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ASSOCIATION OF TWO MANGANESE ATOMS WITH THE REACTION CENTER OF PHOTOSYSTEM II IN A HIGHLY ACTIVE O_2-EVOLVING PHOTOSYSTEM II PREPARATION

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The stoichiometry of manganese atom and the Photosystem (PS) II reaction center was determined in a highly active O_2-evolving PS II preparation from spinach chloroplasts. Two manganese atoms were shown to be associated with a reaction center of PS II. Judging from the sensitivity to various treatments which specifically inhibited O_2 evolution, the two manganese atoms seem to be the minimum and essential amount of manganese required for the O_2-evolution activity of the subchloroplast preparation.

Manganese is an essential component in catalyzing photosynthetic O_2 evolution in chloroplasts [1–3]. Photochemical reaction at PS II induces accumulation of oxidizing equivalents, probably on Mn, and O_2 is produced from the subsequent oxidation of water. Although the chemical cycle of O_2 evolution (S-state) is well documented [2,3], there is still no agreement on the number of Mn atoms involved in the molecular mechanism of O_2 evolution. Early studies indicated the presence of 5–8 Mn/400 Chl in chloroplasts although the value varied depending on the preparation [1]. Recently, a more exact estimation of the minimum amount of Mn associated with PS II was carried out after removing loosely bound nonfunctional Mn atoms from the thylakoid membrane by EDTA or salt treatment, and a level of 4 Mn/400 Chl was suggested to be essential for O_2-evolution activity [4]. However, as far as the broken chloroplasts are used as a sample for estimation of Mn, the contribution of Mn atoms which have no function in O_2 evolution may not be completely excluded even after repeated washings. In this study we used a highly active O_2-evolving PS II subchloroplast preparation from spinach chloroplasts and examined the stoichiometry of Mn and PS II reaction center. It was demonstrated that the PS II preparation required only 2 Mn atoms per reaction center for the high O_2-evolution activity.

Broken chloroplasts were prepared as previously described [5] with a medium containing 0.4 M sucrose, 20 mM Tris and 15 mM NaCl, pH 7.5 (buffer A). Highly active O_2-evolving PS II subchloroplasts were obtained by the method described in Ref. 5 with some modification; a buffer solution containing 0.33 M sorbitol, 10 mM Mes and 4 mM $MgCl_2$, pH 6.2 (buffer B) was used as a medium for grinding and suspending. For EDTA washing, broken chloroplasts and PS II subchloroplasts were incubated in buffer C (0.4 M sucrose and 20 mM Tris, pH 7.5) and buffer D (0.33 M sorbitol and 10 mM Mes, pH 6.2), respectively, which contained 1 mM EDTA. After standing for 10 min, the suspensions were centrifuged at 8000 $\times g$ for 10 min. This washing step was repeated twice and the pellet was suspended in buffer A or buffer B. When the PS II preparation was treated

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Abbreviations: PS, photosystem; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

with Tris, NH_2OH and NaCl, it (0.1 mg Chl/ml) was suspended in either 0.8 M Tris (pH 8.0, adjusted with HCl at 4°C), 5 mM NH_2OH (pH 7.5, adjusted with NaOH) or 250 mM NaCl solution, and allowed to stand for 30 min on ice under the room light. Each suspension was then centrifuged at $25000 \times g$ for 30 min and suspended in buffer B. For the cholate treatment, the PS II preparation suspended in a solution containing 0.33 M sorbitol, 20 mM Hepes and 4 mM NaCl (pH 7.3) was treated with 2% sodium cholate for 10 min at 4°C. Subsequently, the sample was precipitated by centrifugation at $30000 \times g$ for 30 min and the precipitate was suspended in buffer B. For determination of Mn, membrane preparations equivalent to 0.5 mg Chl were heated at 500°C in quartz tubes for 12 h and dry ashed. Three drops of concentrated HCl and 1 ml of deionized water were then added and the samples were heated at 200°C for 30 min. The treatment with concentrated HCl was repeated twice and the samples were diluted with deionized water to 5 ml. To avoid incomplete solubilization of the dried material which might deposit on the surface of the quartz tubes, the contents were carefully suspended by the use of a glass rod. Standard Mn solution was treated in the same way. Mn was determined by the use of a Hitachi model 170-10 atomic absorption spectrophotometer equipped with an acetylene/air burner head. Absorption of Mn was measured at 279.5 nm with a slit width of 0.18 mm. SDS-polyacrylamide gel electrophoresis was carried out as described in Ref. 6. The densitograms of gels were obtained by a Toyo digital densitometer DMU-33C. The amount of PS I and PS II pigment-protein complexes in the preparations was estimated from the integrated values of the corresponding polypeptide bands stained by Coomassie brilliant blue. O_2 evolution was measured with a Rank oxygen electrode at 20°C. The assay medium (2.0 ml) contained buffer B, 0.1 mM phenyl-*p*-benzoquinone, 1 mM potassium ferricyanide and broken chloroplasts or PS II preparation equivalent to 40 μg Chl. NH_4Cl (5 mM) was added as an uncoupler when broken chloroplasts were used as a sample. Chlorophyll was determined using the absorption coefficients reported in Ref. 7. Measurements were repeated five times in each preparation and the variation of the data was within 4%.

