# Chemical Probes for Water-Oxidation: Synthetic Manganese Complexes in Photoactivation of Water Splitting Complex and as Exogenous Electron Donors to Photosystem II

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Z. Naturforsch. **56c,** 755-766 (2001); received April 9/May 28, 2001

Photosystem II, Water Splitting Complex, Synthetic Manganese Complexes

Photoactivation of the water splitting enzyme was performed with 13 different synthetic manganese complexes and characterized by oxygen evolution yield, thermoluminescence and chlorophyll fluorescence induction kinetics. The efficiency of different compounds in photoactivation correlated with the rate of linear electron transport in the presence of these compounds. The organic ligands, associated with the manganese ions, do not prevent the photoactivation of the water splitting complex (WOC). Photoactivation with different manganese complexes depended on the number of the Mn-ions in the complex, their valence state and the nature of their donor atoms. The most efficient restorations were achieved by using tetrameric complexes having a dimer+dimer structure, complexes containing Mn(II) ions, and having 4-6 oxygen and 0-2 nitrogen atoms as donor atoms. Further, the effectiveness of photoactivation depended largely on the structure of the complexes. Our data support the notion that WOC in intact thylakoids requires the cooperation and well determined arrangement of all four manganese ions, and argue against the hypothesis that two manganese ions are sufficient for water splitting. Photoactivation by some complexes led to anomalous flash-oxygen patterns, which are explained by a modified/perturbed water splitting complex.

*Abbreviations:* Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; $F_m$, maximal fluorescence; $F_o$, initial fluorescence; $F_v$, maximal variable fluorescence; $F_v$', increase of maximal variable fluorescence after photoactivation; $L_1$, salicylaldoxime; $L_1^*$, deprotonated $L_1$; $L_2 = L_3$, 3,5-di-*tert*-butyl-1,2-benzoquinone monoxime; $L_4$, 2-hydroxy-1,4-naphthoquinone; $L_5$, 2-hydroxy-3-methyl-1,4-naphthoquinone (phthiocol); $L_6$, acetylphthiocolmonoxime; $L_7$, propionylphthiocolmonoxime; $L_8$, 1,10-phenanthroline; $L_9$, 3-[((2′-pyridyl)methyl)imino)methyl]benzene-1,2-diol; $L_{10}$, 3-[((2-(2′-pyridyl)ethyl)imino)methyl]benzene-1,2-diol; $L_{11}$, 3-[((2-(hydroxymethyl)phenyl)imino)methyl]benzene-1,2-diol; $L_{12}$, 3-[((2′-benzimidazolyl)methyl)imino)methyl]benzene-1,2-diol; $L_{13}$, 3-[((2′-pyridyl)hydrazono)methyl]benzene-1,2-diol; M-1($L_2$), M-2($L_x$) (x = 3-7), M-3($L_8$), M-4($L_y$) (y = 1,9-13), synthetic mono-, bi-, tri- and tetranuclear manganese complexes, respectively; MES, 4-morpholinoethanesulfonic acid; PS II, photosystem II; Q-band, thermoluminescence band associated with $S_2Q_A^-$ charge recombination; $Q_A$ and $Q_B$, primary and secondary quinone acceptors of PS II, respectively; TL, thermoluminescence; WOC, water splitting complex; $Y_D$ and $Y_Z$, redox active tyrosine-161 of $D_2$ and redox active tyrosine-161 of $D_1$ reaction center protein of PS II, respectively.

## Introduction

The catalytic reaction of oxygen evolution, represented by the overall equation $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$, takes place at the four manganese-containing water splitting complex (WOC) located at the donor side of photosystem II (PS II) (for reviews, see Debus, 1992; Britt, 1996; Robblee *et al.*, 2001; Nugent *et al.*, 2001; Renger, 2001; and accompanying reviews in the same issue). Evolution of one molecule of oxygen is preceded by accumulation of four positive charges at the level of WOC, generated by four sequential photoactions of the PS II reaction center. Thus the WOC exists in five different *in vivo* redox states, designated from $S_0$ to $S_4$. Mechanism of water oxidation and many structural detail of the manganese complex is unknown.

One of the methods in studying the WOC is the light-dependent assembly of the manganese com-

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plex, so called photoactivation. Such experiments were carried out on a wide variety of experimental objects from PS II particles to intact plants (Tamura and Cheniae, 1987; Kamachi *et al.*, 1994). During photoactivation two light-dependent events ($Mn^{2+} \rightarrow Mn^{3+}$ oxidations) are linked with a light-independent step (ligation of the second manganese) (Tamura and Cheniae, 1987; Miller and Brudvig, 1990). Recently, Ananyev and his coworkers have carried out intensive quantitative studies on the kinetics of photoactivation and could construct an impressive model for this procedure (see Ananyev *et al.*, 2001; and references therein). This implies detailed interpretation of the first steps and intermediates as well as the elucidation of the role of the $Ca^{2+}$ ion during the photoactivation (Chen *et al.*, 1995). Reactivation of the water oxidation requires $Cl^-$- (Miyao and Inoue, 1991a) and according to recent data (reviewed by Klimov and Baranov, 2001) bicarbonate-ions. The reactivation of WOC is probably accompanied with structural rearrangement(s). The observation, that photoactivation involves a downshift of the redox potential of the primary quinone acceptor, $Q_A$ (Johnson *et al.*, 1995) supports this suggestion.

The bioinorganic approach at the research of manganese-containing enzymes is to model them by structural analogs of synthetic mono-, bi-, tri- and tetranuclear manganese complexes (reviewed by Wieghardt, 1989; Law *et al.*, 1999). In these studies certain physical properties of the structural analogs and native enzymes are compared (e.g. Wells *et al.*, 1993; Schäfer *et al.*, 1998). It is obvious to probe synthetic manganese complexes in photoactivation experiments. Such kind of experiments were carried out by Allakhverdiev *et al.* (1994a, 1999) by using binuclear and mononuclear manganese complexes, respectively. According to their observations these complexes were more effective in photoactivation of manganese-depleted PS II particles than $MnCl_2$ (Allakhverdiev *et al.*, 1994a,b, 1999). Moreover, exogenous electron donation capability of their binuclear Mn(III) complexes exceeded the value belonging to $MnCl_2$. In the present work we have compared the effectiveness of thirteen synthetic complexes not examined in photoactivation experiments before (one mono-, five bi-, one tri- and six tetranuclear complexes) with that of $MnCl_2$ in both types of experiments mentioned above. Tri- and tetranuclear manganese complexes have not probed in photoactivation yet.

