Effect of extraction and re-addition of manganese on light reactions of photosystem-II preparations

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1. INTRODUCTION

Manganese plays an important role in photosynthetic oxidation of H_2O (reviews [1–3]). Reaction centers (RC) of photosystem II (PS II) carry out successive 4-step oxidation of a special (Mn-containing) enzymatic system which in turn oxidizes H_2O [1–3]. The minimal quantity of Mn necessary for O_2 evolution is 5–6 atoms/400 chlorophyll (chl) molecules or /1 RC of PS II [1–4]. The greater part (~2/3rds) of this Mn is 'loosely bound' and can be easily extracted by alkaline Tris, NH_2OH, Triton X-100 or by heating, and the extraction leads to inhibition of O_2 evolution and associated light reactions of PS II [1–10]. 'Firmly bound' Mn (~1/3rd of the pool) which remains in PS II after the extraction procedures seems not to be required for electron transport in PS II [4]. However, up to 70% of Mn can be removed from chloroplasts without essential loss of their ability to evolve O_2 [11]. Reported characteristics of EPR spectra of Mn in chloroplasts [12–15] may indicate participation of either 4 or 2 atoms of Mn in PS II reactions.

In [10] we described a procedure for 'gentle' extraction of practically all (>99%) Mn from PS II preparations after which up to 80% of activity in the oxidizing side of PS II (evaluated from photo-induced changes of chl fluorescence related to photoreduction of the primary electron acceptor of PS II, Q) can be restored by addition of $MnCl_2$ at extremely low concentrations (0.1–0.2 μM in the absence of Mg^{2+} and 0.05–0.1 μM in the presence of Mg^{2+} or any other divalent cation of metals, M^{2+}). New results from a thorough investigation of these effects reported here show that activity of the Mn-containing system in the donor side of PS II requires 4 Mn atoms, 2 of which can be replaced by either Mg^{2+} or some other divalent metal ions (M^{2+}).

2. MATERIALS AND METHODS

Subchloroplast particles (DT-20) enriched in PS II were prepared by treatment of pea chloroplasts with digitonin (0.4%) and Triton X-100 (0.1%) followed by centrifugation (30 min at 20 000 × *g*) as in [16]. Content of PS II in the particles determined from photoreduction of pheophytin (Pheo), the intermediary electron acceptor of PS II [17], was 1 RC/200–220 chl molecules [10]. Hill reaction rate evaluated from both photoreduction of 2,6-dichlorophenol indophenol (DPIP) and O_2 evolution in DT-20 was 10–20% of that observed in chloroplasts [10,16].

The partial (60–80%) extraction of Mn from PS II was carried out by the widely-used method of washing in 0.8 M Tris–HCl (pH 8.0) at 2°C [4,5] as in [10]. The complete (>99%) extraction of Mn from DT-20 was reached as in [10]. DT-20 particles at 50 μg chl/ml were incubated for 1 h at 2°C in the medium containing 1 M Tris–HCl (pH 8.0) as well as 0.5 M $MgCl_2$ (applied sometimes for 'displacement' of Mn in chloroplasts [1,8]). Then the DT-20 particles were precipitated at 20 000 × *g*. The pellet was washed twice, by resuspending to

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10 μg chl/ml and centrifugation, first in 0.8 M Tris–HCl (pH 8.0) then in a medium containing 20 mM Tris–HCl (pH 8.0) plus 35 mM NaCl. The latter medium was used in all measurements of PS II activity, adjusted to 10 μg chl/ml.

Photoreduction of DPIP as well as photoinduced changes of chl fluorescence (ΔF) related to photoreduction of Q and Pheo, measured in a phosphoroscopic set-up as in [9,10,16], were used as tests for PS-II activity. The content of Mn in DT-20 particles (at 1 mg chl/ml) was determined using an atomic-absorption spectrophotometer AAS-1N (Karl Zeiss, Jena) [10].

