complex can be reconstituted in the presence of exogenous manganese in a process called photoactivation. In the present study, mononuclear manganese complexes with ligands derived from either nitrosonaphthol and ethylenediamine (Niten) or from diaminohexane and salicylaldehyde (Salhxn) are used in photoactivation experiments. Measurements of photoinduced changes of chlorophyll fluorescence yield, thermal dissipation using photoacoustic spectroscopy, photoreduction of 2,6-dichorophenolindophenol and oxygen evolution in manganese-depleted and in reconstituted photosystem II preparations demonstrate that photoactivation is more efficient when Niten and Salhxn complexes are used instead of $MnCl_2$. It is inferred that the aromatic ligands facilitate the interaction of the manganese atoms with photosystem II. The addition of $CaCl_2$ and of the extrinsic polypeptide of 33 kDa known as the manganese-stabilizing protein during photoactivation further enhances the recovery of electron transport and oxygen evolution activities. It is proposed that mononuclear manganese complexes are able to contribute to reconstitution of the water-oxidizing complex by sequential addition of single ions similarly to the current model for assembly of the tetranuclear manganese cluster and that these complexes constitute suitable model systems to study the assembly of the water-oxidizing complex.

INTRODUCTION

The water-oxidizing complex (WOC)† of chloroplast photosystem II (PSII) is located on the luminal side of the thylakoid membrane where it performs the decomposition of water into molecular oxygen, protons and electrons. The WOC is composed of a manganese (Mn) cluster that can accumulate four oxidizing redox equivalents that are reduced with the simultaneous oxidation of two water molecules. The electrons are sequentially used to reduce P680, the primary electron donor of the PSII reaction center, that is oxidized upon light-induced charge separations resulting in electron transfer to specific quinone acceptors (1–3).

The precise organization of the Mn cluster and its association with PSII polypeptides is still under debate (1,4,5). It is known that a functional WOC is composed of four Mn atoms that are probably assembled in two binuclear clusters (6,7). Only two Mn seem to be implicated directly in water cleavage, and the other two possibly play a structural role (6–12). The nature of the polypeptides involved in Mn binding is not totally elucidated but it is likely that the Mn cluster is embedded at the luminal face of PSII in or near the polypeptides D1, D2 and the pigment-binding polypeptides CP47 and CP43 (1–3,13). From studies using chemical modification of amino acids and site-directed mutagenesis, several amino acid residues of the polypeptide D1 were suggested to be involved in the formation of Mn binding sites. It has been demonstrated that at least one or two histidine residues, possibly His-190 and His-337 of the polypeptide D1, are bound to Mn (13–15), and it was suggested that a histidine residue is involved in the photooxidation of coordinated Mn^{2+} (16,17). Carboxyl groups were also reported to be involved in photoactivation and Mn binding (15,18,19). More precisely, the residue Glu-69 of the polypeptide D2 and the residue Asp-170 together with other residues in the carboxy-

*To whom correspondence should be addressed at: Groupe de Recherche en Énergie et Information Biomoléculaires, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec G9A 5H7, Canada. Fax: 819-376-5057; e-mail: Robert_Carpentier@uqtr.uquebec.ca

†*Abbreviations:* ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; Chl, chlorophyll; DCPIP, 2,6-dichorophenolindophenol; Niten, nitrosonaphthol and ethylenediamine; PSII, photosystem II; Salhxn, diaminohexane and salicylaldehyde; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; WOC, water-oxidizing complex.

terminal domain of the polypeptide D1 were suggested as ligands for Mn (20–22). The hydrophobic domain (loop E) of the pigment-binding polypeptide CP47 was also suggested to be involved in the formation of a stable WOC (23).

Depletion of the Mn from the WOC fully inhibits water cleavage and oxygen evolution. The WOC can be reconstituted in the presence of exogenous Mn under weak illumination, a process called photoactivation (24). This has been demonstrated in various types of materials, including PSII-enriched membranes (25–29), and constitutes a multistep process that requires two separated short illumination periods (30–32). Besides Mn^{2+}, a maximally effective photoactivation also requires the presence of Ca^{2+} and Cl^- together with the 33 kDa extrinsic polypeptide known as the Mn-stabilizing protein (24,33–37).

In the past few years, several Mn complexes have been synthesized as models to mimic the Mn cluster of the WOC. Most of these complexes are binuclear or tetranuclear; however, little attention has been focused on mononuclear complexes (38,39). Synthetic Mn complexes could provide a powerful system to analyze the assembly of the WOC. In recent reports, photoactivation of Mn-depleted PSII-enriched membranes in the presence of tetranuclear (30) and binuclear (40,41) Mn complexes (with catechol and 2-hydroxy-1,4-naphthoquinone monoxime as ligand, respectively) was shown to be more effective in comparison with photoactivation in the presence of $MnCl_2$. It was proposed that the aromatic ligand could facilitate the interaction of the Mn atoms with PSII, the ligand being stripped off from the Mn atoms only inside the PSII complex where it is substituted by amino acid side chains (40).

