Isolation of an Mn-carrying 33-kDa protein from an oxygen-evolving photosystem-II preparation by phase partitioning with butanol

Yasusi Yamamoto, Hiroshi Shinkai, Yasuhiro Isogai, Katsumi Matsuura* and Mitsuo Nishimura

Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, and **National Institute for Basic Biology, Okazaki 444, Japan*

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An Mn-containing 33-kDa protein was isolated by phase partitioning with 50% *n*-butanol from an O_2-evolving photosystem-II preparation. The Mn content in the 33-kDa protein increased when 1 mM potassium ferricyanide and 0.4 mM diaminodurene were present as oxidants during the butanol treatment and the following dialysis. Under these conditions, 0.1–0.25 atom Mn was detected in one 33-kDa protein molecule. EPR spectra of the Mn protein showed that the Mn atoms were bound to the protein.

O_2 evolution *Mn-protein* *Butanol treatment* *33-kDa protein* *Photosystem II* *Photosynthesis*

1. INTRODUCTION

Photosynthetic O_2 evolution is catalyzed by an enzyme complex containing Mn atoms at its catalytic site. Positive charges produced by photo-induced charge separation at the reaction center of PS-II are transferred to the enzyme complex and accumulated on the Mn atoms. O_2 evolution subsequently occurs with specific binding of water molecules to the oxidized Mn complex. In spite of efforts to unveil the organization of the O_2-evolution enzyme complex, information on Mn and the protein components of the enzyme complex is still limited. Recently, several authors have shown that three proteins with molecular masses of 33, 24 and 18 kDa were released from the O_2-evolving PS-II preparations and the inside-out thylakoid membranes by various treatments that inhibit O_2 evolution [1–3]. However, these proteins did not contain Mn in their isolated forms, probably because the treatments employed for the isolation of the proteins were so harsh that the native conformation of the protein required for retaining Mn in situ was distorted [4]. We used phase partitioning with *n*-butanol for the isolation of an Mn-carrying protein from the O_2-evolving PS-II preparation. A 33-kDa protein partitioned into the aqueous phase after the phase partitioning was shown to contain an appreciable amount of bound Mn.

Abbreviations: PS-II, photosystem II; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FeCy, potassium ferricyanide; DAD, diaminodurene

2. MATERIALS AND METHODS

Broken chloroplasts and a highly active O_2-evolving PS-II preparation were obtained from spinach as previously described [5,6]. For NaCl treatment, the PS-II preparation was incubated with 1 M NaCl for 20 min at 4°C and centrifuged at 35000 × *g* for 20 min. By this treatment the 24- and 18-kDa proteins were removed from the membranes without any effect on the Mn content of the PS-II preparation [7]. After the NaCl treatment the preparation was washed once with 10 mM Mes buffer (pH 6.5). Broken chloroplasts, the PS-II

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preparation and the NaCl-washed PS-II preparation were suspended in 10 mM Mes (pH 6.5) with chlorophyll concentration of 0.5–1.0 mg/ml.

For butanol treatment, cold *n*-butanol and the suspension containing each preparation were mixed in a volume ratio of 1:1, and the whole mixture was shaken vigorously for 10 s. After centrifugation at 3000 × *g* for 10 min at 0°C, the butanol-saturated water phase was collected and dialyzed overnight against 10 mM Mes buffer (pH 6.5) at 4°C. The dialyzed sample was concentrated with an Amicon ultrafiltration cell model 52. Where indicated, 1 mM potassium ferricyanide and 0.4 mM diaminodurene were included in the buffer medium (10 mM Mes, pH 6.5) for the butanol treatment and the following dialysis.

Mn determination with an atomic absorption spectrophotometer was carried out as previously described [8]. EPR signals of Mn were measured at 20°C with a Bruker ER 200D EPR spectrometer operated in the X-band microwave region at 9.79 GHz. The microwave power was set at 50 mW and the field modulation intensity at 20 G. Four signals were accumulated to improve the signal to noise ratio.

