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Reconstitution of the Water-Oxidizing Complex in Manganese-Depleted Photosystem II Complexes by Using Synthetic Binuclear Manganese Complexes[†]

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ABSTRACT: The efficiency of synthetic binuclear manganese complexes in reconstituting PS II electron flow and oxygen-evolution capacity was analyzed in PS II enriched preparations deprived of their manganese and of the extrinsic regulatory subunits. Measurements of the variable fluorescence induced by actinic illumination with continuous light led to the following results: (a) the synthetic binuclear complexes are more efficient than $MnCl_2$ in establishing a PS II electron flow; (b) an almost complete restoration is achieved at concentrations of these complexes that correspond with an overall stoichiometry of two manganese per PS II; and (c) the electron flow restored by the binuclear manganese complexes closely resembles that of normal O_2-evolving PS II preparations in its resistance to addition of 50 μM EDTA, while that supported by $MnCl_2$ is practically completely suppressed at the same chelator concentration. The rate of O_2 evolution was used as a measure of the capability to function as manganese source in reconstitution of the oxygen evolution capacity. It was found that (i) as in the case of PS II electron transport, the synthetic binuclear manganese complexes are significantly more efficient than $MnCl_2$; (ii) with respect to the manganese concentration, the maximum effect is achieved with a μ-oxo bridged binuclear Mn(III) complex (symbolized by M-3) at concentrations corresponding to four manganese per PS II; and (iii) at all concentrations of binuclear manganese complex M-3 a significantly higher restoration of the O_2 evolution rate is achieved if the reconstitution assay contains in addition the extrinsic regulatory 33 kDa protein (PS II-O protein). The implications of these data are discussed in terms of electron donation and photoligation of the functional tetranuclear cluster of the water oxidase.

The crucial process of photosynthetic water cleavage is the oxidation of two H_2O molecules to dioxygen and four protons. This process requires two indispensable prerequisites: (a) the generation of sufficiently oxidizing redox equivalents (holes) and (b) the cooperation of four holes. The former goal is achieved by light-induced electron ejection from the lowest excited singlet state of a special chlorophyll *a* species (designated as P680) with pheophytin (Pheo)[1] as primary acceptor and subsequent rapid electron transfer from $Pheo^-$ to a special plastoquinone, Q_A [for a recent review, see Renger (1992)]. The latter reaction takes place via a sequence of univalent electron abstraction steps from a manganese-containing unit, the water-oxidizing complex (WOC). Oxygen is released after the accumulation of four holes within the WOC. The redox components participating in the process of water oxidation are incorporated into an integral membrane protein complex referred to as photosystem II (PS II) which is anisotropically incorporated into the thylakoid membrane and associated with extrinsic regulatory subunits [for a review of the polypeptide pattern of PS II, see Vermaas et al. (1993)].

Although significant progress has been achieved in understanding the photosynthetic water oxidation, especially with respect to the kinetic pattern [for reviews, see Hansson and Wydrzynski (1990), Rutherford et al. (1992), Debus (1992), and Renger (1993)], key mechanistic questions still remain to be answered [for a list, see Renger (1987, 1993)]. Likewise, the structure of the water oxidase and the nature of its protein matrix are not yet resolved [for a detailed discussion, see Debus (1992)]. With respect to the latter problem, it now seems clear that a functionally competent WOC contains four manganese (Cheniae & Martin, 1970; Klimov et al., 1982a; Yocum et al., 1981; Allakhverdiev et al., 1986; Renger et al., 1989). Several lines of evidence indicate that these four manganese centers are likely not to be functionally equivalent [for a detailed discussion, see Renger (1987, 1993)]. According to previous results, only two manganese are inferred to be indispensable for the oxidative pathway leading to water oxidation to dioxygen, while the other two were reported to be replaceable under certain conditions by other divalent cations and probably play a structural role (Klimov et al., 1982a,b; Allakhverdiev et al., 1986; Allakhverdiev & Klimov, 1992). The idea of manganese heterogeneity within the WOC was elaborated on the basis of selective population of the "superreduced" states S_{-1} and S_{-2} (Messinger & Renger, 1993) and gained further support by recent structural information