The current model for the assembly of the Mn cluster during photoactivation, however, indicates that the first two Mn are integrated sequentially (24–32). The first Mn^{2+} is photooxidized with a high quantum efficiency to Mn^{3+} before a second Mn^{2+} can be ligated resulting in formation of a Mn^{3+}–Mn^{2+} complex. This intermediate is then photooxidized with low quantum efficiency to Mn^{3+}–Mn^{3+}, a reaction that is followed by the relatively slow ligation of two additional Mn^{2+} ions and the formation of an active complex (24,25,31). In the present study, mononuclear Mn complexes, with ligands derived from either nitrosonaphthol and ethylenediamine (Niten) or from diaminohexane and salicylaldehyde (Salhxn), are used for the first time to reconstitute the WOC in spinach Mn-depleted PSII preparations. The mononuclear complexes are proposed to enable the sequential addition of the four individual Mn ions according to the above description of the reconstruction of the WOC.

MATERIALS AND METHODS

Chloroplasts were isolated from deveined spinach leaves following the procedure described by Whatley and Arnon (42). The PSII-enriched membranes were prepared by treatment of chloroplasts with 0.4% digitonin and 0.15% Triton X-100 and centrifugation at 20 000 *g* as described elsewhere (7–10,38). These preparations, referred to as DT20, evolved O_2 at a rate of 250–300 μmol/mg chlorophyll (Chl) h under saturating light with 200 μ*M* phenyl-*p*-benzoquinone plus 300 μ*M* potassium ferricyanide as electron acceptor. The concentration of PSII reaction centers was calculated from the photoreduction of the primary electron acceptor pheophytin and from the photooxidation of the primary electron donor P680 (7,10–12,40,41,43). The DT20 preparations contained 80–100 Chl molecules per P680 or one photoreducible pheophytin and 15 500–16 000 Chl molecules per P700 (10,11,40).

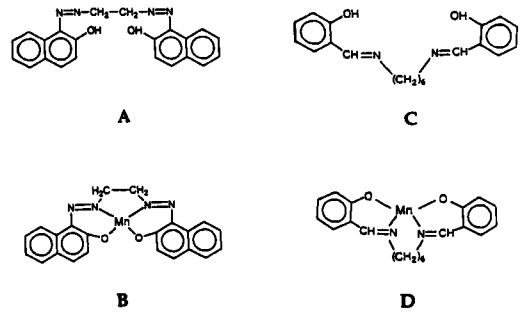

Figure 1. Chemical structure of A, Niten ligand; B, Mn–Niten complex; C, Salhxn ligand; D, Mn–Salhxn complex.

Extraction of Mn (>97%) and of the extrinsic polypeptides of 33, 24 and 18 kDa associated with the WOC from the DT20 preparations was performed as reported previously (40,44). Samples at a concentration of 200 μg Chl/mL were incubated for 10 min at 2°C in a medium containing 20 m*M* Mes-NaOH (pH 6.5), 0.5 m*M* $MgCl_2$ and 20 m*M* *N,N,N',N'*-tetramethylethylenediamine (TEMED). After centrifugation at 20 000 *g*, the pellet was washed twice in 20 m*M* Tris-HCl (pH 8.0) and 35 m*M* NaCl. Photoactivation of the Mn-depleted DT20 samples was performed in the presence of $MnCl_2$ or Mn complexes and four dark/light cycles ($\lambda > 600$ nm, I = 55 W m^{-2}, illumination of 30–40 s periods separated by 30–40 s of dark) as described in detail by Klimov *et al.* (30). The manganese content was assayed with AAS-1 flame atomic absorption spectrophotometer (Carl Zeiss Jena), and the polypeptide composition was verified using sodium dedecyl sulfate–polyacrylamide gel electrophoresis as described previously (7,10–12).

Simultaneous fluorescence and photoacoustic measurements were performed using a laboratory-constructed instrument composed of a photoacoustic cell (MTEC Photoacoustics Inc., Ames, IA) in combination with a PAM-101 chlorophyll fluorometer (Walz, Effeltrich, Germany) as previously described (45). The photoacoustic-measuring beam was provided from a 150 W xenon lamp (ILC Technology, Sunnydale, CA) as reported (45–47). The intensity of this measuring beam (680 nm, 35 Hz) was 3 W m^{-2}. The DT20 samples (200 μL, 200 μg Chl/mL) were prepared by aspiration onto a nitrocellulose filter following a procedure described in detail elsewhere (45–49).

The rate of oxygen evolution was monitored at 20°C with a Clark-type electrode (50). The sample (3 mL) was illuminated by red light (KC 11 filter) passed through a heat filter consisting of a 5% $CuSO_4$ solution. The light intensity at the cell surface was 100 W m^{-2}. The assay medium consisted of DT20 at a concentration of 10 μg Chl/mL, 25 m*M* Mes-NaOH (pH 6.5), 5 m*M* $CaCl_2$, 10 m*M* NaCl, 300 m*M* sucrose and 200 μ*M* phenyl-*p*-benzoquinone/300 μ*M* potassium ferricyanide as electron acceptor.

The photoreduction of 2,6-dichorophenolindophenol (DCPIP) by DT20 preparations was measured at 20°C using a spectrophotometer (SLM-Amino DW-2000 UV–visible) by following the absorbance change at 580 nm with 500 nm as a reference beam. The reaction medium contained DT20 preparations at a concentration of 10 μg Chl/mL, 20 m*M* Tris-HCl (pH 7.8), 10 m*M* NaCl, 2 m*M* $MgCl_2$ and 50 μ*M* DCPIP. The control value (100%) was 150–160 μmol DCPIP reduced/mg Chl h.