Broken chloroplasts prepared with buffer A had an Mn content of 6 Mn/400 Chl (Table I). After washing twice with EDTA, the Mn content in the broken chloroplasts decreased to a level of 4 Mn/400 Chl, although there was no significant loss of O_2 evolution in the chloroplasts. Almost the same Mn:Chl stoichiometry was also reported in Ref. 4 where Mn functionally associated with the O_2-evolution activity was determined by EPR measurement. However, not all of the Mn atoms detected in the washed broken chloroplasts were associated with PS II. In the PS II preparation which had an O_2-evolution activity higher than that in the uncoupled broken chloroplasts, the Mn content was 2 Mn/200 Chl molecules or 2 Mn/PS II reaction center. Here we assumed the presence

TABLE I

O_2-EVOLUTION ACTIVITY AND Mn CONTENT IN BROKEN CHLOROPLASTS AND THE PS II PREPARATION BEFORE AND AFTER EDTA TREATMENT

Preparation	O_2 evolution [b] (μmol O_2/mg Chl per h)	Mn content [c]	
		Mn/400 Chl	Mn/200 Chl
Broken chloroplasts [a]	203 ± 16.0	6.0 ± 0.5	
EDTA-washed broken chloroplasts [a]	187 ± 8.0	3.9 ± 0.8	
PS II preparation	235 ± 10.9		2.1 ± 0.4
EDTA-washed PS II preparation	178 ± 2.4		1.6 ± 0.2

[a] O_2-evolution activity was measured in the presence of 5 mM NH_4Cl.
[b] Data are the average of three measurements ± S.D.
[c] Data are the average of six measurements ± S.D.

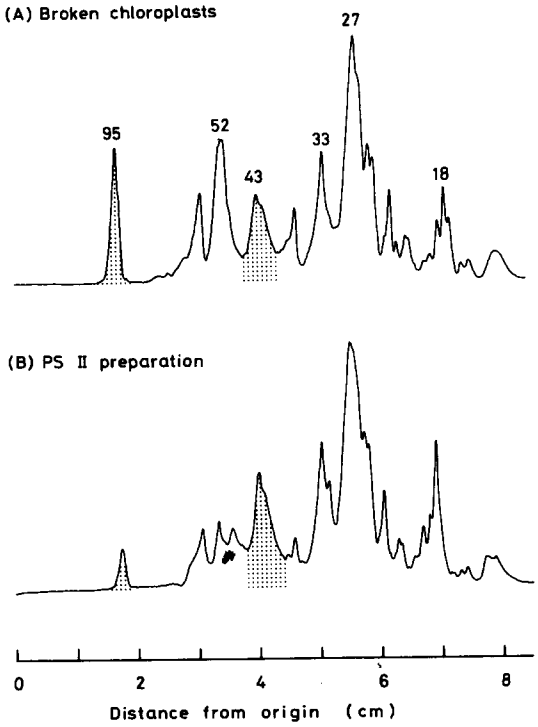

Fig. 1. Densitograms of SDS-polyacrylamide slab gel showing Coomassie blue-stained polypeptides of broken chloroplasts (A) and the PS II preparation (B). The figures show the molecular mass of polypeptide bands in kDa. The polypeptide bands of 95 kDa and 43 kDa (dotted areas) correspond to PS I and PS II pigment-protein complexes, respectively. Each sample contained 10 μg Chl.