3. RESULTS

3.1. *Photoreduction of Q*

Illumination of untreated DT-20 particles by actinic light induces an increase in chl fluorescence yield (related to photoreduction of Q [18]) by a factor of 2.5–3 and the effect does not depend on the presence of Mn^{2+} or other M^{2+} in the medium (fig.1A). (In chloroplasts the fluorescence is increased by a factor of 3–3.5 upon illumination.) The complete extraction of Mn (see section 2) is accompanied by a 15–20-fold decrease of the photoinduced ΔF while the 'constant' component of fluorescence is not affected (fig.1B). Subsequent addition of the cations Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+} (fig.1B) as well as Ba^{2+}, Ca^{2+}, Zn^{2+} (not shown) at 3–150 μM does not influence the photoinduced ΔF, while addition of Mn^{2+} leads to an almost complete restoration of the ΔF (up to 85% of the ΔF-value observed in untreated DT-20). If $MnCl_2$ is added to the totally extracted DT-20 at 0.05–0.08 μM (when the reactivation is negligible), subsequent addition at all the M^{2+} mentioned above leads to considerable activation of ΔF (fig.1C). Upon addition of dithionite (10 mM) to the extracted DT-20, fluorescence increases in the dark up to the level which is reached upon illumination in the samples reactivated by Mn^{2+}. These data as well as activation of the ΔF by ascorbate (10 mM) or by NH_2OH (1 mM) confirm that removal of Mn leads to inactivation namely of the donor side of PS II.

Reactivation of ΔF by any M^{2+} (including Mn) is also seen after the partial (see section 2) extraction of Mn resulted in a 6–8-fold inhibition of ΔF (fig.1D). The monovalent cation Na^+ at up to 0.35 M did not influence the reactivation of ΔF by $MnCl_2$ after removal of Mn.

Fig.1E shows dependence of reactivation of ΔF in the totally depleted DT-20 on $MnCl_2$. The most important facts here are:

(1) Maximal reactivation is observed at extremely low [$MnCl_2$] (0.2 μM) when ≤ 4 Mn atoms/RC are added to the medium;

(2) Addition of Mn^{2+} jointly with Mg^{2+} (fig.1E,○) or with other M^{2+} (not shown) leads to a shift of the dependence to lower [$MnCl_2$] (0.05–0.1 μM) when there are ≤ 2 Mn atoms/RC in the medium.

Fig.1E also shows that the experimental dependences of reactivation of ΔF on concentration of Mn^{2+} added alone or jointly with Mg^{2+} coincide with theoretically predicted dependences for the cases when reactivation of ΔF requires 'filling' of a 4-atomic or a 2-atomic Mn-containing center, respectively.

3.2. *Photoreduction of Pheo*

At a redox potential (E_h) established near −450 mV when Q is reduced in the dark, illumination of PS-II preparations induces a reversible photoreduction of the intermediary electron acceptor of PS II, Pheo, located between P680 and Q (see [17]). This photoreaction can be monitored by a negative photoinduced ΔF (which is actually the disappearance of nanosecond recombination luminescence due to photoaccumulation of the state [P680 $Pheo^{-\cdot}$]$Q^{-\cdot}$ (see [17]). The negative ΔF effect is inhibited upon the total removal of Mn from DT-20 particles (fig.2A); the capability to exhibit a negative ΔF is restored by addition of $MnCl_2$ at 0.2 μM (if added alone) or at 0.1 μM (if added jointly with 3 μM $MgCl_2$). The dependence of restoration of the Pheo photoreduction on [$MnCl_2$], added alone or together with $MgCl_2$ (fig.2B), is close to that observed for photoreduction of Q (fig.1E). In accordance with [9], reactivation of the negative ΔF needs much higher [$MnCl_2$] ($\sim$400 μM) if it is added to the extracted particles after addition of dithionite (fig.2A).

3.3. *Photoreduction of DPIP*

The complete extraction of Mn from DT-20 particles results in a 30–50-fold decrease of the Hill reaction rate monitored by photoreduction of DPIP in the absence of exogenic electron donors