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[1] Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting system; EDTA, ethylenediaminetetraacetic acid; LwOx, 2-hydroxy-1,4-naphthoquinone monoxime; M-2, complex $[Mn(III)_2(LwOx)_4(OAc)_2]$; M-3, complex $[Mn(III){-}O{-}Mn(III)(LwOx)_2(OAc)_2(H_2O)_2]$; Q_A, primary plastoquinone acceptor of PS II; PS II, photosystem II; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; WOC, water-oxidizing complex; Y_Z, redox-active tyrosine of polypeptide D1 that acts as electron donor to $P680^+$; Pheo, pheophytin.

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gathered from EXAFS data [for a recent review, see Yachandra et al. (1993)]. The EXAFS results can be consistently explained by dimer models of two binuclear manganese clusters (Kusunoki et al., 1993; Klein et al., 1993; Yachandra et al., 1993).

In thylakoids and PS II membrane fragments deprived of their manganese, the oxygen evolution capacity can be restored by a process called photoactivation (Radmer & Cheniae, 1977). A sequence of two light-driven reactions separated by a dark process leads to proper incorporation and ligation of manganese (Klimov et al., 1990; Miyao & Inoue, 1991; Tamura et al., 1991; Blubaugh & Cheniae, 1992). Recently, various manganese complexes have been synthesized and analyzed as possible models for the manganese cluster in the WOC (Christou & Vincent, 1987; Vincent & Christou, 1989; Wieghardt, 1989; Bossek et al., 1990; Pecoraro, 1992; Armstrong, 1992). Many of these complexes contain binuclear or tetranuclear manganese centers. It therefore appeared interesting to use synthetic binuclear manganese complexes for reconstitution experiments on the oxygen evolution capacity of PS II preparations lacking a functionally competent WOC. The present study describes the reconstitution of the WOC with the binuclear manganese complexes $[Mn_2(LwOx)_4(OAc)_2]$ and $[Mn_2O(LwOx)_2(OAc)_2(H_2O)_2]$, symbolized by M-2 and M-3, respectively, where LwOx is the ligand 2-hydroxy-1,4-naphthoquinone monoxime and OAc is acetate. It is shown that these compounds are more efficient than $MnCl_2$ in reconstituting O_2 evolution. The implications of these results are discussed.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves according to a procedure described by Whatley & Arnon (1963). PS II enriched preparations were obtained by treatment of chloroplasts with 0.4% digitonin and 0.15% Triton X-100 and centrifugation at 20000*g* using a method described recently (Allakhverdiev et al., 1992; Allakhverdiev & Klimov, 1992). These preparations, referred to as DT-20, exhibited O_2 evolution rates of 250–300 μmol/[(mg of Chl) h] under saturating light and in the presence of 200 μM phenyl-*p*-benzoquinone (Ph-p-BQ) plus 300 μM $K_3[Fe(CN)_6]$ as electron acceptor. Spectroscopic analyses revealed that the DT-20 preparations contained 80–100 Chl per PS II [measured via photoreduction of pheophytin in the presence of $Na_2S_2O_4$; see Klimov et al. (1982b)] and 15 500–16 000 Chl per PS I (measured as photooxidation of P700). On the basis of this data the "PS I contamination" of the DT-20 preparations was estimated to be below 1%.