The mononuclear complexes used in this study were prepared using the following procedures: The ligand bis(α-nitroso-β-naphthol)ethylenediamine (Niten) (Fig. 1A) was prepared by mixing 2.5 mol of α-nitroso-β-naphthol and 1 mol of ethylenediamine in chloroform solution and refluxing for about 24 h. A bright green product was collected by filtration. This precipitate was washed with chloroform and benzene and dried over silica gel. The Mn complex with this ligand (Fig. 1B) was obtained by refluxing stoichiometric amounts of the ligand Niten and Mn(II) acetate in chloroform solution for 24 h under nitrogen atmosphere. The dark brown crystals formed were filtered and washed with chloroform, then dried in a desiccator over silica gel. The second ligand, a quadridentate Schiff base (Salhxn) (Fig. 1C), was prepared by condensation of 1,6-di-

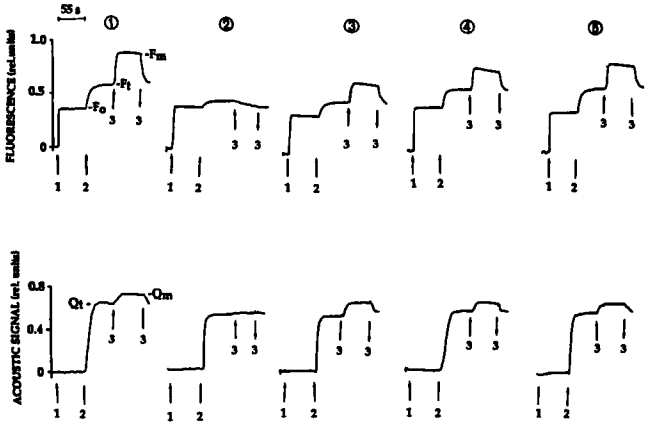

Figure 2. Simultaneous measurement of fluorescence and thermal emissions in DT20 preparations. Up and down arrows indicate lights on and off, respectively. Numbers under the arrows: 1, fluorescence probe beam; 2, photoacoustic measuring beam; 3, saturating background illumination. Trace 1, control; trace 2, Mn depleted; trace 3, reconstituted with 20 μM $MnCl_2$; trace 4, reconstituted with 20 μM Mn–Salhxn complex; trace 5, reconstituted with 20 μM Mn–Niten complex. Details are given in the Materials and Methods.

aminohexane with salicylaldehyde in a 1:2 molar ratio and crystallized from ethanol. The Mn complex (Fig. 1D) was prepared by mixing the ligand Salhxn (0.05 mol) and solid NaOH (0.1 mol) with Mn(II) acetate (0.05 mol) in ethanol under nitrogen atmosphere. The resulting brown crystals were filtered, washed with ethanol and dried under vacuum. Spectral and magnetic measurements indicated that the above complexes have a tetrahedral structure (the author's unpublished results) (51,52).

RESULTS

Simultaneous fluorescence and photoacoustic measurements

Typical traces corresponding to the simultaneous measurement of fluorescence and thermal emission in a DT20 preparation are shown in Fig. 2. The 1.6 kHz excitation beam (beam 1, Fig. 2) from the Walz fluorometer induces the initial fluorescence rise to F_o without generating any measurable thermal dissipation (see acoustic signal, Fig. 2). The latter is obtained by the subsequent addition of the 680 nm modulated light (35 Hz, 3 W/m^2, beam 2), which procures the thermal signal Q_t analogous to the fluorescence intensity F_t obtained simultaneously due to the closure of part of the PSII reaction centers by the 35 Hz modulated light. Total closure of the reaction centers is achieved following further addition of the white nonmodulated saturating beam 3 from the Walz KL1500 illuminator that provides the maximal fluorescence and thermal intensities F_m and Q_m respectively. In control samples (Fig. 2, trace 1), the saturating beam induced a three- to four-fold increase over the initial value of F_o. On the other hand, the thermal energy storage measured as $[(Q_m - Q_t)/Q_m] \times 100\%$ (41) was 10–14%. Variable fluorescence (both F_t and F_m levels) and thermal dissipation were dramatically reduced after TEMED treatment due to the loss of the oxygen-evolving activity that results in a limited electron donor capacity (Fig. 2, trace 2).

Significant restoration of the variable part of fluorescence and thermal dissipation, indicating the recovery of electron transport capacity and reduction of the primary quinone acceptors of PSII, was effected by photoactivation in the presence of exogenous $MnCl_2$ at a concentration of 20 μM (Fig. 2, trace 3). A similar restoration was achieved with the Mn complexes with both ligands. However, photoactivation with the mononuclear Mn complexes was more effective in comparison with $MnCl_2$ and the best restoration of variable fluorescence was obtained with the Mn–Niten complex (Fig. 2, trace 6).

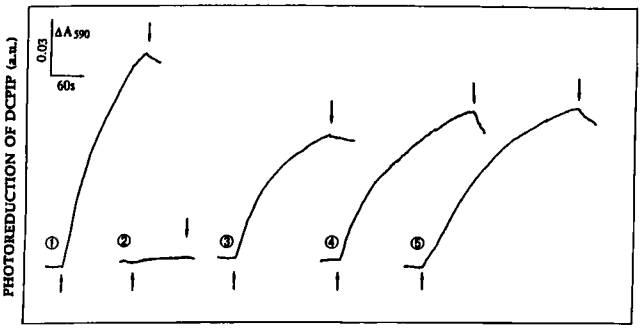

Figure 3. Photoreduction of DCPIP measured at 590 nm in DT20 preparations. Trace 1, control [65]; trace 2, Mn depleted [8]; trace 3, reconstituted with 1 μM $MnCl_2$ [36]; trace 4, reconstituted with 1 μM Mn–Salhxn complex [52]; trace 5, reconstituted with 1 μM Mn–Niten complex [20]. The initial rates of photoreduction (μmol DCPIP/mg Chl h) are given in backets. Conditions are given in the Materials and Methods.