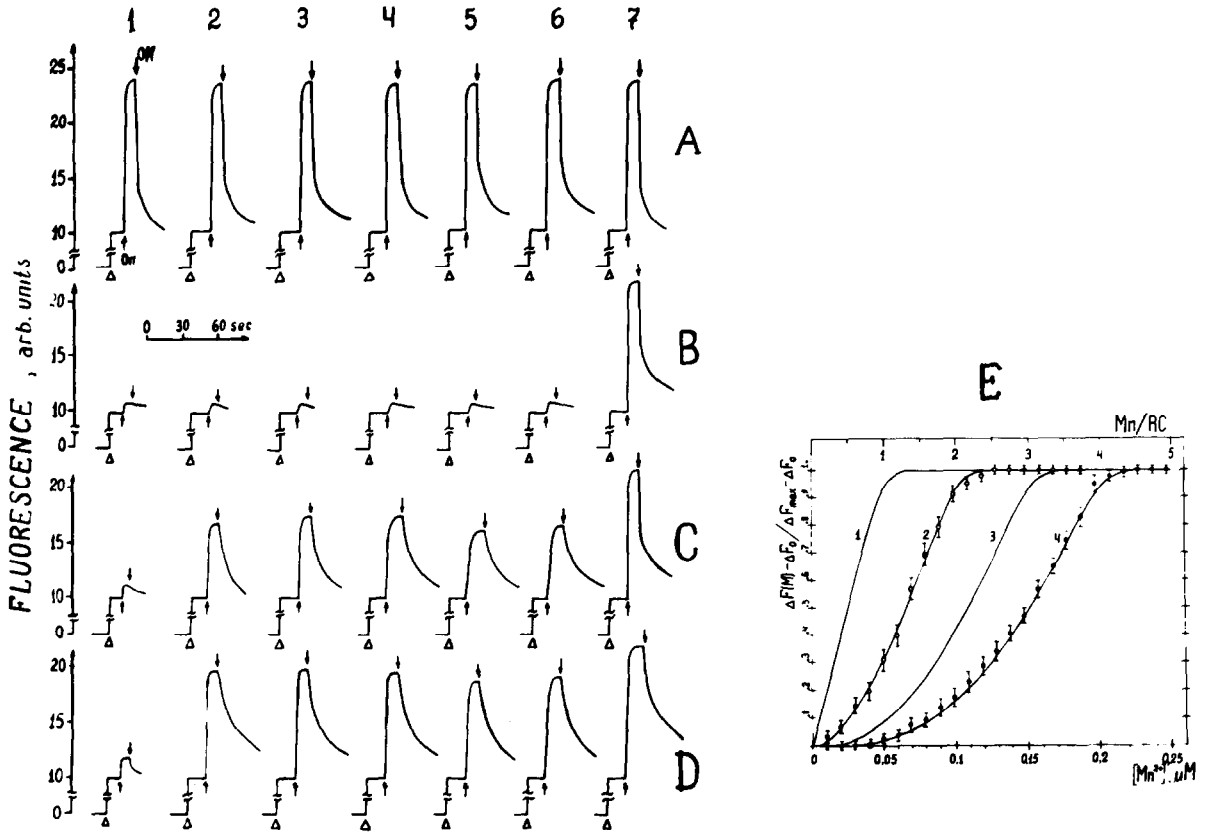

Fig.1. (A–D) Kinetics of photoinduced ΔF in DT-20 particles before (1) and after (2–7) addition of 3 μM cations: Mg^{2+} (2), Co^{2+} (3), Ni^{2+} (4), Fe^{2+} (5), Cu^{2+} (6) and Mn^{2+} (7); (A) untreated DT-20; (B,D) after the complete and partial (section 2) extraction of Mn, respectively; (C) after addition of 0.07 μM $MnCl_2$ to completely extracted DT-20. Measuring light (480 nm, ~100 erg . cm^{-2} . s^{-1}) exciting Chl fluorescence ($\lambda > 660$ nm), on (△); actinic light ($\lambda > 600$ nm, 0.5 . 10^5 erg . cm^{-2} . s^{-1}) on (↑) and off (↓) (20°C); (E) Magnitude of photoinduced ΔF in completely extracted particles as a function of [$MnCl_2$] added to the medium in the absence (•) and in the presence (○) of 3 μM $MgCl_2$. (Measurements were made after 10-min incubation with the added salts; $MgCl_2$ at 3 μM (which was saturating for the effect) was added after addition of corresponding concentration of $MnCl_2$.) The 2 experimental curves are the results of an average of 15 expt. done as in fig.1B. The height of each vertical bar corresponds to the average statistic error for average value of measured ΔF. ΔF, ΔF_{max} and ΔF (M) are ΔF values observed before addition of Mn^{2+}, after maximal reactivation by Mn^{2+} and at given [Mn^{2+}], respectively. Curves (1–4) are theoretically expected dependences for the cases when restoration of a Mn-containing center in the oxidizing side of PS II requires specific binding of 1, 2, 3 or 4 atoms of Mn, respectively [19]. The dependence was calculated [19] assuming that Mn is bound practically irreversibly [10] and that binding constants for each Mn atom in the Mn-containing cluster are nearly the same.