To extract manganese and the three extrinsic regulatory proteins with apparent molecular masses of 33, 24, and 18 kDa (on the basis of their encoding genes these polypeptides are now designated as PS II-O, PS II-P, and PS II-Q proteins, respectively; for a review, see Vermaas et al. (1993)] from DT-20 preparations, the following procedure was used: Samples at 200 μg/mL Chl were incubated for 10 min at 2 °C in a suspension containing 20 mM *N,N,N′,N′*-tetramethylethylenediamine (TEMED), 0.5 M $MgCl_2$, and 20 mM MES–NaOH, pH = 6.5 [see Ananyev et al. (1992)]. After centrifugation at 20000*g*, the pellet was washed twice in a buffer solution of 35 mM NaCl and 20 mM Tris-HCl, pH = 8.0. Photoactivation of the DT-20 samples deprived of their oxygen-evolving complexes was performed in the presence of different manganese-containing compounds and three or four cycles of continuous light ($\lambda > 600$ nm, $I = 55$ W m^{-2}, illumination of 30–40-s periods separated by 30–40 s of dark). The details of this procedure were described previously (Allakhverdiev & Klimov, 1992).

The chlorophyll content of the samples was determined according to Arnon (1949). The manganese content was assayed with an AAS-1 flame atomic absorption spectrophotometer (Carl Zeiss Jena), and the polypeptide composition was measured via SDS–polyacrylamide gel electrophoresis as described in Allakhverdiev & Klimov (1992).

A single-beam differential spectrophotometer with a phosphoroscope similar to that described previously (Klimov et al., 1982b; Allakhverdiev et al., 1992) was used for monitoring (i) the light-induced changes of the fluorescence quantum yield at 682 nm and (ii) the photoreduction of pheophytin at 650 nm. The suspension for fluorescence measurements contained differently treated DT20 samples at 10 μg/mL Chl, 10 mM NaCl, 2 mM $MgCl_2$, and 20 mM Tris-HCl, pH = 7.8.

The rate of oxygen evolution was measured with a Clark-type electrode in a 3-mL cell. The sample was illuminated by red light (KC 11 filter) passed through a heat filter consisting of a 5% $CuSO_4$ solution (light intensity at the cell surface ~ 100 W m^2). The assay mixture contained DT-20 preparations suspended in a buffer solution of 10 mM NaCl, 5 mM $CaCl_2$, 300 mM sucrose, 25 mM MES–NaOH, pH = 6.5, and 200 μM Ph-p-BQ/300 μM $K_3[Fe(CN)_6]$ as electron acceptor.

The binuclear complexes used in this study were synthesized as follows: Ligand LwOx (2-hydroxy-1,4-naphthoquinone monoxime) was prepared according to the method described previously (Rane et al., 1990). The synthesis and characterization of the manganese complexes were carried out according to procedures described by Khan (1993). The thermochromic manganese complex M-2 was obtained via a modified procedure of Charles (1963) by cooling the reaction mixture for 2 weeks at about –50 °C, while the complex M-3 was prepared at 0 °C following a modified procedure of Wieghardt et al. (1985).

RESULTS

In order to characterize the functional state of the samples reconstituted with different manganese compounds, comparative measurements were performed with control and TEMED/$MgCl_2$-treated DT-20 preparations using different analytical tools.

Fluorescence Measurements. Figure 1 shows typical traces of the relative fluorescence quantum yield enhancement induced by illumination of different DT-20 preparations with actinic light. In control samples a pronounced increase of the fluorescence yield is observed due to the light-induced reduction of Q_A. The extent of ΔF amounts to a 3–4-fold increase over the initial value, F_0 (see Figure 1, trace 1). ΔF remains virtually invariant after addition of 50 μM EDTA to the control sample (see dotted curve). This finding indicates that the chelator at this concentration does not affect the electron transport at the donor side of PS II; i.e., the binding of the functional manganese to the protein is resistant to complexation by external EDTA [for EDTA effects, see Wydrzynski & Renger (1986)]. A markedly different pattern arises in preparations deprived of their oxygen evolution capacity due to loss of manganese by the TEMED treatment. In this case ΔF is largely reduced (Figure 1, trace 2) because of the very limited electron donor capacity. Addition of small amounts of $MnCl_2$ corresponding to about two Mn^{2+} per PS II caused a significant restoration of ΔF (up to about 50% of the control value) as is shown in trace 3 of Figure 1. An almost complete recovery of ΔF can be achieved by addition of more than four

