Biochimica et Biophysica Acta 933 (1988) 165–171
Elsevier

BBA 42727

Analysis of the relationship between the extrinsic 30-kDa protein, manganese and oxygen evolution in the thylakoid of *Chlamydomonas reinhardtii* grown under manganese-deficient conditions

Yasusi Yamamoto

Department of Biology, Faculty of Science, Kyushu University, Fukuoka (Japan)

(Received 17 August 1987)
(Revised manuscript received 26 November 1987)

Key words: 30 kDa protein; Manganese; Oxygen evolution; Photosynthesis; Photosystem II; (*C. reinhardtii*)

The content and stability of an extrinsic protein in the thylakoid of a green alga *Chlamydomonas reinhardtii* which has a relative molecular weight of 30 000 and corresponds to the '33-kDa' protein involved in oxygen evolution in spinach chloroplast were studied with the cells grown in a manganese-deficient medium. The amount of the 30-kDa protein in the thylakoid did not change even when the content of manganese in the membrane was decreased significantly and the activity of oxygen evolution was reduced concomitantly under manganese-depleted conditions. The stability of the 30-kDa protein in the membrane, which was determined by the susceptibility of the protein to the treatment with an alkaline buffer and sonication, also showed no significant change between the cells grown with and without manganese. The thylakoid membrane of the cells grown under manganese-deficient conditions was devoid of the loosely bound manganese atoms located at the hydrophilic environment of the membrane probably at the interface between the 30-kDa protein and the reaction center complex of Photosystem II, judging from the partitioning behavior of manganese atoms on butanol/water phase partitioning of the thylakoid. These results suggest that the manganese atoms involved in oxygen evolution are not required for the binding of the 30-kDa protein to the Photosystem II membrane.

Introduction

In photosynthetic oxygen evolution in higher plants and algae, an extrinsic protein of thylakoid of molecular weight 30 000–33 000, manganese atoms and the reaction center polypeptides of PS II are indispensable components [1–3]. These components organize a special catalytic domain which is referred to as 'the oxygen-evolution enzyme' and carry out light-induced oxidation of water molecules, i.e., oxygen evolution in chloroplast. Of these membrane components involved in oxygen evolution, the extrinsic protein has mainly been studied with spinach PS II particles. The '33-kDa' extrinsic protein in spinach chloroplast is associated with PS II with a stoichiometry of one molecule of protein to the PS II reaction center [4], and was released from the membrane concomitant with manganese when the PS II particles were treated with 0.8 M Tris (pH 8.4), which inhibited oxygen evolution specifically [5]. Four

Abbreviations: PS II(I), Photosystem II(I); PS II particles, membrane preparation enriched with Photosystem II; Mes, 4-morpholineethanesulfonic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DCIP, 2,6-dichlorophenolindophenol.

Correspondence: Y. Yamamoto, Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan.

atoms of manganese are associated with PS II [4], two of them are loosely bound to the membrane and are suggested to be located in the hydrophilic environment of the membrane, probably at the interface between the 33-kDa protein and the reaction center polypeptide(s) of PS II [6]. The close association of a part of the manganese atoms and the extrinsic 33-kDa protein was also shown by the isolation of the 33-kDa protein carrying an appreciable amount of Mn [7–9]. The major function of the manganese atoms in PS II is to accumulate the oxidizing equivalents produced by the photochemical reactions at the reaction center. Evidence for involvement of manganese atoms in redox reaction in oxygen evolution was obtained by low-temperature EPR spectroscopy [10]. It is, however, argued that the manganese atoms also participate in the binding of extrinsic protein to the membrane [11].

As the organization of the manganese atoms in the oxygen evolution system is especially important for understanding the mechanism of oxygen evolution, we have studied the possible role of manganese in binding of the extrinsic protein to the PS II membrane by analyzing the quantity and stability of the extrinsic 30-kDa protein which corresponds to the spinach 33-kDa protein with the cells of *Chlamydomonas reinhardtii* grown under manganese-deficient conditions.