## Materials and Methods

### *Sample preparation*

Oxygen-evolving thylakoid membranes were isolated from spinach, as described by Völker *et al.* (1985) and stored at 77 K in a buffer containing 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM MES (pH = 6.5) (= buffer A). Manganese-depleted PS II particles were isolated by the method of Tamura and Cheniae (1987) in buffer A in the presence of 5 mM $NH_2OH$, followed by washing twice in $NH_2OH$-free medium. In photoactivation experiments the $NH_2OH$-treated PS II particles were suspended in a buffer containing 20 mM $CaCl_2$, 110 mM NaCl, 0.4 M sucrose and 20 mM MES (pH = 6.5) (= buffer B) and 100, 50, 33 or 25 μM Mn mononuclear, binuclear, trinuclear or tetranuclear complexes, respectively, in the presence of 10 μM 2,6-dichlorophenol indophenol (DCIP) as electron acceptor ([Chl] = 125 μg/ml). The suspension was illuminated by twenty short cycles (20 s, separated by 20 s of dark) of weak (1 $W/m^2$) red light of 102 L LED lamp of PAM chlorophyll fluorometer (Walz, Effeltrich, Germany; peak wavelength: 650 nm, half bandwidth: 25 nm) at room temperature. After illumination the samples were centrifuged and suspended in buffer B.

### *Measurements of photosynthetic activity*

The photosynthetic oxygen evolution induced by continuous illumination was measured with a Clark-type electrode (Hansatech, Norfolk, UK) at room temperature at saturating light intensity ([Chl] = 50 μg/1.5 ml). Flash-induced oxygen evolution was monitored with a home-built Joliot-type electrode ([Chl] = 10 μg/100 μl). The thermoluminescence (TL) profiles were measured as described in Demeter *et al.* (1985). The dark-adapted samples were excited at −40 °C with continuous white light; the heating rate was 20 °C/min. The fluorescence measurements were carried out on a PAM-101 chlorophyll fluorometer (Walz) at 25 μg/ml Chl-concentration. At the analysis of data the average of 3 independent measurements were taken.

*Synthetic manganese complexes*

$[Mn^{II}(L_2)Cl_2(H_2O)_2]$ complex [= M-1($L_2$)] was synthesized by reacting $MnCl_2{\cdot}2H_2O$ 3,5-di(*tert*-butyl)-1,2-benzoquinone monoxime (= $L_2$ = $L_3$) in aqueous ethanolic solution in 1:2 molar ratio for 3 hrs when the green crystalline compound was precipitated out. It was washed with aqueous ethanol and dried in vacuum while the reaction of $L_2$= $L_3$ with $Mn(Oac)_2{\cdot}2H_2O$ in 1:2 molar ratio yielded the green compound $[Mn^{II,II}(L_3)(Oac)_2](Oac)_2$ symbolised as M-2($L_3$).

The interaction of $Mn(Oac)2{\cdot}2H_2O$ with 2-hydroxy-1,4-naphthoquinone (= $L_4$) in 1:2 metal to ligand ratio under nitrogen atmosphere in methanol-water medium yielded the compound $[Mn(L_4)_2(H_2O)_2]$. Replacement of the two water molecules by 1,10-phenanthroline (= $L_8$) produced the $[Mn^{II,II}(L_4)(L_8)]_2$ complex [= M-2($L_4$)]. $Na_2[Mn^{II,II}(L_5)(Oac)_2]_2(H_2O)_2$, $[Mn^{II,III}(L_6)_2]_2$ and $[Mn^{II,III}(L_7)_2]_2$ complexes [= M-2($L_5$), M-2($L_6$) and M-2($L_7$), respectively] were synthesized as per literature procedures (Padhye *et al.*, 1978). $[Mn^{III}{}_3O_4(L_8)_4(H_2O)_2](NO_3)_4{\cdot}2.5H_2O$ complex [M-3($L_8$)] was synthesized according to Reddy *et al.* (1996).

$[Mn^{III}{}_4(L_1)(L_1{}^*)]_4{\cdot}3CHCl_3$ complex [= M-3($L_8$)] was synthesized as described by Aggarwal *et al.* (1984). $[Mn^{II}{}_2L_x(OAc)_2(MeOH)]_2$ (x = 9–13) tetranuclear complexes [= M-4($L_x$)] were synthesized following a general procedure reported by Theil *et al.* (1997).

The purity of the synthesized manganese compounds was checked by comparing their analytical and spectral profiles with the authentic samples.

## Results and Discussion

The electron donation capacity of $MnCl_2$ and thirteen manganese complexes was investigated in manganese-depleted spinach thylakoid membranes by fluorescence induction measurements. Addition of complexes was performed in the dark therefore photoactivation of the samples could be excluded. The dissociation of $MnCl_2$ and the complexes was compared in aqueous solution by EPR measurements. The amplitude of the six-line EPR signal exhibited by the complexes was smaller with several orders of magnitude than for $MnCl_2$ (data not shown). Consequently, these complexes can be considered fairly stable in solution.