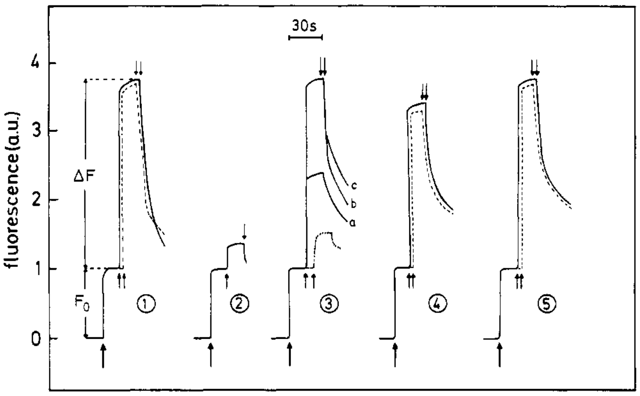

FIGURE 1: Time course of fluorescence changes, ΔF, induced by illumination of different types of DT-20 samples with actinic light. The assay suspension contained PS II preparations (10 μg/mL chlorophyll), 35 mM $MgCl_2$, and 25 mM Tris-HCl, pH = 7.8. Trace 1, control DT20 in the absence (solid line) and presence (dashed curve) of 50 μM EDTA; trace 2, manganese-depleted DT20; trace 3, the same as trace 2 with addition of 0.2 (a), 0.4 (b), and 0.8 μM $MnCl_2$ (c) corresponding to two, four, and eight Mn per PS II, respectively, in the absence (solid line) and presence (dashed curve) of 50 μM EDTA; trace 4, the same as trace 2 with addition of 0.1 μM complex M-2 (corresponding to two manganese per PS II) in the absence (solid line) and presence (dashed line) of 50 μM EDTA; trace 5, the same as trace 2 with addition of 0.1 μM complex M-3 (corresponding to two manganese per PS II) in the absence (solid line) and presence (dashed curve) of 50 μM EDTA. The arrows at the bottom indicate the switching on of the modulated measuring light beam ($\lambda \approx 480$ nm, intensity ≈ 0.15 W m^{-2}), arrows in the middle and at the top symbolize the switching on and off, respectively, of the actinic light ($\lambda > 600$ nm, intensity ≈ 100 W m^{-2}).

Mn^{2+} per PS II (trace 3 of Figure 1). However, in marked contrast to the control sample, the Mn^{2+}-mediated ΔF is highly susceptible to inhibition by EDTA (see Figure 1, trace 3, dotted curve). The experiments were performed without the preillumination with actinic light required for oxidative photoligation. Therefore, the EDTA effect shows that Mn^{2+} acts as electron donor of PS II without being tightly ligated by the protein matrix. A surprisingly different feature arises when instead of $MnCl_2$ the dimeric manganese complexes M-2 and M-3 are used for reconstitution of ΔF. In this case, a high recovery can be achieved with only one molecule of the manganese complex per PS II, where compound M-3 is more efficient than M-2 (compare traces 4 and 5 of Figure 1).

Apart from the high efficiency, however, the most interesting phenomenon is the finding that EDTA virtually does not affect the extent of ΔF increase due to addition of M-2 and M-3 (see dotted curves of traces 4 and 5 of Figure 1). This behavior, closely resembling that of PS II preparations with a functionally competent manganese cluster of the water oxidase, strikingly differs from the action of $MnCl_2$. Accordingly, compounds M-2 and M-3 very likely bind as binuclear manganese complexes to TEMED/$MgCl_2$-treated DT-20 preparations thereby permitting an electron transport without getting disrupted by the exogenous chelator EDTA. Furthermore, these results with M-2 and M-3 also support the previous conclusion that in DT-20 preparations two manganese per PS II is sufficient to sustain photoinduced electron transfer (Klimov et al., 1982a; Allakhverdiev et al., 1986).