Materials and Methods

Culture of Chlamydomonas reinhardtii and isolation of thylakoid membranes. The cells of *C. reinhardtii* wild type strain 137C + were grown in the medium described by Sager and Granick [12] with or without manganese at 24 °C under the illumination of the white fluorescent lamps (3500 lx). The number of the cells in the growth medium was counted microscopically with a hemocytometer after fixation of the cells with formaldehyde. The concentration of chlorophyll of the cells collected by centrifugation at $10000 \times g$ for 10 min was determined spectrophotometrically with an 80% acetone extract of the cells. The *Chlamydomonas* cells in the late log phase of growth were harvested by centrifugation at $5000 \times g$ for 5 min and washed twice with a medium containing 0.33 M sorbitol, 10 mM Mes and 120 mM NaCl (pH 6.5) (hereafter the solution is referred to as buffer A). The cells were suspended in a solution containing 0.33 M sorbitol and 10 mM Mes (pH 6.5) at a chlorophyll concentration of 2–3 mg/ml and broken with a French Pressure Cell (Ohtake Seisakusho Co., Japan). The chloroplasts were obtained by centrifugation of the broken cell suspension at $10000 \times g$ for 10 min and suspended in a minimum volume of buffer A.

Preparation of antibody against spinach 33-kDa protein and Western-blotting analysis. The extrinsic 33-kDa protein of spinach chloroplast was obtained as previously described [13]. 1 mg of the purified 33-kDa protein was suspended in 1 ml of 10 mM Mes (pH 6.2), mixed with an equal volume of a complete Freund adjuvant and injected by syringe to a rabbit.

Booster doses were given with the same antigen. Antiserum for the immunized rabbit was purified by precipitation procedure with ammonium sulfate and stored at −80 °C before use. Western-blotting was carried out with a Bio-Rad blotting apparatus. Proteins in the thylakoid of *Chlamydomonas* were separated by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane at 70 V for 3 h at 0 °C. The protein cross-reacted with the antiserum against spinach 33-kDa protein was detected on the nitrocellulose membrane by the peroxidase activity of goat anti-rabbit immunoglobulin.

Treatment of the thylakoid membranes with alkaline buffer and sonication. The thylakoid membranes of *Chlamydomonas* were suspended in 50 mM Mes-NaOH or Tris-HCl buffer at pH 6–9.5 with a chlorophyll concentration of 0.1 mg/ml. After incubation of the thylakoid membranes in the buffer solution for 30 min at 4 °C, the membranes were sonicated with an ultrasonic disruptor (Tomy Seiko Co., Japan) at 40 W for 30 s at 0 °C. The treated membranes were then centrifuged at $35000 \times g$ for 90 min and the precipitate and the supernatant were separated from each other. The supernatant fractions were concentrated by lyophilization and used for SDS-PAGE and Western-blotting analysis.

Butanol / water phase partitioning of the thylakoid membranes. The thylakoid membranes of *Chlamydomonas* were suspended in buffer A and

subjected to butanol/water phase partitioning as previously described [14]. The butanol-saturated aqueous phase was collected carefully and manganese in the fraction was assayed. The content of manganese in the membrane preparations and the aqueous fraction obtained by the butanol/water phase partitioning of the membranes was determined according to Ref. 15. SDS-PAGE was carried out as previously described [16]. Oxygen evolution activity of the thylakoid was measured with a Rank oxygen electrode. The assay medium (2 ml) contained buffer A, 1 mM potassium ferricyanide, 50 μM phenyl quinone and thylakoid membrane equivalent to 40 μg chlorophyll. DCIP photoreduction was measured with a Hitachi 356 dual-wavelength spectrophotometer with a cross illumination attachment. The assay medium contained 20 mM Tricine-KOH (pH 8.0), 50 mM NaCl, 10 mM NH_4Cl, 10 μM DCIP and thylakoid membrane equivalent to 40 μg chlorophyll in a total volume of 4.0 ml. Diphenylcarbazide (1 mM) was used as an artificial electron donor to PS II where indicated.