Fig. 1A shows the effects of $MnCl_2$ on the fluorescence induction curves of manganese-depleted thylakoid membranes. After turning on the measuring beam (thin arrow) the fluorescence promptly jumped up to its initial level, $F_o$. The yield elevated to the maximum level, $F_m$ after switching on the actinic beam (thick arrow). After manganese depletion the $F_m$ value significantly decreased in comparison with the control while the $F_o$-level did not change (Fig. 1A, curve 1 and 2) similarly as

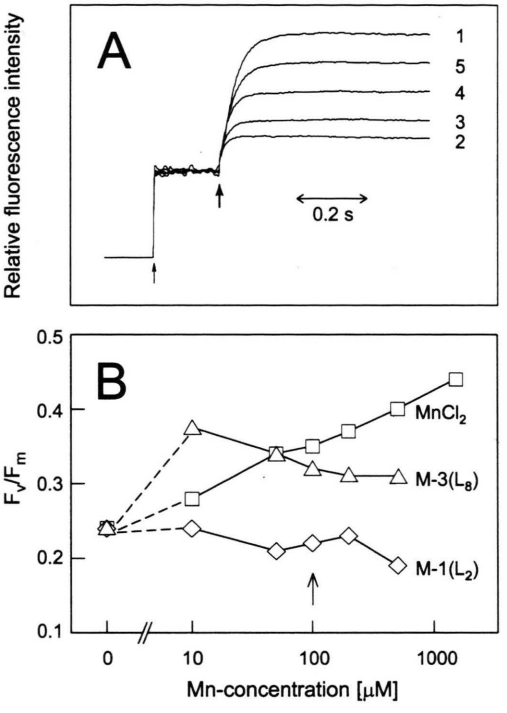


Fig. 1. Effect of $MnCl_2$, M-1($L_2$), a synthetic mono-, and M-3($L_8$), a synthetic trinuclear manganese complex, respectively, on the $F_v/F_m$ ratio of manganese-depleted thylakoid membranes. (A) Effect of depletion and subsequent addition of manganese on the fluorescence induction curves of thylakoid membranes: (1) control; (2) manganese-depleted membranes without addition; manganese-depleted membranes with addition of (3) 10 μM; (4) 200 μM and (5) 5000 μM $MnCl_2$. Thin and thick arrows indicate the onset of the measuring- (0.1 W/m$^2$) and actinic beam (10 W/m$^2$), respectively. Simultaneously with the onset of actinic light the signal to noise ratio of the signal was decreased by modulation of the measuring light with 100 kHz. (B) Effect of the probed mononuclear and trinuclear manganese complexes on the normalized maximal variable fluorescence of manganese-depleted thylakoid membranes. Squares, diamonds and triangles represent the values belonging to $MnCl_2$, M-1($L_2$) and M-3($L_8$), respectively. The arrow indicates the applied concentration in the case of photoactivation.

observed by Klimov *et al.* (1982) and Allakhverdiev *et al.* (1994a, 1999). The remaining part of variable fluorescence ($F_v = F_m$-$F_o$) is probably associated with electron transport from $Y_Z$ and (to lesser extent) cytochrome $b_{559}$ and $Y_D$ (Magnuson *et al.*, 1999). Addition of 10 μM $MnCl_2$ (Fig. 1A, curve 3) induced a significant increase in the fluorescence level in agreement with Klimov *et al.*, (1982) and Allakhverdiev *et al.* (1994a). Increasing amount of added $MnCl_2$ (Fig. 1A, curve 4, 5) resulted in a further gradual enhancement in the $F_v$ value due to larger probability of electron donation.

The effect of manganese compounds was checked in manganese-containing membranes (not shown). Addition of increasing concentration of $MnCl_2$ (10–5000 μM) did not cause any alteration in the fluorescence yield. On the other hand the synthetic manganese complexes slightly decreased the $F_o$ and $F_m$ level but the $F_v/F_m$ ratio ($F_v/F_m \approx 0.67$) remained constant. We assume that the decrease of fluorescence yield is caused by light-absorption of the ligands and lowering of the light-harvesting capability of the chlorophyll antenna system.

The concentration dependence of $F_v/F_m$ in manganese-depleted samples reactivated with mono- and trinuclear manganese complexes is shown in Fig. 1B. Similarly to $MnCl_2$, equimolar amounts of the trinuclear complex, M-3($L_8$) also induced an enhancement in the $F_v/F_m$ ratio, whereas the applied mononuclear complex, M-1($L_2$) did not (Fig. 1B). It means that, from these two complexes, that one [M-3($L_8$)] has the better electron donation capability to PS II in which Mn ions exist at higher (+3) valence state, has larger size and contains a ligand, 1,10-phenanthroline (= $L_8$), which is a well-known inhibitor to PS II. The data concerning the Mn valence state supports the findings that Mn(III) containing complexes also can donate electron to PS II (Allakhverdiev *et al.*, 1994a). According to our present data this capability depends less on the size of the complex than expected. Due to the action site of 1,10-phenanthroline at the non-heme iron of PS II between $Q_A$ and $Q_B$, the primary and secondary quinone acceptor of PS II, respectively (Klimov *et al.*, 1980), it does not influence the restoration of variable fluorescence indicating the reduction of $Q_A$. Further, $L_8$ is stable bound to Mn-ions, thus probably unable to interact with non-heme iron. Although these complexes did not inhibit the $F_v/F_m$ ratio of fluorescence in manganese-containing thylakoid membranes, at high concentrations they induced some inhibition in the manganese-depleted membranes as represented by the descending tendency of the corresponding curves after reaching their maxima. The different electron donation capability of these compounds, especially at low concentration, can be explained by their different redox potential and/or steric reasons (e.g. accessibility to the site of electron donation). Surprisingly, at low concentration (< 50 μM) the trinuclear manganese complex (triangles) had higher electron donation capacity than $MnCl_2$.