Oxygen Evolution. In TEMED/$MgCl_2$-treated DT-20 preparations the oxygen evolution capacity is virtually completely eliminated as shown in the bottom trace of Figure 2. A partial restoration of the activity can be achieved by photoactivation with $MnCl_2$. A more pronounced effect was obtained in the presence of the binuclear manganese complexes M-2 and M-3. A comparison of the traces in Figure 2 reveals that the initial rate of O_2 evolution in the presence of two molecules of M-3 per PS II, i.e., four manganese/PS II, is 5–6 times higher than that of samples reconstituted with 10 $MnCl_2$ per PS II. The rate can be further enhanced if in addition the PS II-O protein is used for the reconstitution of the O_2 evolution activity. The traces in Figure 2 additionally exhibit a strong bending. At first glance, one might interpret this phenomenon as a "photoinhibition"-type decline of the oxygen evolution capacity. This, however, is not the case. As can be seen, after the actinic light is turned off a marked O_2 uptake takes place. It is therefore concluded that during the illumination an increasing O_2 uptake is superimposed on the O_2 evolution, leading to a truncation of the signal. The origin of this phenomenon remains to be elucidated in future studies.

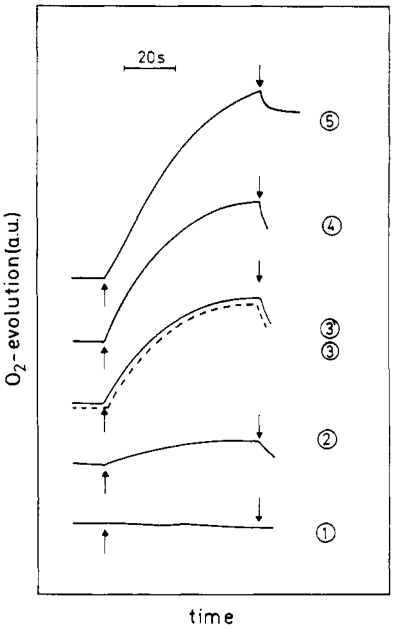

FIGURE 2: Polarographic traces of oxygen evolution in DT20 preparations deprived of their manganese and extrinsic polypeptides and reactivated under different conditions. The assay suspension contained DT-20 samples (20 μg/mL chlorophyll), 10 mM NaCl, 5 mM $CaCl_2$, 300 mM sucrose, 50 μM EDTA, 25 mM MES–NaOH, pH = 6.5, and 0.2 mM Ph-p-BQ/0.3 mM $K_3[Fe(CN)_6]$ as electron acceptor. Trace 1, Mn-depleted DT20 sample; trace 2, Mn-depleted DT20 sample reconstituted with 2 μM $MnCl_2$ (corresponding to 10 manganese per PS II); trace 3, Mn-depleted DT20 sample reconstituted with 0.4 μM complex M-3 (corresponding to four manganese per PS II); trace 3′, the same as trace 3 but without $CaCl_2$ in the assay medium; trace 4, Mn-depleted DT20 sample reconstituted with 0.4 μM complex M-3 and in the presence of the PSII-O protein; trace 5, untreated control DT 20 sample. The following O_2 evolution activities [in (μmol of O_2)/(mg of Chl) h] were obtained: trace 5, 250–300; trace 4, 200–250; traces 3 and 3′, 160–180; trace 2, 40–50; trace 1, <10.

The effect of compound M-3 and the PS II-O protein on the reconstitution was analyzed in more detail. The results obtained are presented in Figure 3. The data reveal two interesting phenomena: (i) the maximum effect in terms of the manganese stoichiometry is already achieved at a ratio of four Mn/PS II i.e., a further increase of the M-3 concentration does not lead to higher O_2 evolution rates in the reconstituted samples), and (ii) addition of the PS II-O protein leads to a marked stimulatory effect at all concentrations of M-3. Under optimum conditions more than 80% of the control activity can be restored in the presence of M-3 and the PS II-O protein. The implications of these findings will be outlined in more detail in the Discussion.