(fig.3). The photoreaction recovers up to 85% of the control after addition of either 0.2 μM $MnCl_2$ or 0.1 μM $MnCl_2$ added jointly with 3 μM $MgCl_2$. Separate addition of 0.1 μM $MnCl_2$ or 3 μM $MgCl_2$ does not result in such reactivation (fig.3A). Dependence of this restoration on [Mn^{2+}] (added alone or together with 3 μM $MgCl_2$) is quite similar to that observed for ΔF (fig.3B).

3.4. *Oxygen evolution*

Preliminary experiments have shown that some restoration by Mn^{2+} is also seen for photoinduced oxygen evolution measured in the completely extracted DT-20 in the presence of 1 mM ferricyanide or 50 μM DPIP. However, the reactivation effect was much lower than that for photoreduction of Q, Pheo and DPIP (probably due to inter-

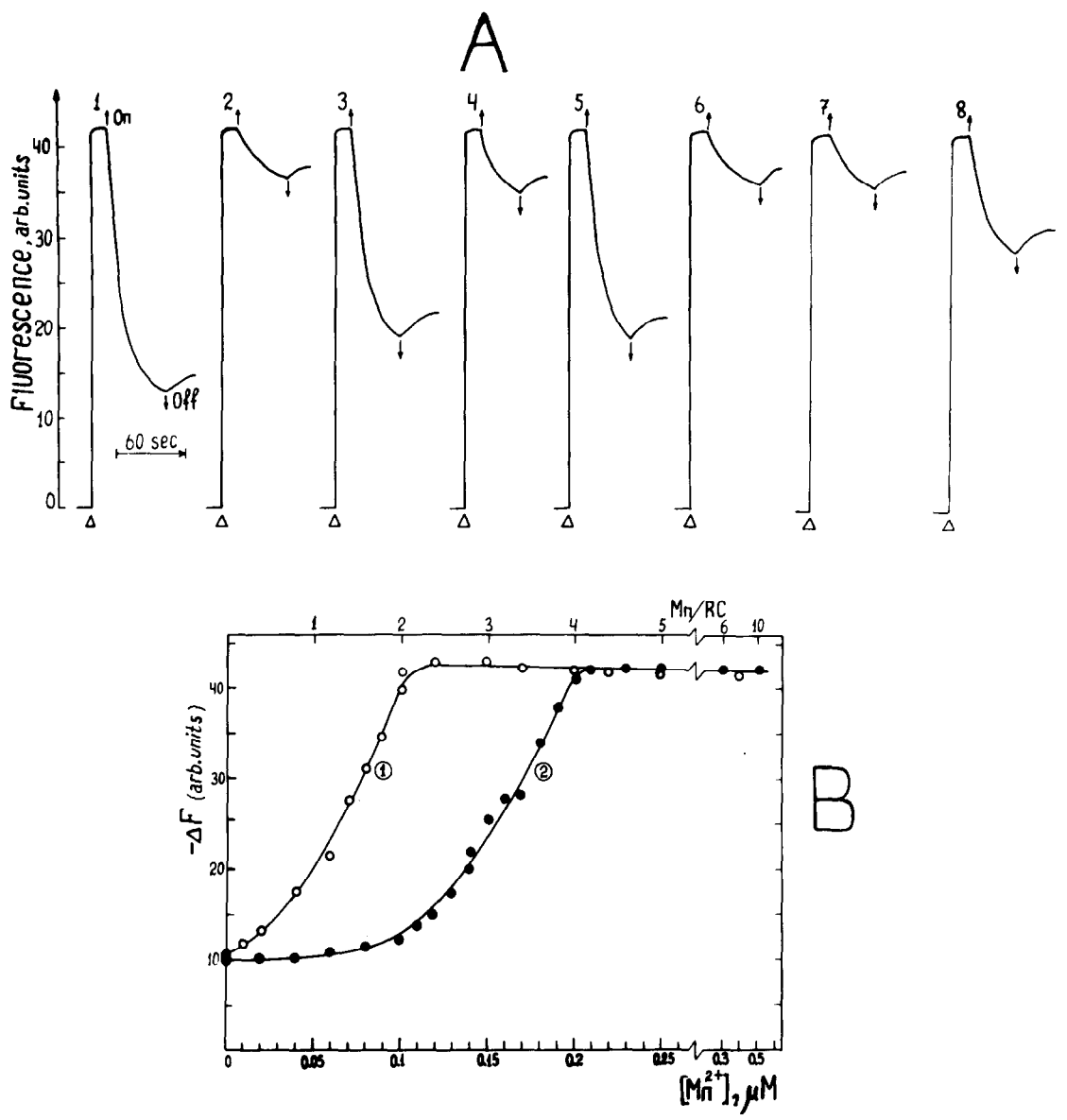

Fig.2. (A) Kinetics of photoinduced negative ΔF, related to photoreduction of Pheo, at $E_h \approx -450$ mV in: untreated (1); completely extracted (2–8) DT-20 particles with no additions (2); and in the presence of 0.2 μM $MnCl_2$ (3), 0.1 μM $MnCl_2$ (4), 0.1 μM $MnCl_2$ plus 3 μM $MgCl_2$ (5), 3 μM $MgCl_2$ (6) added before addition of dithionite, and in the presence of 0.2 μM (7) and 400 μM (8) $MnCl_2$ added after addition of dithionite. Measuring light (480 nm, ~ 100 erg . cm^{-2} . s^{-1}) on (△); actinic light ($\lambda > 600$ nm, 1.2 . 10^5 erg . cm^{-2} . s^{-1}) on (↑) and off (↓) (20°C). (B) Dependence of magnitude of the negative photoinduced ΔF, related to photoreduction of Pheo, at $E_h \approx -450$ mV during the first 30 s illumination in completely extracted DT-20 particles (see fig.3A) on concentration of $MnCl_2$ added to the medium alone (•) or jointly with 3 μM $MgCl_2$ (○).