Electron donation experiments were carried out by using synthetic bi- and tetranuclear manganese complexes, too. Fig. 2A shows the effects of five different binuclear complexes. At low concentrations (5–50 μM) all of the complexes had normal electron donation capability, whereas at higher concentration (> 100–200 μM), due to the mentioned unidentified inhibition, only the donation of the M-2($L_4$) complex exceeded the value belonging to manganese-depleted samples. This is surprising, since M-2($L_4$) binuclear complex, in addition to 2-hydroxy naphthoquinone, also contains 1,10-phenanthroline with a molar ratio of 2:2 ($L_8$ to Mn) which ratio is close to the $L_8$ to Mn ratio of M-3($L_8$) (4:3). Nevertheless, their electron donation capability significantly differs from each other. M-2($L_4$) donates electron toward P680 similarly as $MnCl_2$ in the whole examined concentration range, while M-3($L_8$) behaves differently at low and at high concentrations: electron donation capability of the trinuclear manganese complex is higher at low concentrations and lower at high concentrations than belonging to $MnCl_2$. This different behaviour cannot be explained by the different valence state of the Mn in M-3($L_8$) and M-2($L_4$) [Mn(III) and Mn(II), respectively] (see the decrease the electron donation capability of all but one [M-2($L_4$)] mono-, bi- and trinuclear complexes), thus the efficiency of the manganese complexes in electron donation highly depends on not only the nature of the ligands but on the structure of the complex, too. The sufficient electron donation capability of M-($L_4$) complex supports the data that binuclear Mn(III) complexes containing 2-hydroxy-1,4 naphthoquinone monoxime, which differs from the $L_4$ (= 2-hydroxy-1,4 naphthoqui-

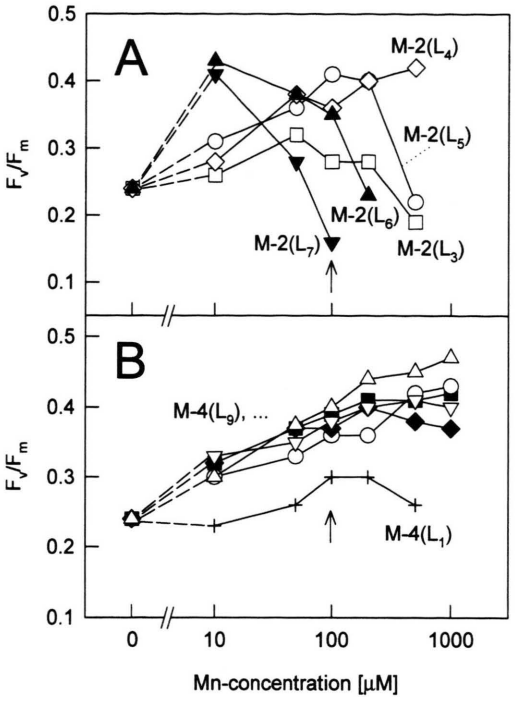


Fig. 2. Effect of binuclear (A) and tetranuclear (B) manganese complexes on the normalized maximal variable fluorescence of manganese-depleted thylakoid membranes. Squares, diamonds, circles, filled standing and filled reversed triangles on panel A represent the $F_v/F_m$ values belonging to M-2($L_3$), M-2($L_4$), M-2($L_5$), M-2($L_6$) and M-2($L_7$), respectively. Crosses, filled squares, circles, filled diamonds, standing and reversed triangles on panel B show the values after electron donation with M-1($L_1$), M-4($L_9$), M-4($L_{10}$), M-4($L_{11}$), M-4($L_{12}$) and M-4($L_{13}$), respectively. The arrows indicate the applied concentration in the case of photoactivation.

none) ligands only by one functional group, also have high efficiency in electron donation (Allakhverdiev *et al.*, 1994a).

Similarly to M-3($L_8$) trinuclear complex, electron donation capacity of two binuclear complexes, M-2($L_6$) and M-2($L_7$) was higher than that of $MnCl_2$ at low concentration (< 20–50 μM). In these complexes having a very similar structure manganese ions exist in mixed valence state [Mn(II) and Mn(III)]. Thus it is possible that the reason of the observed high efficiency in electron donation at low concentrations in the case of M-3($L_8$), M-2($L_6$) and M-2($L_7$) is the higher valence state of the manganese ions in these complexes.

In addition to 1,10-phenanthroline there is another ligand existing not only in binuclear manganese complexes: both M-1($L_2$) and M-2($L_3$) complexes contains 3,5-di-*tert*-butyl-1,2-benzoquinone monoxime (= $L_2$ = $L_3$). By comparing the certain curves in Fig. 1A and in Fig. 2A it can be observed that the electron donation capability of M-2($L_3$) binuclear complex (which has lower electron donation capability than that of other binuclear complexes) is significantly better than belonging to M-1($L_2$) mononuclear complexes (which is approximately zero). This observation also indicates the relationship between the structure and efficiency in electron donation of the complexes.

Although M-2($L_6$) and M-2($L_7$) complexes, due to the close similarity between $L_6$ and $L_7$ ligands (= acetylphthiocolmonoxime and propionylphthiocolmonoxime, respectively, they differ from each other only by a methylene group), have analog chemical structure, their electron donation capabilities are different: $F_v/F_m$ values belonging to M-2($L_7$) complex having larger ligand and consequently larger total size decreases faster after reaching the maximum appearing around 10 μM. Thus not only the structure alone, but also the size of the complexes has effect on the efficiency in electron donation.

Among the probed synthetic manganese complexes the largest group is the tetranuclear compounds. They can be divided into two parts: the salicylaldoxime (= $L_1$) containing M-4($L_1$) complex has tetragonal, while the remaining five complexes have an analog linear, dimer+dimer structure (Theil *et al.*, 1997). This analogy is caused by the similarity of the $L_9$-$L_{13}$ complexes: all of them are cathecolimines substituted by different aromatic groups. Due to this reason the concentration dependence of the $F_v/F_m$ ratios after addition of these compounds to manganese-depleted thylakoid membranes do not show a high-grade heterogeneity as in the case of binuclear complexes. Their concentration dependence also can be divided into two groups: the M-4($L_1$) complex proved to be weak, the cathecolimine containing complexes proved to be strong electron donors (Fig. 2B). In equimolar concentrations all of the manganese-cathecolimine complexes had similar $F_v/F_m$ values and the tendency of change was also identical. Within this homolog group, at high concentrations (> 200 μM) the M-4($L_{12}$) complex

proved to be the most potent electron donor: the corresponding $F_v/F_m$ ratio was higher than in the presence of $MnCl_2$. Two explanations can be provided for this phenomenon. According to the first one, the chemical structure of $L_{12}$ is significantly different from that of the other $L_x$ (x = 9,10,11,13) ligands: instead of pyrimidyl ($L_9$, $L_{10}$, $L_{13}$) or substituted phenyl group ($L_{11}$) it contains a benzimidazolyl group which may modify the redox potential of the M-4($L_{12}$) complex. The second, more possible explanation is that the $L_{12}$ ligand itself can also donate electrons to PS II, which was experimentally proven (data not shown). This fact should also be considered in the interpretation of the observations reported by Allakhverdiev *et al.* (1994a). According to their results higher $F_v/F_m$ ratio can be reached by addition of the same amount of binuclear manganese complexes as $MnCl_2$ (naturally, normalised to the manganese content). It cannot be excluded that in their experiments the applied complexing agent, 2-hydroxy-1,4-naphthoquinone monoxime also acted as electron donor. Supporting our assumption the 4-hydroxy-2-oxo tautomeric form of the oxime greatly resembles the structure of 1,4-quinols, which proved to be efficient donors towards P680, the primary electron donor of PS II, in manganese-depleted PS II particles (data not shown).