Photoreduction of DCPIP

The above observations clearly show that mononuclear complexes could reconstitute the Mn-depleted DT20 preparations in terms of electron transport to the primary quinone acceptors. Reconstitution of electron transport capacity is further demonstrated by the experiments using DCPIP as the final electron acceptor (Fig. 3). The initial rate of DCPIP photoreduction in Mn-depleted DT20 preparations (Fig. 3, trace 2) was severely reduced in comparison with the rate in control samples (Fig. 3, trace 1). Photoreduction was greatly restored in the samples photoactivated in the presence of either $MnCl_2$ or mononuclear Mn–Niten or Mn–Salhxn complexes (Fig. 2, traces 3, 4 and 6). However, the rates were not as greatly sustained as in control samples, which indicates that some photoinhibition may occur in these samples as they are not fully functional on the oxidizing side of the photosystem.

Oxygen evolution

Recovery of the electron transport capability of Mn-depleted DT20 preparations as measured by the recovery of variable fluorescence and thermal dissipation and by the photoreduction of DCPIP does not discriminate between electron donation by Mn bound to DT20 particles and the reconstruction of an active WOC. The oxygen-evolving activity of these preparations is reported in Table 1. The oxygen-evolving activity is almost completely inhibited in Mn-depleted DT20 samples. This activity is partially recovered after photoactivation with $MnCl_2$. Photoactivation in the presence of added 33 kDa polypeptide, the extrinsic polypeptide known as the Mn-stabilizing protein, was more efficient in comparison with photoactivation in its absence. However, the addition

Table 1. Reactivation of oxygen evolution in Mn-depleted DT20 preparations

	Initial rate of oxygen evolution (μmol O_2/mg Chl h)
Native preparations	275
Mn-depleted	0
Reconstituted with*	
$MnCl_2$	44
+ 33 kDa polypeptide	57
+ 33 kDa polypeptide and $CaCl_2$	92
Mn–Niten complex	76
+ $CaCl_2$	81
+ $CaCl_2$ and 33 kDa polypeptide	98
Mn–Salhxn complex	69
+ $CaCl_2$	76
+ $CaCl_2$ and 33 kDa polypeptide	89

*$MnCl_2$ or Mn complexes were added at a concentration of 2 μ*M*, the 33 kDa polypeptide at 40 μg/mL and $CaCl_2$ at 20 m*M*.

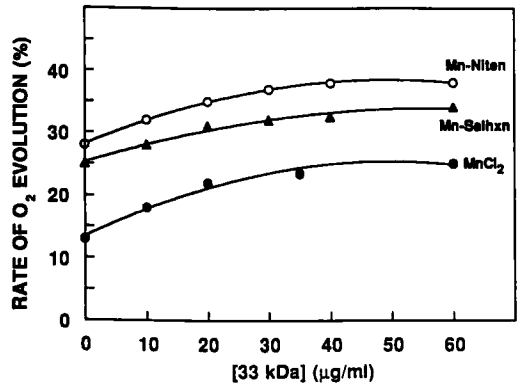

Figure 5. Reactivation of oxygen evolution in Mn-depleted DT20 preparations reconstituted with Mn in the presence of various concentrations of the 33 kDa protein. (–●–), $MnCl_2$; (–○–), Mn–Niten complex; (–▲–), Mn–Salhxn complex. The reaction medium without $CaCl_2$ contained 20 μg Chl/mL and 10 Mn (as $MnCl_2$ or Mn complex) per PSII reaction center. The rates are given as a percentage of the optimal rates obtained in native DT20 preparations.

of 20 m*M* $CaCl_2$ that supplemented the 5 m*M* $CaCl_2$ already present in the assay medium used for oxygen evolution measurements further increased the efficiency of the oxygen-evolving activity and therefore aided in the reconstruction of an active WOC.

Photoactivation in the presence of the Niten or Salhxn complexes is also shown in Table 1. In both cases, the Mn complexes yielded a greater initial rate of oxygen evolution in the reconstructed preparations than $MnCl_2$. Similar to photoactivation with $MnCl_2$, photoactivation with the mononuclear Mn complexes was clearly enhanced by the addition of 20 m*M* $CaCl_2$ and the addition of the 33 kDa polypeptide elicited a further enhancement.

A more detailed description of the reactivation of oxygen evolution is described in Figs. 4–6. The effect of the concentration of $MnCl_2$ and mononuclear Mn complexes on photoactivation is presented in Fig. 4. With the Mn complexes, about four Mn per reaction center were required to obtain optimal photoactivation. It is also evident from these traces that the Niten complexes participated in a more effective reactivation of the rates of oxygen evolution in comparison with the Salhxn complexes. The concentration of the 33 kDa polypeptide required in the medium for optimal photoactivation was similar for $MnCl_2$ and for Mn complexes and was about 40 μg/mL (Fig. 5). The action of the Mn-stabilizing protein was more important when photoactivation was performed with $MnCl_2$. In comparison, the presence of 60 μg/mL of the 33 kDa polypeptide raised the yield of photoactivation by 100% when $MnCl_2$ was used for reactivation while the rise was only 35–40% when the Mn–Niten or Mn–Salhxn complexes were used. The requirement for $CaCl_2$ during photoactivation in preparations already supplemented with 40 mg/mL 33 kDa polypeptide is clearly illustrated in Fig. 6. However, the presence of Ca^{2+} that was optimal at 10 m*M* had much more impact when $MnCl_2$ was used for reactivation. From Fig. 6, it can be calculated that the presence of 15 m*M* $CaCl_2$ raised the yield of photoacti-