Another cofactor of a functionally competent water oxidase is Ca^{2+} [for reviews, see Rutherford et al. (1992) and Debus

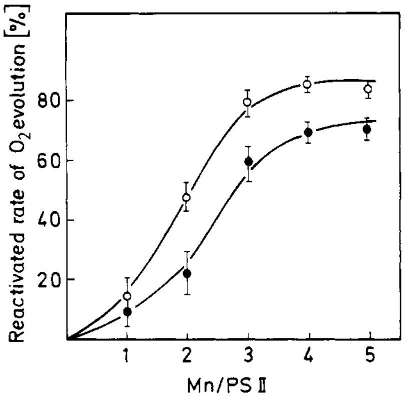

FIGURE 3: Oxygen evolution rate of Mn-depleted DT20 samples as a function of the concentration of complex M-3 in the reconstitution medium in the absence (●) and presence (○) of the PS II-O protein. Assay conditions were as described in Figure 2.

(1992)]. Therefore, its role for the photoligation of M-3 appears worth analyzing. In previous studies it was shown that the reconstitution of O_2 evolution in DT-20 preparations lacking manganese and the extrinsic regulatory proteins required not only $MnCl_2$ (or other manganese-containing substances) but also $CaCl_2$ at comparatively high concentrations (20–80 mM) (Klimov et al., 1987, 1990). In order to check for a possible role of Ca^{2+} in the case of using M-3 as manganese source, comparative experiments were performed in samples containing two molecules of M-3/PS II and two PS II-O proteins/PS II together with 5–50 mM $CaCl_2$. The extent of restoration of the O_2 evolution capacity did not exceed that of samples lacking Ca^{2+} during the reactivation process (data not shown). Accordingly, Ca^{2+} is not required for the photoligation of the dimeric manganese complex M-3. Normally, the assay medium for measuring the oxygen evolution contained Ca^{2+} at concentrations of 5 mM. This might be sufficient to satisfy a Ca^{2+} demand in O_2 evolution under these conditions. In order to check for this possibility, the same experiment was performed with the M-3-reconstituted sample in the absence of Ca^{2+}. No significant differences were observed, as shown by the dotted curve in Figure 2. Accordingly, a comparatively high Ca^{2+} concentration is not required for O_2 evolution in samples photoactivated with M-3. However, it has to be emphasized that a special treatment for Ca^{2+} depletion was not applied. Therefore, a small amount of Ca^{2+} bound to the PS II in the preparation could satisfy a low Ca^{2+} demand.

DISCUSSION

The results of the present study reveal that the binuclear manganese complexes are highly efficient PS II electron donors and powerful substances for establishing inside the protein matrix a manganese cluster that is fully competent for photosynthetic water oxidation.

With respect to the function as PS II donor, it is well established that Mn^{2+} can satisfy the necessary conditions. However, this donor capacity of Mn^{2+} is eliminated by complexation with EDTA as shown by the fluorescence data presented in Figure 1. With respect to the EDTA effect it is important to note that this chelator suppresses the typical six-line EPR signal of manganese released due to inactivation of the WOC (Wydrzynski et al., 1990). This indicates that practically all Mn^{2+} is complexed probably in the form of $[Mn(EDTA)]^{2-}$. Accordingly, the EDTA effect can be explained by two different underlying mechanisms: (i) a structural constraint and/or (ii) a modification of the energetic parameters (redox potential, reorganization energy) that dominate the electron transfer [for a review, see Marcus & Sutin (1985)]. In the former case, the Mn^{2+} complexes with EDTA could be prevented for steric and/or electrostatic reasons from binding at the site of oxidation. Steric effects of PS II donors on the fluorescence rise have been previously observed in Tris-washed thylakoids for bulky derivatives of hydroxylamine and hydrazine (Radmer, 1983). The order of efficiency of these compounds to shift the oscillation pattern of flash-induced oxygen evolution in dark-adapted thylakoids was used in an attempt to study the topology of the water-oxidizing site (Radmer, 1983). The idea of a steric and/or an electrostatic hindrance also would be in line with the latest studies in mutants from *Synechocystis* sp. PCC 6803. It was shown that the capability of manganese to act as donor to Y_Z^{ox} is markedly affected by structural constraints [see Chu et al. (1994) and references therein). However, as shown in this study, the size of the manganese complexes per se is obviously not the dominating factor because the binuclear manganese complexes M-2 and M-3, with ligands of significant size, exhibit a more efficient donor capacity than hydrated Mn^{2+}. The inefficiency of EDTA in preventing the effect of the donor function of these complexes (M-2, M-3) indicates that their ligand binding appears to be rather strong. Therefore, it has to be concluded that the site of manganese oxidation is able to interact with complexes containing comparatively bulky ligands. On the basis of these findings, it seems more likely that the inefficiency of Mn^{2+}–EDTA complexes to act as PS II electron donor relates to changes of energetic parameters. Alternatively, it could also be possible that the accessibility of the site of oxidation of manganese complexes depends not only on the total size of these species but also on their hydrophobic properties (vide infra).