The M-4($L_1$) complex differs from the other tetranuclear complexes not only by structurally but by the valence state of its manganese ions [Mn(III)], too. Its electron donation capability does not exceed the efficiency of $MnCl_2$ nor at low neither at high concentrations. Thus it can be excluded that the high electron donation capacity of M-3($L_8$), trinuclear and M-2($L_6$) and M-2($L_7$) complexes can be explained only by the valence state of manganese ions. However, this characteristics is observed only in the case of Mn(III)-containing complexes, thus it is possible, the higher valence state of the Mn-ions is indispensable for this phenomenon. According the different behaviour of M-4($L_1$), existence of other unidentified factor(s) is (are) also necessary.

The synthesized mono-, bi-, tri- and tetranuclear manganese probes were also tested in photoactivation experiments. The photosynthetic activity of the reactivated samples was measured by fluorescence induction technique as well as by continuous and flash oxygen polarography and thermoluminescence. It is important to emphasise that in these cases the restoration of the photosynthetic parameters was achieved by the incorporation of the manganese ions to PS II, not by single (exogenous) electron donation of them. The applied concentration was 100 μM of mononuclear or equivalent amounts of bi-, tri- or tetranuclear complexes (represented as arrows in Fig. 1B, 2A and 2B). Fig. 3A illustrates the incorporation of $Mn^{2+}$ followed by fluorescence induction. The photosynthetic activity was estimated by the differences of $F_v$ values of reactivated and manganese-depleted samples (marked with $F_v'$). Normalized values are

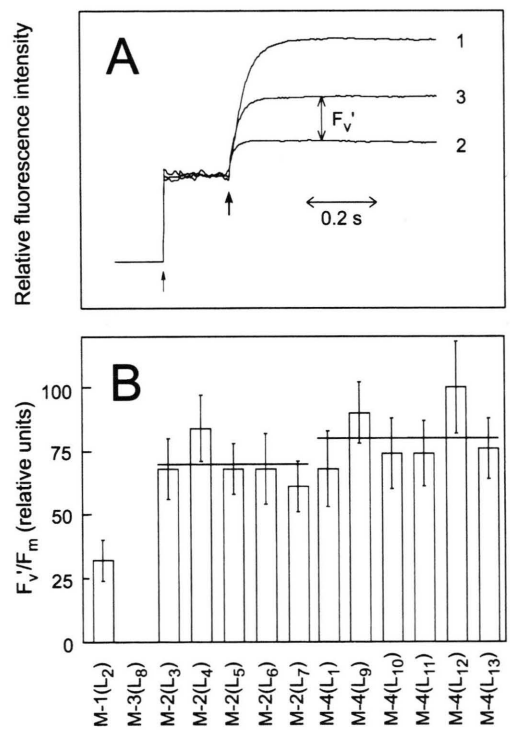


Fig. 3. Manganese depletion and subsequent photoactivation of the WOC using synthetic manganese complexes as monitored by fluorescence induction. (A) Fluorescence induction curves of (1) control thylakoids; (2) manganese-depleted thylakoids and (3) thylakoid membranes photoactivated by $MnCl_2$. Switching on the measuring- (0.1 W/m$^2$) and actinic beam (10 W/m$^2$) are indicated by thin and thick arrows, respectively. (B) Normalized $F_v'/F_m$ values of thylakoid membranes photoactivated by 100 μM $MnCl_2$ or equivalent amounts of different manganese complexes ($MnCl_2$ = 100%). The horizontal lines over the columns represent the averages of two different groups (bi- and tetranuclear complexes) of the synthetic manganese compounds. Error bars are indicated.

shown in Fig. 3B (The $F_v/F_m$ ratio is 0.24 and 0.48 in manganese-depleted and $MnCl_2$-reactivated samples, respectively). The reason of the partial restoration can be explained by partial photoinhibition during the photoactivation procedure (Rova *et al.*, 1996). [The enhanced sensitivity of manganese-depleted PS II particles against donor side photoinhibition which takes place even at low light regime was reviewed by Aro and her coworkers (1992).] As can be seen the efficiency of the synthetic complexes in the restoration of the $F_v$' values follows the M-3($L_8$) < M-1($L_2$) < binuclear complexes < tetranuclear complexes order. With one exception [M-3($L_8$) cannot restore the photosynthetic activity] the orders of the efficiency within the three groups (mono-/tri-, bi- and tetranuclear complexes) in the photoactivation is the same as in the electron donation experiments at 100 μM Mn-concentration (Fig. 1B, 2A and 2B, respectively). This cannot be considered as an obvious fact because the site of electron donation is not necessarily identical with the *in vivo* binding site of manganese. Especially, if we take into account that during photoactivation the protein structure of the lumenal side of PS II is rearranged (Tamura and Cheniae, 1987; Ananyev *et al.*, 2001). However, in Ghirardi's works (Ghirardi *et al.*, 1998; and references therein) it was suggested that the action sites of 1,5-diphenylcarbazide (DPC) photooxidation was identical with the physiological Mn-binding sites of WOC.