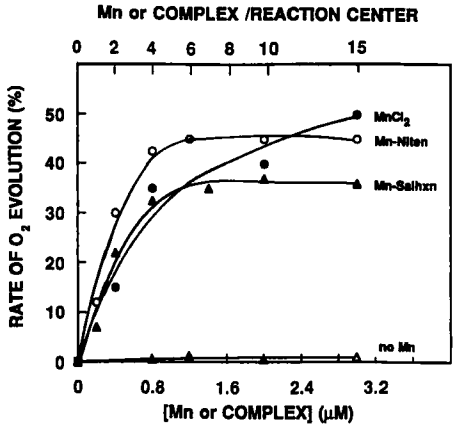

Figure 4. Reactivation of oxygen evolution in Mn-depleted DT20 preparations reconstituted with various concentrations of Mn: (–●–), $MnCl_2$; (–○–), Mn–Niten complex; (–▲–), Mn–Salhxn complex; (–△–), without Mn. The reactivation medium contained 20 μg Chl/mL, 20 m*M* $CaCl_2$ and 40 μg/mL 33 kDa protein. The rates are given as a percentage of the optimal rates obtained in native DT20 preparations. Other details are as in the Material and Methods.

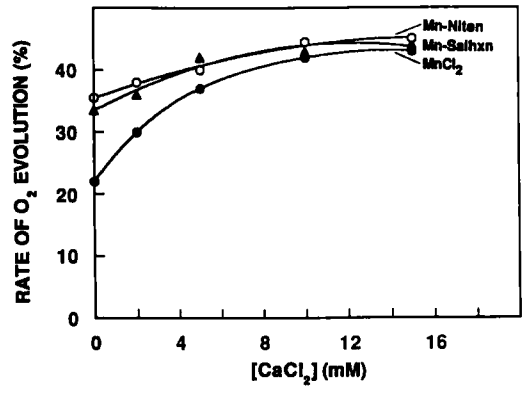

Figure 6. Reactivation of oxygen evolution in Mn-depleted DT20 preparations reconstituted with Mn in the presence of various concentrations of $CaCl_2$. (–●–), $MnCl_2$; (–○–), Mn–Niten complex; (–▲–); Mn–Salhxn complex. The reaction medium contained 20 μg Chl/mL, 10 Mn (as $MnCl_2$ or Mn complex) per PSII reaction center and 40 μg/mL 33 kDa protein. The rates are given as a percentage of the optimal rates obtained in native DT20 preparations.

vation by about 95% if $MnCl_2$ was used and by only 25% with Mn–Niten or Mn–Salhxn complexes.

DISCUSSION

The above results demonstrate that mononuclear Mn complexes using Niten and Salhxn ligands can reconstitute the WOC in Mn-depleted PSII preparations and restore significant rates of electron transport and oxygen evolution. Photoactivation using Mn complexes was more efficient in comparison with the results obtained with $MnCl_2$. Binuclear and tetranuclear Mn complexes were also more efficient electron donors than $MnCl_2$ (30,40,41). Similarly, several secondary amines with bulky aromatic substituents and acidic –NH groups were reported to be strong catalysts of the ADRY effect in an intact WOC (53). It is likely that the ligands Niten and Salhxn that contain, respectively, four and two aromatic rings facilitate interaction with the exposed donor side of PSII. Accordingly, Mn–Niten complexes having four aromatic rings were significantly more efficient than Mn–Salhxn complexes in restoring fluorescence induction and DCPIP photoreduction (Figs. 2 and 3). In the case of DCPIP photoreduction the initial rates were higher with the Salhxn ligand but they declined rapidly while the initial rate of photoreduction remained stable for a much longer period of time in the case of the Niten ligand. Niten complexes were thus more competent electron donors. The superiority of Niten complexes indicates that the complexes formed with this ligand gained more accessibility to the Mn oxidation sites in the protein matrix. This also brings about higher rates of oxygen evolution (Table 1, Fig. 4) owing to the effective reconstruction of an active WOC.

In the case of binuclear Mn complexes, there was no special requirement for addition of exogenous Ca^{2+} during photoactivation (40). It was inferred that the Ca^{2+} retained in the isolated DT20 preparations was sufficient or that Ca^{2+} was not required for insertion of binuclear complexes. In contrast, in the present experiments where mononuclear complexes are used, the addition of 20 m*M* $CaCl_2$ during photoactivation significantly stimulated the initial rates of oxygen evolution in the samples photoactivated in the presence of Mn complexes using either Niten or Salhxn ligands (Table 1, Fig. 6). The use of mononuclear complexes, in contrast to the binuclear or tetranuclear complexes, should allow the reconstruction of the WOC through a sequential addition of single Mn ions according to the current model for assembly of the tetranuclear Mn cluster (24–37). It was suggested that during reconstruction with binuclear complexes, Mn was bound to PSII as a binuclear cluster (40).