As an extension of these ideas, the protein matrix also seems to be surprisingly flexible in accepting comparatively bulky complexes like M-2 and M-3 as manganese source for photoligation and establishing a functional competent water oxidase. These compounds are markedly more efficient than Mn^{2+} in photoactivation of the oxygen evolution capacity (see Figure 2). Therefore, it is inferred that the complex M-3 (also M-2) is taken up by PS II as a preformed binuclear cluster. The aromatic ligand probably favors the transport into the neighborhood of the redox-active tyrosine Y_Z and the binding domain of the WOC. This idea is supported by previous findings. It was shown that the highly lipophilic anion tetraphenylboron (TPB^-) acts as a powerful PS II donor that competes with very high efficiency with water as the natural PS II donor without destroying the functionality of the WOC. The oxygen evolution recovers to a significant extent after irreversible oxidation of TPB^- (Erixon & Renger, 1973). Likewise, some secondary amines with rather bulky aromatic substituents and an acidic NH group are extremely efficient catalysts of the S_2 and S_3 decay in an intact WOC [ADRY effect; see Renger (1972), Renger et al. (1973), and Hanssum et al. (1985)]. The concentrations required to elicit a significant effect are several orders of magnitude below those that are necessary for reducing the WOC by hydrophilic redox-active amines of small molecular size [NH_2OH, NH_2-NH_2; see Messinger et al. (1991) and references therein]. On the basis of these considerations, it is inferred that the ligand LwOx facilitates the interaction with the PS II donor side. The aromatic ligands (LwOx) are probably stripped off only inside the PS II complex and substituted by amino acid residues of the matrix. It seems likely that the binuclear core is not disrupted and becomes incorporated as an entity. Furthermore, the Mn(III) valence state of the manganese in complex M-3 is probably also in support of a suitable ligation that eventually leads to a functionally competent WOC. In this respect it is interesting to note that in the intact WOC of the dark-relaxed redox state S_1 one of the two binuclear groups is assumed to attain the oxidation state Mn(III) Mn(III) (Kusunoki et al., 1993; Yachandra et al., 1993).

The titration curve presented in Figure 3 indicates that a maximum level of restoration is achieved at four manganese per PS II. This finding provides an independent line of evidence for a tetranuclear manganese cluster forming the core of an intact WOC. In addition, the data of Figure 3 also indicate that the extrinsic PS II-O protein is essential in achieving maximum activity of O_2 evolution. On the other hand, the presence of this protein in the reconstitution assay does not affect the manganese stoichiometry. It is therefore concluded that the PS II-O protein plays an essential role in regulating the reaction pathway in the WOC.

In summary, the present study shows that synthetic binuclear manganese complexes provide an invaluable complementary tool to analyze the assembly of the water-oxidizing complex. Experiments are in progress to analyze this process in more detail.

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