Results and Discussion

The cells of wild type strain of *C. reinhardtii* grown in the Sager and Granick's medium for photoautotrophic growth were inoculated to the same medium but with no manganese. The inoculation-growth cycle was repeated and the cells at the late log phase in the fifth cycle were harvested. From the growth curves of the cells, it is apparent that the cells in the manganese-depleted medium grew considerably slower than those in the medium with manganese (Fig. 1). As number of the cell and the amount of chlorophyll synthesized changed in parallel during the course of growth of the cells both in the presence and absence of manganese in the growth medium, it is probable that the amount of chlorophyll per chloroplast and the antenna size of the reaction center of PS

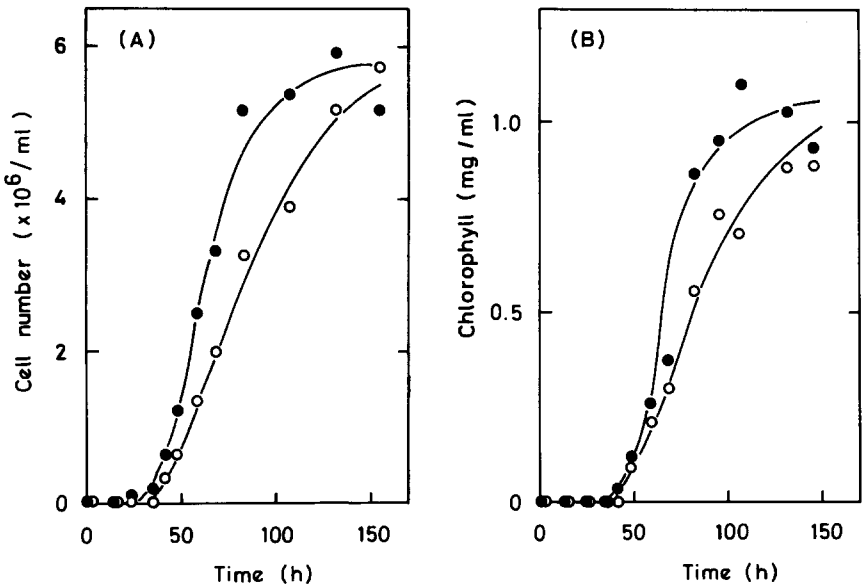

Fig. 1. The growth curves of the cells of *Chlamydomonas reindardtii* wild type strain 137C+. (A) The number of cells grown in the Sager and Granick's medium with (●) or without (○) manganese was counted microscopically with a hemocytometer. In the growth medium with manganese, $MnSO_4$ was added at a concentration of 0.17 mM. For the growth of cells under manganese-deficient conditions, cells were inoculated to the medium not containing manganese and the inoculation-growth cycle was repeated. The growth curve of the cells in the manganese-deficient medium was obtained in the fifth inoculation-growth cycle. The number of cells at the start of the growth was $1.2 \cdot 10^4$ cells/ml in the medium either with or without manganese. (B) The concentration of chlorophyll was determined with cells grown in the presence (●) or absence (○) of manganese. The other conditions were the same as those described in A.

TABLE I

CHARACTERISTICS OF THE THYLAKOID MEMBRANES OBTAINED FROM THE CELLS OF *CHLAMYDOMONAS REINHARDTII* GROWN IN MEDIUM WITH OR WITHOUT MANGANESE

Relative values are shown in the parenthesis.

	Cells grown with manganese	Cells grown without manganese
Content of manganese (Mn/400 chlorophylls)	5.8 (100)	1.7 (29)
Oxygen evolution (μmol/mg chlorophyll per h)	120 (100)	34 (28)
DCIP photoreduction (μmol/mg chlorophyll per h)		
$H_2O \rightarrow$ DCIP	35 (100)	8.1 (23)
Diphenylcarbazide $\rightarrow$ DCIP	41 (117)	26 (74)
Amount of the 33-kDa protein (relative amount)	100	120
Manganese atom(s) partitioned into the aqueous phase on butanol/water phase partitioning (Mn/400 chlorophylls)	2.3	0.28

II were also not affected significantly by the manganese-deficient condition in spite of decrease in the growth rate of the cells.