In photoactivation experiments using $MnCl_2$, it has been inferred that a Ca^{2+} ion is involved in the conversion of the first Mn^{2+} mononuclear intermediate to the Mn^{3+}–Mn^{2+} intermediate (37). Accordingly, the formation of a carboxylate bridge between Mn and Ca^{2+} has been suggested from Fourier-transform infrared spectroscopy (54). Further, site-directed mutagenesis experiments indicated that several amino-acid residues of the carboxyl-terminal domain and of the lumenal interhelical domains of the D1 polypeptide either ligate with Ca^{2+} or influence its interaction with the WOC (20). Chloride is another cofactor that may influence photoactivation (35). Even though it was reported that Cl^- is not absolutely essential (24), Cl^- concentrations above 3 m*M* were suggested to increase the quantum yield of photoactivation (1,33–35). The extrinsic 33 kDa polypeptide identified as the Mn-stabilizing protein was shown to decrease the Cl^- requirement and to stimulate photoactivation (1,6,8,33–35,40). The presence of this polypeptide during photoactivation with the mononuclear complexes also increased oxygen evolution in the photoactivated samples (Table 1, Fig. 5). Hence, the presence of exogenous Ca^{2+} and Cl^- during photoactivation using the mononuclear complexes accelerates the photoassembly of a competent Mn cluster and reduces the photoinactivation caused by the ligation of nonfunctional Mn^{3+} ions as reported for experiments using $MnCl_2$ (1,7,34–37). Interestingly, though the optimal concentrations of $CaCl_2$ and 33 kDa polypeptide for photoactivation were similar with $MnCl_2$ and Mn complexes, these cofactors increased the yield of photoactivation to a greater extent when $MnCl_2$ was used to reconstitute an active WOC (Figs. 5 and 6). It is probable that the organic ligands participated in the stabilization of the forming oxygen-evolving Mn cluster.

In conclusion, the present study demonstrates that synthetic mononuclear Mn complexes were able to reconstitute the WOC with a greater efficiency than $MnCl_2$. Photoactivation by mononuclear complexes was characterized by a cofactor requirement similar to what is necessary for optimal photoactivation with $MnCl_2$. These Mn complexes thus constitute suitable model systems to study the assembly of the WOC.

Acknowledgements—This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) to R.C. and grant-in-aid for specially Promoted Research from the Japanese Ministry of Education, Science and Culture (08/02011) to N.M. S.I.A. was recipient of an International Scientific Exchange Award from NSERC. We thank A. Tessier for his professional assistance in some of the experiments.

REFERENCES

1. Murata, N. and M. Miyao (1989) Photosystem II and oxygen evolution. In *Photosynthesis* (Edited by W. R. Briggs), pp. 59–30. Alan R. Liss, New York.
2. Renger, G. (1992) Energy transfer and trapping in photosystem II. In *Topics in Photosynthesis, the Photosystems: Structure, Function and Molecular Biology* (Edited by J. Barber), pp. 45–99. Elsevier, Amsterdam.
3. Govindjee and W. J. Coleman (1992) Oxidation of water to molecular oxygen. In *Photosynthesis: Photoreactions to Plant Productivity* (Edited by Y. P. Abrol, P. Mohanty and Govindjee), pp. 83–108. Kluwer Academic Publishers, Dordrecht.
4. Rutherford, A. W., J.-L. Zimmermann and A. Boussac (1992) Oxygen evolution. In *Topics in Photosynthesis, the Photosystems: Structure, Function and Molecular Biology* (Edited by J. Barber), pp. 179–229. Elsevier, Amsterdam.
5. Britt, R. D. (1996) Oxygen evolution. In *Oxygenic Photosynthesis: the Light Reactions* (Edited by D. R. Ort and C. F. Yocum), pp. 137–164. Kluwer Academic Publishers, Dordrecht.
6. Murata, N., M. Miyao, T. Omata, H. Matsunami and T. Kuwabara (1984) Stoichiometry of components in photosynthetic oxygen evolution system of photosystem II particles prepared with Triton X-100 from spinach chloroplasts. *Biochim. Biophys. Acta* **765**, 363–369.
7. Klimov, V. V., S. I. Allakhverdiev, V. A. Shuvalov and A. A. Krasnovski, (1982) Effect of extraction and re-addition of manganese on light reactions of photosystem-II preparations. *FEBS Lett.* **148**, 307–312.
8. Kuwabara, T. and M. Murata (1983) Quantitative analysis of