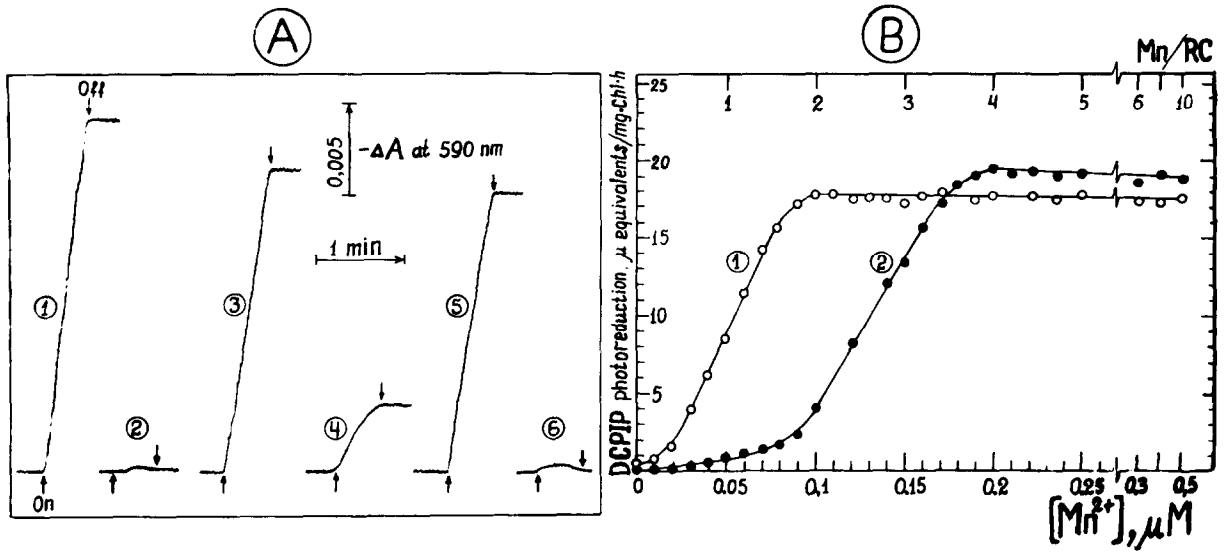

Fig.3. (A) Kinetics of photoreduction of DPIP in untreated (1), in completely extracted (2–6) DT-20 particles with no additions (2), and after addition of 0.2 μM $MnCl_2$ (3), 0.1 μM $MnCl_2$ (4), 0.1 μM $MnCl_2$ plus 3 μM $MgCl_2$ (5), 3 μM $MgCl_2$ (6) (20°C). (B) Dependence of the rate of DPIP photoreduction in completely extracted DT-20 particles on [$MnCl_2$] added alone (•) or jointly with 3 μM $MgCl_2$ (○).

ference with the effect of photoinduced oxygen consumption which was much higher in the extracted than in untreated DT-20.

3.5. *Content of Mn*

Determination of Mn content in DT-20 samples used in the experiments has shown that they contain 3.4±0.5 Mn atoms/1 RC of PS II. The partial and complete extraction of Mn results in a decrease of the ratio Mn/RC to ~0.7 and <0.02, respectively. Addition of 0.2 μM $MnCl_2$ to the totally extracted DT-20 at 10 μg chl/ml (followed by centrifugation) leads to binding of ~3.1 Mn atoms/RC.

4. DISCUSSION

The results reported here strongly support the idea [1–15] that Mn is a highly essential element for providing electron donation to RC from the oxidizing side of PS II. In fact, all the activities of PS II tested here require a high rate of electron donation (to prevent charge recombination in the RC after primary photoreaction), and they are inhibited upon removal of Mn and are reconstituted by added Mn^{2+} (but not by other M^{2+}). An advantage of these data (in comparison with previous similar experiments [6–9]) is the possibility to observe the reactivation at very low [Mn^{2+}] (nearly stoichiometric with that of RC) in preparations where >98% of RC do not contain Mn at all. This allows a more explicit investigation of the minimal Mn requirement and its specificity.