The characteristics of the thylakoid membranes prepared from the cells grown in the manganese-depleted medium were studied and the results are shown in comparison with those obtained with the cells grown in the medium with manganese (Table I). Both the content of manganese and the oxygen evolution activity of the thylakoid obtained from the cells grown under manganese-deficient conditions were about one fourth of those from the cells grown with manganese.

The parallel relationship between the amount of manganese in the thylakoid membrane and oxygen evolution activity was shown previously by studying the effect of extraction of manganese on the oxygen evolution in the spinach PS II preparation [17]. Manganese is an essential component in the catalysis of water oxidation and it has been suggested that four atoms of manganese associated with PS II form a tetrameric complex and undergo a structural change depending on the redox state of manganese [18]. Depletion of manganese in the membrane is, therefore, expected to have a significant effect on the organization of the oxygen-evolution enzyme complex. The depletion of manganese in the membranes seemed to have a primary effect on the oxygen-evolution system under these conditions, because the photochemical reaction of PS II monitored by photoreduction of DCIP with diphenylcarbazide as an electron donor was not inhibited (Table I). As complete loss of manganese in the thylakoid may induce a secondary effect on the activity of oxygen evolution due to inhibition of the other possible manganese-dependent enzymatic or photochemical reactions, we did not extend our study to such an extreme condition.

Although the activity of oxygen evolution was decreased significantly in the thylakoid of the cells grown under manganese-deficient conditions, there was no apparent difference in the profile of the protein in the thylakoid between the cells grown with and those grown without manganese (Fig. 2, lanes 1 and 2). In spinach chloroplast, there is an extrinsic protein of molecular weight 33000 as estimated by SDS-PAGE [5], and the protein participates in stabilization of the manganese atoms in the oxygen evolution apparatus [19]. In *C. reinhardtii*, the protein corresponding to the spinach 33-kDa protein was identified as a 30-kDa protein by Western-blotting analysis with the rabbit antiserum against the spinach 33-kDa protein (Fig. 2, lanes 3 and 4). As was observed with spinach PS II particles, the 30-kDa protein was liberated from the thylakoid membrane of *Chlamydomonas* by the treatment with Tris buffer although the following sonication was required to disrupt the thylakoid and to release the trapped protein in the lumenal side of the thylakoid (Fig. 2, lanes 5 and 6). The amount of 33-kDa protein in the thylakoid membrane determined from the densitograms of SDS-PAGE gels of both the thylakoid membranes and the supernatant fraction of Tris/sonication treatment of the membranes did not change significantly between the cells grown in the medium with manganese and those grown in the medium without manganese (Table I).

The stability of the 30-kDa protein in the thylakoid membrane was studied by assaying the amount of the protein released from the membrane by the treatment with buffer solutions of