the inactivation of photosynthetic oxygen evolution and the release of polypeptides and manganese in the photosystem II particles of spinach chloroplasts. *Plant Cell Physiol.* **24**, 741–747.
9. Yachandra, V. K., V. J. DeRose, M. J. Latimer, I. Mukerji, K. Sauer and M. P. Klein (1993) Where plants make oxygen: a structural model for the photosynthetic oxygen-evolving manganese center. *Science* **260**, 675–679.
10. Allakhverdiev, S. I., M. A. Shafiev and V. V. Klimov (1986) Effect of reversible extraction of manganese on photooxidation of chlorophyll P_{680} in photosystem II preparations. *Photobiochem. Photobiophys.* **12**, 61–65.
11. Allakhverdiev, S. I. and V. V. Klimov (1992) Photoreduction of $NADP^+$ in photosystem II of higher plants: requirements for manganese. *Z. Naturforsch.* **47c**, 57–62.
12. Allakhverdiev, S. I., I. Yruela, R. Picorel and V. V. Klimov (1997) Bicarbonate is an essential constituent of the water-oxidizing complex of photosystem II. *Proc. Natl. Acad. Sci. USA* **94**, 5050–5054.
13. Tamura, N., M. Ikeuchi and Y. Inoue (1989) Assignment of histidine residue in D1 protein as possible ligands for functional manganese in photosynthetic water-oxidizing complex. *Biochim. Biophys. Acta* **973**, 281–289.
14. Tang, X.-S., B. A. Diner, B. S. Larsen, M. L. Gilchrist, G. A. Lorigan and R. D. Britt (1994) Identification of histidine at the catalytic site of the photosynthetic oxygen-evolving complex. *Proc. Natl. Acad. Sci. USA* **91**, 704–708.
15. Magnuson, A. and L.-E. Andréasson (1997) Different manganese binding sites in photosystem II probed by selective chemical modification of histidyl and carboxylic acid residues. *Biochemistry* **36**, 3254–3261.
16. Ono, T.-A. and Y. Inoue (1991) A possible role of redox-active histidine in the photoligation of manganese into a photosynthetic O_2-evolving enzyme. *Biochemistry* **30**, 6183–6188.
17. Allakhverdiev, S. I., V. V. Klimov and S. Demeter (1992) Thermoluminescence evidence for light-induced oxidation of tyrosine and histidine residues in manganese-depleted photosystem II particles. *FEBS Lett.* **297**, 51–54.
18. Tamura, N., K. Noda, K. Wakamatsu, H. Kamachi, H. Inoue and K. Wada (1997) Involvement of carboxyl groups of the PS II reaction center proteins in photoactivation of the apo-water-oxidizing complex. *Plant Cell Physiol.* **38**, 578–585.
19. Noguchi, T., T.-A. Ono and Y. Inoue (1995) Direct detection of a carboxylate bridge between Mn and Ca^{2+} in the photosynthetic oxygen-evolving center by means of Fourier transform infrared spectroscopy. *Biochim. Biophys. Acta* **1228**, 189–200.
20. Chu, H.-A., A. P. Nguyen and R. Debus (1995) Amino acid residues that influence the binding of manganese or calcium to photosystem II. 2. The carboxy-terminal domain of the D1 polypeptide. *Biochemistry* **34**, 5859–5882.
21. Nixon, P. J., J. T. Trost and B. A. Diner (1992) Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the Cyanobacterium Synechocystis sp. PCC 6803: assembly requires a free carboxyl group at C-terminal position 344. *Biochemistry* **31**, 10859–10871.
22. Vermaas, W., J. Charité and G. Shen (1990) Glu-69 of the D2 protein in photosystem II is a potential ligand to Mn involved in photosynthetic oxygen evolution. *Biochemistry* **29**, 5325–5332.
23. Gleiter, H. M., E. Haag, J.-R. Shen, J. J. Eaton-Rye, A. G. Seeliger, Y. Inoue, W. F. Vermaas and G. Renger (1995) Involvement of CP47 protein in stabilization and photoactivation of a functional water-oxidizing complex in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* **34**, 6847–6856.
24. Tamura, N. and G. M. Cheniae (1987) Photoactivation of the water-oxidizing complex in photosystem II membranes depleted of Mn and extrinsic proteins. 1. Biochemical and kinetic characterization. *Biochim. Biophys. Acta* **890**, 179–194.
25. Miller, A.-F. and G. W. Brudvig (1990) Electron-transfer events leading to reconstitution of oxygen-evolution activity in manganese-depleted photosystem II membranes. *Biochemistry* **29**, 1385–1392.
26. Tamura, N., H. Kamachi, N. Hokari, H. Masumoto and H. Inoue (1991) Photoactivation of the water-oxidizing complex of photosystem II core complex depleted of functional Mn. *Biochim. Biophys. Acta* **1060**, 51–58.
27. Miyao, M. and Y. Inoue (1991) An improved procedure for photoactivation of photosynthetic oxygen evolution: effect of artificial electron acceptors on the photoactivation yield of NH_2OH-treated wheat photosystem II membranes. *Biochim. Biophys. Acta* **1056**, 47–56.
28. Ananyev, G. M. and G. C. Dismuked (1996) Assembly of the tetra-Mn site of photosynthetic water oxidation by photoactivation: Mn stoichiometry and detection of a new intermediate. *Biochemistry* **35**, 4102–4109.
29. Tamura, N., M. Kuwahara, Y. Sasaki, K. Wakamatsu and T. Oku (1997) Redox dependence for photoligation of manganese to the apo-water-oxidizing complex in chloroplasts and photosystem II membranes. *Biochemistry* **36**, 6171–6177.
30. Klimov, V. V., G. Ananyev, S. I. Allakhverdiev, S. K. Zharmukhamedov, M. Mulay, U. Hedge and S. Padhye (1990) Photoreaction and photoinactivation of photosystem II after a complete removal of manganese from pea subchloroplast particles. In *Current Research in Photosynthesis* (Edited by M. Baltscheffsky), pp. 247–254. Kluwer Academic Publishers, Dordrecht.
31. Blubaugh, D. and G. M. Cheniae (1992) Photoassembly of the photosystem II manganese cluster. In *Research in Photosynthesis* (Edited by N. Murata), pp. 361–364. Kluwer Academic Publishers, Dordrecht.
32. Miyao-Tokutomi, M. and Y. Inoue (1992) Improvement by benzoquinones of the quantum yield of photoactivation of photosynthetic oxygen evolution: direct evidence for the two-quantum mechanism. *Biochemistry* **31**, 526–532.
33. Kuwabara, T. and N. Murata (1982) An improved purification method and a further characterization of the 33-kilodalton protein of spinach chloroplasts. *Biochim. Biophys. Acta* **680**, 210–215.
34. Miyao, M. and N. Murata (1983) Partial reconstitution of the photosynthetic oxygen evolution system by rebinding of the 33-kDa polypeptide. *FEBS Lett.* **164**, 375–378.
35. Miyao, M. and N. Murata (1984) Role of the 33-kDa polypeptide in preserving Mn in the photosynthetic oxygen-evolution system and its replacement by chloride ions. *FEBS Lett.* **170**, 350–354.
36. Tamura, N., Y. Inoue and G. M. Cheniae (1989) Photoactivation of the water-oxidizing complex in photosystem II membranes depleted of Mn, Ca and extrinsic proteins. II. Studies on the functions of Ca^{2+}. *Biochim. Biophys. Acta* **976**, 173–181.
37. Chen, C., J. Kazimir and G. M. Cheniae (1995) Calcium modulates the photoassembly of photosystem II $(Mn)_4$-clusters by preventing ligation on nonfunctional high-valency states of manganese. *Biochemistry* **34**, 13511–13526.
38. Pecoraro, V. L., M. J. Baldwin and A. Gelasco (1994) Interaction of manganese with dioxygen and its reduced derivatives. *Chem. Rev.* **94**, 807–826.
39. Hage, R. (1996) Oxidation catalysis by biomimetic manganese complexes. *Recl. Trav. Chim. Pays-Bas* **115**, 385–395.
40. Allakhverdiev, S. I., M. S. Karacan, G. Somer, N. Karacan, E. M. Khan, S. Y. Rane, S. Padhye, V. V. Klimov and Renger (1994) Reconstitution of the water-oxidizing complex in manganese-depleted photosystem II complexes by using synthetic binuclear manganese complexes. *Biochemistry* **33**, 12210–12214.
41. Allakhverdiev, S. I., M. S. Karacan, G. Somer, N. Karacan, E. M. Khan, S. Y. Rane, S. Padhye, V. V. Klimov and G. Renger (1994) Binuclear manganese (III) complexes as electron donors in D1/D2/cytochrome b559 preparations isolated from spinach photosystem II membrane fragments. *Z. Naturforch.* **49c**, 587–592.
42. Whatley, F. R. and D. I. Arnon (1963) Photosynthetic phosphorylation in plants. *Methods Enzymol.* **6**, 308–330.
43. Klimov, V. V., E. Dolan, E. R. Shaw and B. Ke (1980) Interaction between the intermediary electron acceptor (pheophytin) and a possible plastoquinone–iron complex in photosystem II reactions centers. *Proc. Natl. Acad. Sci. USA* **77**, 7227–7231.
44. Ananyev, G., T. Wydrzynski, G. Renger and V. V. Klimov (1992) Transient peroxide formation by the manganese-containing, redox-active donor side of photosystem II upon inhibition