There are similarities between the results of electron donation and photoactivation in respect of the efficiency of Mn(II)- and Mn(III)-containing complexes in these experiments, too: the efficiencies of Mn(III)-containing complexes is lower than Mn(II)-containing ones. The possible reason of this the different chemical nature of these complexes which render a more difficult incorporation of their manganese ions into WOC. The partial or total incorporation of these complexes also cannot be excluded. In this case the modified WOC may donate electrons toward P680 with lower efficiency than the normal one. A subsequent similarity between the results of electron donation and photoactivation experiments the significant difference between M-1($L_2$) mononuclear and M-2($L_3$) binuclear complexes which contain same kind of ligands. This indicates that not only the exogenous electron donation depends on the structure of the complex but also the incorporation and/or the function of the reactivated water splitting complex.

After photoactivation the photosynthetic activity was also investigated by oxygen polarography. The relatively high yield of oxygen evolution of thylakoid membranes (Fig. 4A, curve 1) is almost completely eliminated by extraction of the four manganese ions of WOC (Fig. 4A, curve 2). Execution of photoactivation experiment by different manganese-containing compounds resulted in a partial restoration of the photosynthetic activity

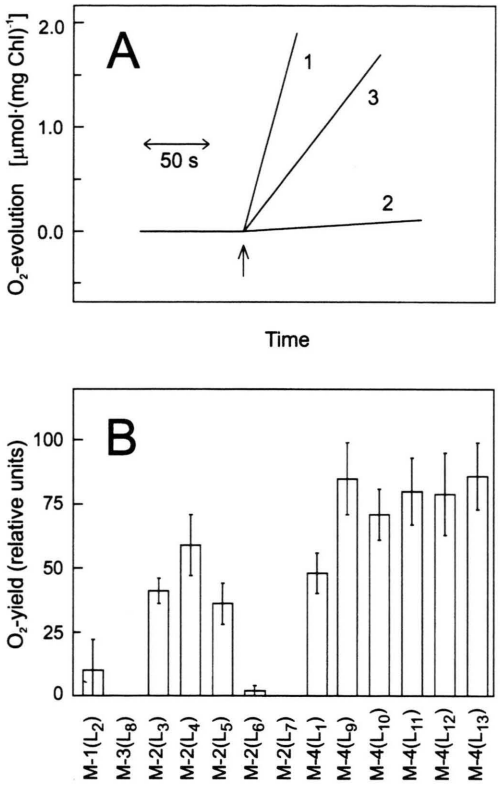


Fig. 4. Manganese depletion and subsequent photoactivation of the WOC using synthetic manganese complexes as monitored by Clark-type oxygen electrode. (A) Rate of oxygen evolution of (1) control thylakoid; (2) manganese-depleted thylakoid and (3) thylakoid membranes photoactivated by 100 μM $MnCl_2$. The arrow indicates the onset of the saturating white light. ([Chl] = 50 μM/1.5 ml). 500 μM phenyl-*p*-benzoquinone was used as electron acceptor. The $O_2$ yield of the control samples (the slope of curve 1) was 170 μmol·(mg Chl)$^{-1}$·h$^{-1}$. (B) Normalized $O_2$ yields of thylakoid membranes photoactivated by 100 μM $MnCl_2$ or equivalent amounts of different manganese complexes ($MnCl_2$ = 100% = 120 μmol·(mg Chl)$^{-1}$·h$^{-1}$). Error bars are indicated.

(Fig 4A, curve 3). Using 100 μM $MnCl_2$ up to 70% restoration could be achieved which can be considered satisfactory (Miyao and Inoue, 1991b).

Fig. 4B shows the relative restoration of the steady state oxygen evolution by the tested complexes during photoactivation (restoration by $MnCl_2$ is considered as 100%). In all cases the relative activity is slightly smaller than that obtained by fluorescence induction since oxygen evolution is a more complex phenomenon and rather *in vivo* property than fluorescence. No detectable oxygen evolution could be measured after restoration by M-3 ($L_8$) and M-2 ($L_7$) complexes and only a small one in the case of M-2($L_6$) reconstituted samples. We have tried to carry out photoactivation by these binuclear complexes at lower concentration, too, when their electron donation is more pronounced (see Fig. 1B and 2A), but the attempts were unsuccessful. The order of the restoration is almost the same as mentioned in fluorescence induction: tetranuclear manganese complexes were better than mono-, bi- and trinuclear ones. Within bi- and tetramers, complexes having only Mn(II) ions proved to be better than those (also) having Mn(III). The M-4($L_1$) complex having a tetragonal Mn setting had a lower capability in restoration the oxygen evolving properties of manganese depleted thylakoid membranes.

For getting more detailed information about the oxygen evolution of the samples polarographic signals were also detected by a Joliot-type oxygen electrode before and after photoactivation. The pattern of the control sample followed the well-known oscillation with a period of four with the first maximum at the 3rd flash (Fig. 5A, curve 1). After manganese depletion the signal was almost totally abolished (Fig. 5A, curve 2). The photoactivated samples exhibited two very different flash oxygen yield patterns (Fig. 5A, curves 3 and 4).

All of the probed synthetic tetra-, mono- and one of the binuclear complexes (Fig. 5A, curve 3) had a partial restoration with the same period four oscillation pattern. The absolute values were relatively low in comparison with the control sample (maximum 20%) due to the limited size of the plastoquinone pool. The relative values of oxygen yields obtained with this type of complexes are presented in Fig. 5B ($MnCl_2$ = 100%). With one exception [M-4($L_{11}$)] the relative flash oxygen yields were proportional with or larger than the