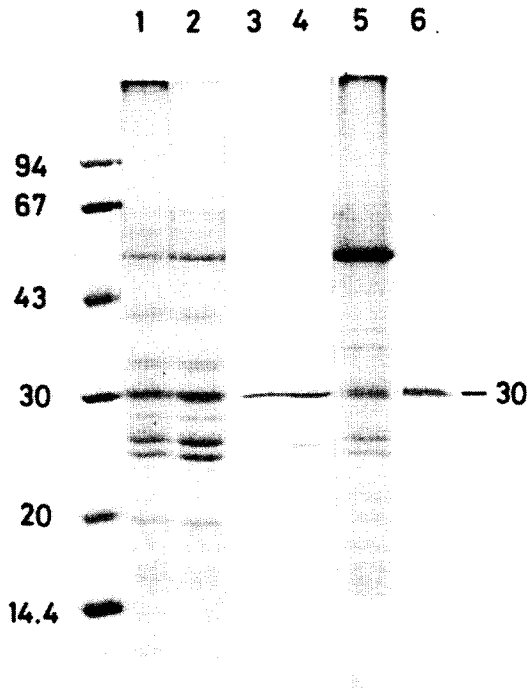

Fig. 2. SDS-PAGE and Western-blotting of the proteins in the thylakoids from *Chlamydomonas* cells grown under manganese-deficient conditions. Lane 1, the thylakoid membranes of cells grown in the medium with manganese. Lane 2, the thylakoid membranes of the cells grown in the manganese-deficient medium. Lane 3, Western-blotting of the proteins of lane 1 with the antibody against spinach 33-kDa protein. Lane 4, Western-blotting of the proteins of lane 2 with the antibody against spinach 33-kDa protein. Lane 5, the proteins released from the thylakoid membranes of *Chlamydomonas* grown in the medium with manganese by the treatment with 50 mM Tris-HCl (pH 9.5) and a subsequent brief sonication. Lane 6, Western-blotting of the proteins of lane 5 with the antibody against spinach 33-kDa protein. Lanes 1–4 contained the thylakoid membranes equivalent to 2 μg chlorophyll. The lane farthest to the left shows the molecular weight marker proteins. Relative molecular masses in kDa are given on the left.

pH in the range 6–9 and subsequent sonication (Fig. 3). The amount of 30-kDa protein released from the membrane was increased when the pH of the buffer used for the treatment was increased with the thylakoid membranes from the cells grown both with and without manganese (Fig. 4). The similarity of the profile of the protein release between the membranes from either types of cell at the various pH values suggests that the stability of the 30-kDa protein in the membrane does not change even when the amount of manganese in the membrane was decreased significantly.

The nature of the manganese atoms associated with membranes of cells grown in the manganese-deficient medium was studied by butanol/water phase partitioning and the following atomic absorption analysis. With spinach PS II particles, it was shown previously that four atoms of manganese are associated with PS II and two of them are partitioned into the aqueous phase on the butanol/water phase partitioning of the PS II particles [6]. A similar situation was observed with the *Chlamydomonas* cells grown with manganese. With thylakoid membranes of *Chlamydomonas,* the amount of manganese was estimated on the basis of 400 chlorophylls, which are assumed to be present in the photosynthetic unit, containing one of each reaction center of PS I and II. Of the 5–6 atoms of manganese detected per 400 chlorophylls in the wild type strain of *Chlamydomonas,* two or three were partitioned into the aqueous phase on the butanol/water phase partitioning. Partitioning of the manganese atom into the aqueous phase reflects that the environment where the manganese atom is located is relatively hydrophilic.

The interface between the extrinsic 30-kDa protein and the PS II membrane, or more accurately the reaction center complex of PS II, is probably the site where the hydrophilic environment is provided for manganese atoms. These manganese atoms, referred to as the 'loosely bound manganese' in Ref. 19, were shown to be stabilized by the extrinsic 30–33-kDa protein. The removal of the extrinsic protein from the membrane induced a release of these manganese atoms from the catalytic site of oxygen evolution and a concomitant inhibition of oxygen evolution [5]. On butanol/water phase partitioning of the thylakoid membranes from the *Chlamydomonas* cells grown in the manganese-deficient medium, only a small portion of manganese (less than 0.3 Mn/400 chlorophylls) out of 1.7 Mn/400 chlorophylls in the membranes was partitioned into the aqueous phase. These results suggest that the manganese atoms in the hydrophilic environment in the membrane were mostly lost under manganese-deficient growth conditions. The tendency observed here of the easy depletion of manganese atoms in the hydrophilic environment