SDS–PAGE was carried out as described in [4]. Concentration of proteins was determined by the method of [9].

3. RESULTS

By the phase partitioning with *n*-butanol, the hydrophobic proteins and the hydrophilic proteins in broken chloroplasts and the PS-II preparation were separated according to their hydrophobicity. As shown in fig.1, the aqueous phase of the phase partitioning of broken chloroplasts contained 10–15 polypeptides ranging from 10 to 100 kDa in molecular mass. In the aqueous fraction obtained after the butanol treatment of the PS-II preparation, the 33-, 24- and 18-kDa proteins and a minor polypeptide band at 55–60 kDa were detected. As the proteins with molecular masses of 55–60, 24 and 18 kDa were removed from the membranes by prewashing of the PS-II preparation with 1 M NaCl, the butanol phase partitioning of the NaCl-treated PS-II preparation showed only one polypeptide band at 33 kDa. In the fractions obtained by butanol treatment of broken chloroplasts and the PS-II preparation, we detected the

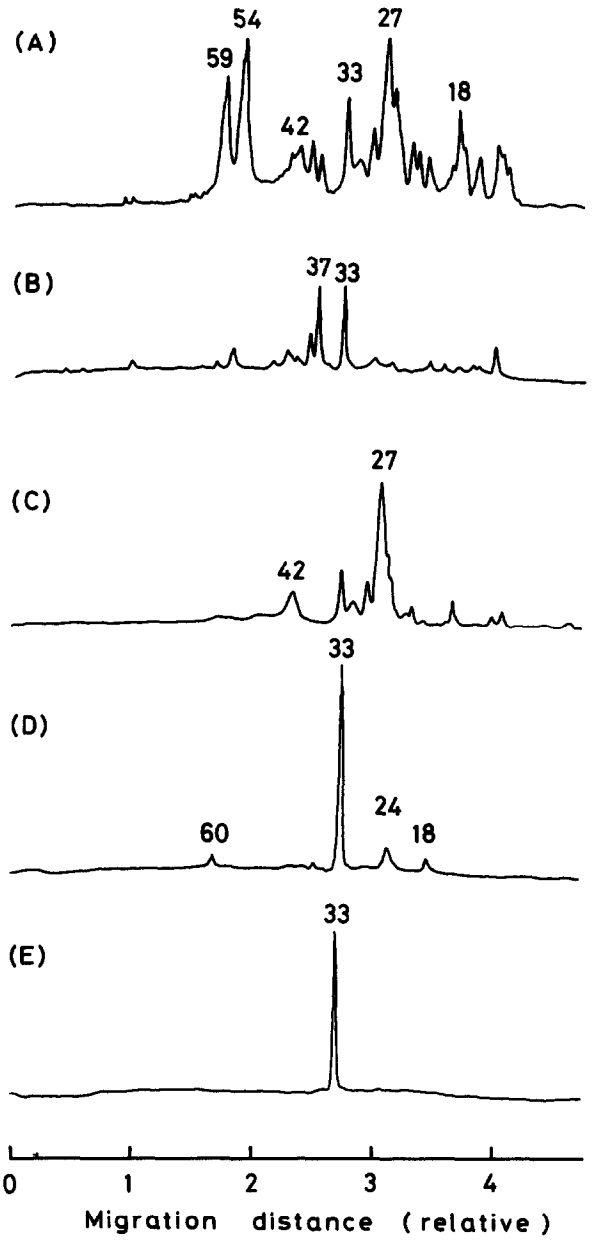

Fig.1. Densitograms of SDS–PAGE gels showing Coomassie blue stained polypeptide bands. The figures show the relative molecular masses of polypeptides in kDa. (A) Broken chloroplasts; (B) the aqueous phase obtained by the phase partitioning of broken chloroplasts with *n*-butanol; (C) the PS-II preparation; (D) the aqueous phase obtained after the phase partitioning of the PS-II preparation; (E) the aqueous phase obtained after the phase partitioning of the NaCl-washed PS-II preparation. The amounts of samples applied to the gel were equivalent to 4 μg chlorophyll for (A) and (C), and 2.7 μg protein for (B), (D) and (E).