of O_2 evolution with lauroylcholine chloride. *Biochim. Biophys. Acta* **1100**, 303–311.
45. Allakhverdiev, S. I., V. V. Klimov and R. Carpentier (1994) Variable thermal emission and chlorophyll fluorescence in photosystem II particles. *Proc. Natl. Acad. Sci. USA* **91**, 281–285.
46. Velitchkova, M. and R. Carpentier (1994) Variable thermal dissipation in a photosystem I submembrane fraction. *Photosynth. Res.* **40**, 263–268.
47. Allakhverdiev, S. I., V. V. Klimov and R. Carpentier (1997) Evidence for the involvement of cyclic electron transport in the protection of photosystem II against photoinhibition: influence of a new phenolic compound. *Biochemistry* **36**, 4149–4154.
48. Markovic, D. and R. Carpentier (1995) Relationship between quenching of variable fluorescence and thermal dissipation in isolated thylakoid membranes: similar terminology and mathematical treatments may be used. *Biochem. Cell Biol.* **73**, 247–252.
49. Yahyaoui, W., D. Markovic and R. Carpentier (1997) Application of modulated photoacoustic spectroscopy to measurement of photochemical quenching of variable thermal emission in thylakoid membranes during heat or light stress. *Opt. Eng.* **36**, 337–342.
50. Carpentier, R., B. LaRue and R. M. Leblanc (1984) Photoacoustic spectroscopy in *Anacystis nidulans*. III. Detection of photosynthetic activities. *Arch. Biochem. Biophys.* **228**, 534–543.
51. Titus, S. J. E., W. M. Barr and L. T. Talor (1979) Oxygenation studies of manganese (II) complexes employing tetradentate ligands derived from salicylaldehyde and long chain diamines. *Inorg. Chim. Acta* **32**, 103–106.
52. Cotton, F. A., D. M. L. Goodgame and M. Goodgame (1961) The electronic structures of tetrahedral cobalt (II) complexes. *J. Am. Chem. Soc.* **83**, 4690–4695.
53. Hanssum, B., G. Dohnt and G. Renger (1985) On the mechanism of ADRY agent interaction with photosystem II donor site. *Biochim. Biophys. Acta* **806**, 210–220.
54. Noguchi, T., T.-A. Ono and Y. Inoue (1995) Direct detection of a carboxylate bridge between Mn and Ca^{2+} in the photosynthetic oxygen-evolving center by means of Fourier transform infrared spectroscopy. *Biochim. Biophys. Acta* **1228**, 189–200.