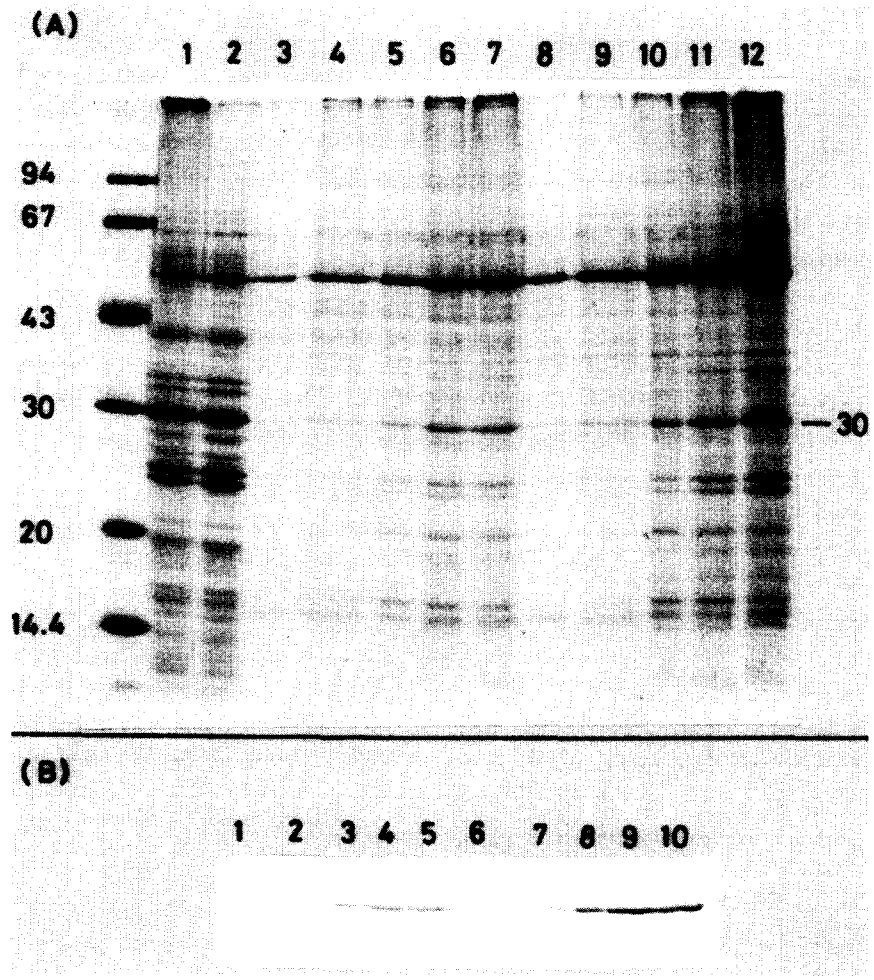

Fig. 3. (A) A gel of SDS-PAGE showing the release of proteins from the thylakoids of *Chlamydomonas reinhardtii* by treatment with alkaline buffers and sonication. The thylakoid membranes of the cells grown in the medium with manganese (lane 1) were incubated with 50 mM of Mes-NaOH, pH 6 (lane 3), Mes-NaOH, pH 7 (lane 4), Tris-HCl, pH 7 (lane 5), Tris-HCl, pH 8 (lane 6) or Tris-HCl, pH 9 (lane 7), for 30 min at 4° C and sonicated for 30 s at 0° C. The treated membranes were centrifuged at 35000 × *g* for 90 min and the supernatant fractions concentrated were put onto the gel. The same procedure was applied to the thylakoid membranes of the cells grown in the manganese-deficient medium, in which the membranes (lane 2) were incubated with 50 mM of Mes-NaOH, pH 6 (lane 8), Mes-NaOH, pH 7 (lane 9), Tris-HCl, pH 7 (lane 10), Tris-HCl, pH 8 (lane 11) or Tris-HCl, pH 9 (lane 12). The lane farthest to the left shows the molecular weight marker proteins. (B) Western-blotting of the SDS-PAGE shown in A. The thylakoid membranes of the cells grown in medium with or without manganese were treated as described in A. The antibody against spinach 33-kDa protein was used to detect the 30-kDa protein in the supernatant fraction of the thylakoid membranes treated with buffers and sonication. Lanes 1–10 correspond to lanes 3–12 of A.

of the membrane under the manganese-deficient condition may be related to the organization of the manganese cluster in the catalytic site and further analysis is required.