Table 1

Amounts of Mn in the aqueous fractions obtained by the phase partitioning with butanol of broken chloroplasts, the PS-II preparation and the NaCl-washed PS-II preparation

Samples	Mn (ng Mn/ mg protein)
Broken chloroplasts	110
Broken chloroplasts (+ FeCy, DAD)	260
PS-II preparation	53
PS-II preparation (+ FeCy, DAD)	380
NaCl-washed PS-II preparation	70
NaCl-washed PS-II preparation (+ FeCy, DAD)	160

Mn was assayed with a flame type atomic absorption spectrophotometer. The data were the average of 3 measurements. Variation of the data was within 5%

presence of Mn ranging from 50 to 100 ng Mn/mg protein by atomic absorption measurement (table 1).

When 1 mM potassium ferricyanide and 0.4 mM diaminodurene were included as oxidants in the buffer medium during butanol treatment and the following dialysis, the content of Mn per mg protein increased 2–7 times without affecting the SDS–PAGE profile of each fraction (table 1). The 33-kDa protein fraction obtained in the presence of the oxidants contained 0.1 Mn atom per protein molecule. In the 24- and 18-kDa proteins, isolated by DEAE-Sepharose column chromatography of the aqueous fraction obtained after the butanol treatment of the PS-II preparation, we did not detect Mn (not shown).

The Mn-containing 33-kDa protein was subjected to EPR measurement at room temperature (fig.2). Before the addition of HCl, the EPR spectrum did not show any structure suggesting the presence of free Mn in the protein fraction. Addition of 0.5 M HCl to the protein induced a typical 6-lined EPR signal of hexaquo Mn^{2+}, which showed the presence of bound Mn atoms in the protein. The amount of Mn detected by the EPR measurement was 0.25 Mn atom per molecule of the 33-kDa protein.

4. DISCUSSION

The phase partitioning with *n*-butanol employed

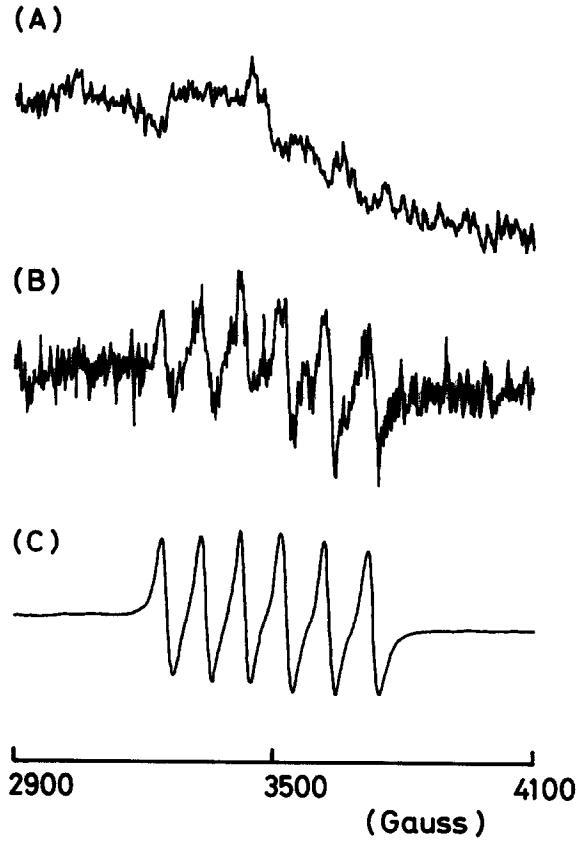

Fig.2. EPR spectra of the 33-kDa protein at room temperature. (A) The 33-kDa protein with no addition; (B) the 33-kDa protein with 0.5 M HCl; (C) 100 μM $MnCl_2$ in 0.5 M HCl. The concentration of the protein was 490 μg/ml. Background signal observed with 0.5 M HCl was corrected in each measurement. Gain settings of the instrument were 5×10^4 for (A) and (B), and 2.5×10^3 for (C).