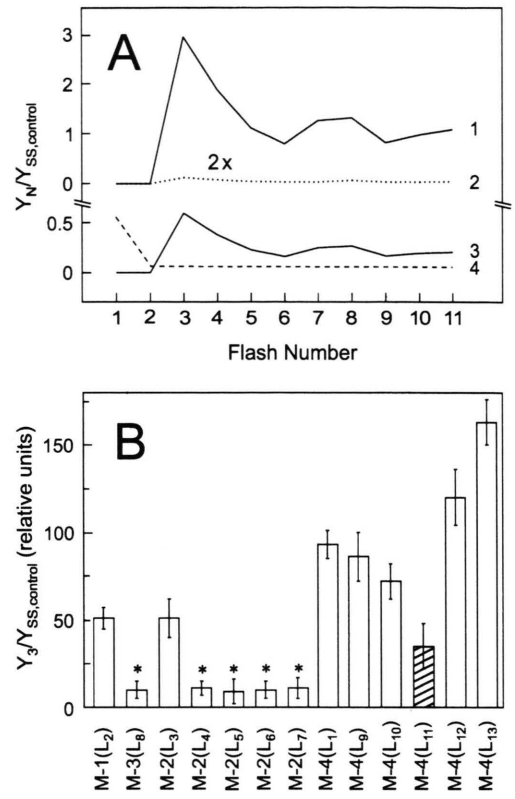


Fig. 5. Manganese depletion and subsequent photoactivation of the WOC using synthetic manganese complexes as monitored by Joliot-type oxygen electrode. (A) Patterns of oxygen evolution induced by one-turnover-flashes of (1) control thylakoid; (2) manganese-depleted thylakoid and thylakoid membranes photoactivated by (3) $MnCl_2$ and (4) M-3($L_8$). (B) Normalized $O_2$ yields of thylakoid membranes after the 3rd flashes photoactivated by 100 μM $MnCl_2$ or equivalent amounts of different manganese complexes ($MnCl_2$ = 100%). The strange flash oxygen patterns (curve 3 in panel A) are signed by stars. Since oxygen evolution of the M-4($L_{11}$)-reconstituted samples is accompanied with respiration the value of the striped column should be considered carefully. Error bars are indicated.

continuous yields (Fig. 4B). There was close correlation with the M-1($L_2$) < M-2($L_3$) < tetranuclear complexes order obtained in the fluorescence induction and steady state oxygen experiments. In the case of M-4($L_{11}$) complex, respiration was observable which distorted the results, which may be explained by the different, O-O-N-O donor atom set of the $L_{11}$ ligand, contrary to O-O-N-N set of the other cathecolimine ligands. Thus the oxygen evolution belonging to M-4($L_{11}$) complex is probably higher than that can be read from Fig. 4. It is re-

markable that in two cases [M-4($L_{12}$) and M-4($L_{13}$)] the observed activity was higher than that obtained with $MnCl_2$. This result provided indirect evidence that not only the dissociated manganese ions as in the case of $MnCl_2$ but the whole M-4($L_{12}$) and M-4($L_{13}$) complex can (partially or totally) be incorporated into WOC. Similarly, photoactivation of spinach PS II particles (depleted of extrinsic proteins) by tetranuclear complex resulted in higher (continuous) oxygen yield than by using $MnCl_2$ (data not shown). These complexes can probably produce better restoration energetically or kinetically than simple $Mn^{2+}$ but in the intact thylakoid membranes the water-soluble extrinsic proteins hinder their binding sterically. In accordance with this it has been published that the *Synechocystis* ΔpsbO mutant lacking 33 kDa manganese stabilizing protein presented higher $Mn^{2+}$ accessibility than the wide type (Burnap *et al.*, 1995).

The remaining four bi- and the investigated trinuclear complex produced a strange oxygen yield pattern without any oscillation (Fig. 5A, curve 4). These patterns show large oxygen release after the first flash. After the second flash the oxygen yield was very low and almost completely disappeared after subsequent flashes. This indicates that these reconstituted centers are trapped in stable $S_3$ state after dark incubation and one ore more subsequent S-state transition(s) ($S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$) is (are) inhibited. It has been published that oxygen produced in cyanobacteria after the first (and partially the second) flash. This oxygen release is also attributed to metastable $S_3$ redox state (Bader *et al.*, 1983; Bader, 1994). Oxygen evolution from special PS II preparations after the first flash has also been reported (Lavorel and Seibert, 1982). It is an interesting parallelism that three of the probed Mn(III) containing complexes produced such strange oxygen pattern. Thus, complexes in which the manganese ions exist in higher oxidation state can build up water splitting complex having larger stability in higher S-states. Thus, we concluded that these bi- and trinuclear complexes can be incorporated into WOC, but it cannot perform normal photosynthesis: the reconstituted WOC is modified or perturbed.

It is a remarkable that three of the complexes exhibiting unusual oxygen yield pattern [M-3($L_8$), M-2($L_6$) and M-2($L_7$)] at low concentration have higher electron donation capacity than $MnCl_2$ (Fig. 1B and 2A), but cannot exhibit steady state oxygen evolution (Fig. 4B). All of them contains Mn(III) ions. This may suggest that the valence state of Mn-ions, evidently, highly influence the redox properties of the complexes. However, role of the chemical nature of the ligands and the structure of the complexes are not to be doubt [see the strange flash oxidation patterns of the Mn(II)-containing M-2($L_4$) and M-2($L_5$) complexes or the normal pattern of Mn(III) containing M-4($L_1$) complex].

For the investigation of the redox potential changes of WOC during the photoactivation procedure TL glow curves also were recorded. Thermoluminescence and oxygen polarimetry give more reliable data about manganese binding than fluorescence measurements because the main TL bands and the $O_2$ signal can be obtained only from incorporated manganese ions. The position of a TL peak is determined by the redox distance between the interacting oxidized donor and reduced acceptor (reviewed by Vass and Inoue, 1992). Any change in the redox potential of the positively charged donor (S-state) can shift the peak position of the corresponding TL band. We investigated the effect of manganese complexes on the amplitude and peak position of the Q TL band ($S_2Q_A^-$ charge recombination) which appears in the glow curve at about 0 °C in the presence of DCMU (an inhibitor acting between $Q_A$ and $Q_B$). Fig. 6A, curve 1 and 2 represent the TL glow curve of thylakoid membranes before and after manganese depletion, respectively. Fig 6A, curve 3 represents the TL spectra of a reactivated sample. After manganese depletion (Fig. 6A, curve 2) the Q-band was considerably decreased and a new TL-band appeared at around −25 °C ($A_T$-band) which can be associated with $His^+Q_A^-$ recombination (Ono and Inoue, 1991). Reactivation of manganese-depleted thylakoid membranes by equimolar amount of $MnCl_2$ resulted in a partial reappearance of the Q-band and a total disappearance of the $A_T$-band (Fig 6A, curve 3). Similar phenomenon has been described by Tamura *et al.* (1989). The peak position of the Q-band has not changed during the reactivation processes indicating that binding of the complexes resulted in the formation of the ordinary $S_2$ state.