Our results indicate that the mangano-center acting in the donor side of PS II can comprise 4 Mn atoms. In fact, the maximal restoration of both ΔF and photoreduction of DPIP is observed upon re-addition of ≤4 Mn atoms/RC, and the experimental dependence of ΔF reactivation on [$MnCl_2$] (fig.1E) corresponds to 'filling' of a tetra-atomic manganese center. Not all of these Mn atoms are equivalent. Half of them (2 atoms) can be replaced by any M^{2+} without loss of the activities so that in the presence of Mg^{2+} the maximal reaction occurs at addition of ~2 Mn atoms/RC, and dependence of the reactivation on [Mn] corresponds to a 'filling' of a 2-atomic manganese system. So, the quantity of Mn which is strictly

necessary and irreplaceable in the donor side of PS II is 2 atoms/RC. This Mn is likely to correspond to so-called 'firmly bound' Mn since it remains after the partial extraction of Mn (fig.1D) used for removal of the 'loosely bound' Mn [1–11]. Our results also show that the irreplaceable (firmly bound) rather than the replaceable Mn is more likely to be involved in intrinsic redox processes of PS II (probably into catalytic oxidation of water). In fact, addition of 2 Mn/RC (in the presence of Mg^{2+}) is sufficient for restoration of Hill reaction in which the quantity of electrons transferred to DPIP exceeds the quantity of added Mn^{2+} at least by a factor of 50. Moreover, the reactivation effects of Mn remain after separation of the reconstituted particles from solution of $MnCl_2$ [10]. So, the effects of reactivation are related to formation of a stable Mn-complex in the functionally active structure of PS II, and Mn (or a donor system reactivated by Mn), being repeatedly oxidized by PS II, is capable of re-reduction (more likely using water as an electron donor).

The role of 2 Mn atoms which can be replaced by Mg^{2+} or by any other M^{2+} (but not by monovalent cations) may be considered as satisfying a special structural requirement of the manganoprotein that contributes to its catalytic activity.

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