for broken chloroplasts and the PS-II preparation is a mild procedure for separating the hydrophilic proteins from the hydrophobic proteins. The 33-kDa protein is probably identical with the lysine-rich 33-kDa protein that was shown to be released from the O_2-evolving PS-II preparation by various treatments [2–4,7]. Molecular mass (33 kDa), hydrophilic nature and behavior in ion-exchange column chromatography are common to the two proteins. Both these proteins were easily stained by Coomassie blue in SDS–PAGE gels, which is in contrast with the 30–34-kDa herbicide-binding protein that has been shown to be poorly stained by the dye [10]. Preliminary results on pep-

tide mapping for the lysine-rich 33-kDa protein and the Mn-carrying 33-kDa protein also confirmed the identity of the two proteins (not shown).

An EPR signal showed the presence of bound Mn atoms in the 33-kDa protein. As the oxidants used in the isolation procedure of the protein had a significant effect in increasing the yield of Mn-protein, it is likely that the oxidants somehow stabilized the binding of Mn atoms to the protein. Authors in [11] suggested from the comparison of stability constants in model compounds that Mn(III) and Mn(IV) are stabilized much more than Mn(II) by coordination to proteins. The oxidants may protect the higher oxidation state of Mn from reduction by endogenous reductants [11], or alternatively, Mn(II) may be oxidized by light reaction in the presence of the oxidants. As we carried out the isolation of the protein under room light, there is a possibility that the population of Mn(III) and Mn(IV) was increased to a certain extent. The 33-kDa protein has been suggested to be an Mn-carrying protein from the work on algal mutants which lacked the ability to bind Mn to the membranes [12], and from that on inhibition of O_2 evolution and concomitant release of proteins and Mn from the O_2-evolving PS-II preparation [7]. However, a recent result [13] showed that the 33-kDa protein could be removed from the membranes of the PS-II preparation by 1 M $CaCl_2$ treatment, with Mn preserved in the membranes. The apparent discrepancy between the results in [13] and here might be explained in terms of stability of Mn binding to proteins depending on the oxidation state of Mn. Here we suggest the presence of the 'resting' and 'active' states of Mn atoms functioning in O_2 evolution. The Mn atoms are probably located at the interface of the core complex of the PS-II reaction center and the 33-kDa protein, and Mn(II) (in the 'resting' state) may have a tendency to bind to the core complex of PS-II, whereas Mn(III) and Mn(IV) (in the 'active' state) are probably stabilized by binding to the 33-kDa protein.

In the PS-II preparation treated with 1 M $CaCl_2$, transition from S_1 to S_2 state in the S cycle of O_2 evolution was inhibited and Mn was kept in the lower oxidation state (unpublished). As O_2 evolution of the $CaCl_2$-treated PS-II preparation was reconstituted by adding back the 33-kDa protein [14], it is possible that the 33-kDa protein is required for stabilization of higher oxidation states of Mn responsible for the S_2 and S_3 states. Earlier results that O_2 evolution in chloroplasts was inhibited by Tris more significantly in the light than in the dark and that the target of Tris inhibition was the S_2 state [15,16] seem to be compatible with our idea; Mn in the 'active' state, probably bound to the 33-kDa protein, was removed easily by Tris treatment, which facilitated inhibition of O_2 evolution in chloroplasts under the illumination.

Further study on the effects of the oxidants and light illumination on the yield of Mn-protein is being undertaken to examine the possible 'resting' and 'active' states of Mn.

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