of one PS II reaction center per 200 Chl molecules in the PS II subchloroplasts as compared with one PS II reaction center/400 Chl molecules in chloroplasts. The assumption was confirmed by the amount of PS I and PS II pigment-protein complexes in the PS II preparation and broken chloroplasts. In the densitogram of Coomassie blue-stained polypeptide bands after SDS-polyacrylamide gel electrophoresis, PS I and PS II complexes which appeared at the molecular mass ranges of 95 kDa [8] and 43 kDa [9], respectively, were determined from the integrated values of the stained bands (Fig. 1 and Table II). The amount of PS I complex in the PS II preparation was only 15% of that in broken chloroplasts, whereas the PS II complex in PS II subchloroplasts was twice as much as that in broken chloroplasts on a chlorophyll basis.

TABLE II

RELATIVE AMOUNT OF PS I AND PS II PIGMENT-PROTEIN COMPLEXES IN BROKEN CHLOROPLASTS AND THE PS II PREPARATION

Data (%) are the average of four measurements ± S.D.

Preparation	PS I pigment-protein complex	PS II pigment-protein complex
Broken chloroplasts	100 (±17)	100 (±9)
PS II preparation	14.6 (±1.7)	188 (±4)

The two Mn atoms associated with the PS II preparation were partly removed by EDTA treatment, which was accompanied by a partial loss of O_2 evolution (Table I). This result suggests that two atoms of Mn are the minimum and essential amount of Mn required for the O_2-evolution activity of the preparation. Mn atoms in the PS II preparation were also removed by other treatments which are known to inhibit O_2 evolution specifically (Table III). Tris and NH_2OH were most effective in removing Mn; 80–90% of the total Mn was released from the membranes. Thus, the Mn atoms rather 'tightly' associated with PS II even after detergent treatment of the membranes were quite sensitive to the Tris and NH_2OH treatments, and seem to play a crucial role in O_2 evolution. NaCl and sodium cholate affected the Mn content only slightly. It was reported that cholate treatment of the broken chloroplasts did not release Mn significantly [10].

The requirement of two Mn atoms for each

TABLE III

INHIBITION OF O_2 EVOLUTION AND RELEASE OF Mn FROM MEMBRANES OF THE PS II PREPARATION BY VARIOUS TREATMENTS

Treatment	O_2 evolution (relative)	Mn content [a] (Mn/200 Chl)
Untreated	100 [b]	1.90±0.03
Tris (0.8 M)	14	0.29±0.06
NH_2OH (5 mM)	13	0.42±0.06
NaCl (250 mM)	41	1.45±0.10
Sodium cholate (2%)	60	1.54±0.10

[a] Data are the average of three measurements ± S.D.
[b] Control activity was 190 μmol O_2/mg Chl per h.

reaction center of PS II seems to be consistent with the result of Klimov et al. [11], although they could not correlate the two Mn atoms with O_2-evolution activity. Our PS II subchloroplast particles were prepared in the presence of 4 mM $MgCl_2$ during the whole preparation procedure including detergent treatments, suggesting the possibility that nonfunctional Mn atoms were replaced by the externally added cations. Although the mode of interaction of Mn involved in the O_2-evolution enzyme complex is still unknown, the stoichiometry of 2 Mn/PS II reaction center obtained here suggests the possible existence of a binuclear Mn center in the O_2-evolution enzyme complex. Models for a binuclear complex of Mn were proposed previously [12,13]. Further experiments, such as flash yield and quantum yield studies, are necessary for an unequivocal establishment of the minimum Mn requirement for O_2 evolution. We will focus our effort also to elucidate the organization of the catalytic Mn in PS-II subchloroplasts.

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