Fig. 6B shows the normalized ($MnCl_2$ = 100%) average values of the Q-band after reactivation by

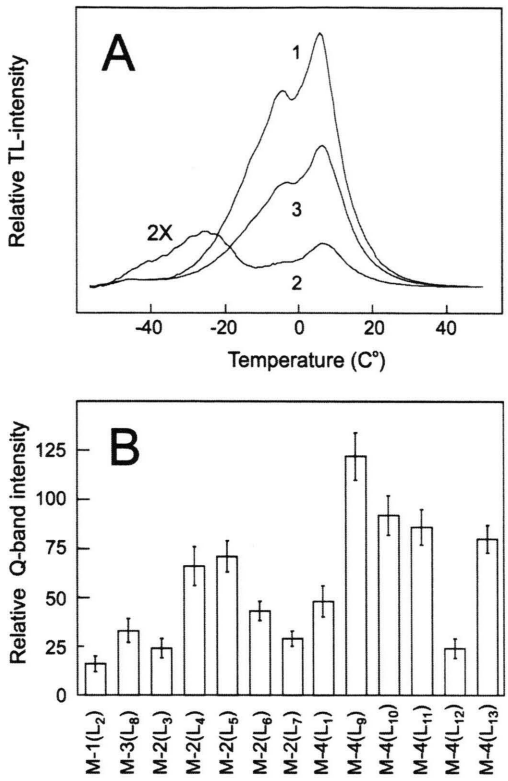


Fig. 6. Manganese depletion and subsequent photoactivation of the WOC using synthetic manganese complexes as monitored by thermoluminescence. The thermoluminescence glow curves were measured in the presence of 10 μM DCMU. (A) TL glow curves of (1) control thylakoids; (2) manganese-depleted thylakoids and thylakoid membranes photoactivated by (3) 100 μM $MnCl_2$. (B) Normalized Q-band intensity of thylakoid membranes photoactivated by 100 μM $MnCl_2$ or equivalent amounts of different manganese complexes ($MnCl_2$ = 100%). Error bars are indicated.

different manganese complexes. The maximum of the corresponding Q-band intensities [M-4($L_9$)] is about 70% of the control thylakoid amplitude, the same as the yield observed in oxygen polarographic measurements. This means that this complex were more effective in the reconstitution of the Q band than $MnCl_2$. The efficiency of photoactivation showed the same sequence (tetra- > bi- > mononuclear, trinuclear complexes) as obtained in fluorescence induction (Fig. 3) and oxygen yield (Fig. 4) measurements. Surprisingly, the Q-band could be reactivated by the binuclear and trinuclear complexes which could not restore the steady state oxygen evolution (Fig. 4B) and the period-4 flash oxygen yield pattern (Fig. 5). This indicates, that the $S_2 \rightarrow S_3$ transition was inhibited in a higher extent than the $S_1 \rightarrow S_2$ transition. This can be reconciled with the data that the same complexes proved to be very efficient donors to WOC in fluorescence induction experiments (Fig. 1B and 2A).

By comparison of the results of the fluorescence induction, polarographic and TL measurements, it can be concluded that tetranuclear complexes were more effective than mono-, bi- and trinuclear complexes in all of these types of experiments. It may indirectly indicate that all four manganese ions are required for water splitting in contradiction with the suggestions of Klimov *et al.* (1982), Saygin and Witt (1987) as well as Allakhverdiev *et al.* (1994a). High efficiency of some binuclear complexes in these experiments can be explained by the concerted action of two binuclear complexes. The very low efficiency of the M-3($L_8$) trinuclear complex supports this idea. It is important that the more efficient five tetranuclear complexes have a dimer+dimer structure resembling the natural structure of WOC.

The efficiency of the complexes in photoactivation experiments can also be compared by the types of the ligands. In most of the probed 13 complexes the manganese ions were ligated by 4–6 oxygen and 0–2 nitrogen atoms. Two exceptions are the M-1($L_2$) mononuclear complex where $Cl^-$ ions also plays role as donor atoms and the M-3($L_8$) trinuclear complex in which manganese ions have four nitrogen and two oxygen donor atoms. Both of these complexes had low efficiency at the restoration of photosynthetic functions. Thus, the donor atom set of the manganese ions may also play role in the efficiency of photoactivation. Although the amino acid residues and donor atoms participating in the binding of the manganese ions of WOC are to be answered, based on the sequence analysis of D1 and D2 reaction center proteins and on characterization of numerous point-mutants, there are several indications for them (for reviews, see Debus, 1992, 2001; Diner, 2001). Most of the donor atoms to manganese in WOC is probably oxygen, which are resistant against Mn(IV) existing in higher S-states. However there are indications that histidine residues also participate in the ligation of manganese ions. Thus, the O-N donor atom distribution of WOC and the

probed synthetic complexes are close to each other.

Thirteen synthetic manganese complexes were probed in electron donation and photoactivation experiments. They can be divided into groups by several ways: by number and valence state of central manganese ions, by donor atom distribution and structure of the complexes. By comparison of the number of the variable parameters and of the probed complexes it is evident that our conclusions were drawn by a few samples. Thus, in several cases it was impossible to decide which factor was the dominant (if several factors were changed at the same time or in the cases of types which were represented by only one or two samples). Let it be said in the excuse of the authors of this article that composition of the complexes are cannot be chosen freely and cannot be altered continuously. Probing of other synthetic manganese complexes in electron donation and photoactivation experiment will serve additional valuable information about photoactivation and structure of WOC.

*Acknowledgements*

This work was supported by the Hungarian National Science Foundation (OTKA: T 025684 and F 029144). The authors thank Drs. Stenbjörn Styring and Fikret Mamedov for critical reading of the manuscript.

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