The manganese atoms in PS II were shown to interact with both the reaction center complex and the extrinsic 30–33 kDa protein [7–9,20]. The manganese atoms may be coordinated via appropriate ligands to both the reaction center complex and the extrinsic protein at the interface of the two components. As to the role of the manganese atoms, one argument exists that manganese atoms have a significant role in the binding of the extrinsic 30–33 kDa protein to the

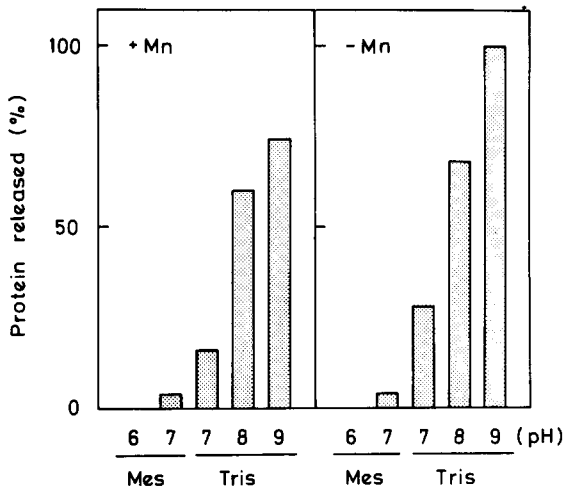

Fig. 4. The amount of the 30-kDa protein released from the thylakoid membranes of *Chlamydomonas* cells by treatment with alkaline buffers and sonication. Data were obtained from the densitograms of the gel shown in Fig. 3(A).

PS II membrane. Previously, the efficiency of reconstitution of the spinach 33-kDa protein and PS II membrane was shown to be reduced considerably by the loss of two atoms of loosely bound manganese in the membrane [11]. However, nonspecific binding of the protein to the membrane upon reconstitution is always inevitable in that approach and an accurate estimation of the role of manganese is difficult. The results obtained in this study using *Chlamydomonas* cells grown under manganese-deficient conditions showed that even when the amount of manganese atom in the membrane was decreased significantly and the activity of oxygen evolution was reduced concomitantly, there was no apparent change in the amount and stability of the extrinsic 30-kDa protein in the membrane. Thus, it is concluded that the manganese atoms involved in oxygen evolution of thylakoid membranes are not required for the binding of the extrinsic protein to the PS II membrane although they interact closely in the catalytic domain of the oxygen-evolution enzyme complex.

Acknowledgements

This work was supported by Grants 61540490 and 62621004 from the Ministry of Education, Science and Culture of Japan. The author is grateful to Dr. Hiroshi Inoue of Toyama University for providing the wild type strain of *Chlamydomonas reinhardtii* and for his suggestions concerning the cultures. Thanks are also due to Dr. Koh Iba for assistance in the preparation of antibody against spinach 33-kDa protein, and to Professor Mitsuo Nishimura for his generous support.

References

1 Ghanotakis, D.F. and Yocum, C.F. (1985) Photosynthesis Res. 7, 97–114.
2 Govindjee, Kambara, T. and Coleman, W. (1985) Photochem. Photobiol. 42, 187–210.
3 Dismukes, G.C. (1986) Photochem. Photobiol. 43, 99–115.
4 Yamamoto, Y., Tabata, K., Isogai, Y., Nishimura, M., Okayama, S., Matsuura, K. and Itoh, S. (1984) Biochim. Biophys. Acta 767, 493–500.
5 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 265–268.
6 Yamamoto, Y. (1988) J. Biol. Chem. 263, 497–500.
7 Abramowicz, D.A. and Dismukes, G.C. (1984) Biochim. Biophys. Acta 765, 318–328.
8 Yamamoto, Y., Shinkai, H., Isogai, Y., Matsuura, K. and Nishimura, M. (1984) FEBS Lett. 175, 429–432.
9 Yamamoto, Y. (1987) in Progress in Photosynthesis research (Biggins, J., ed.), Vol. I, pp. 593–596, Martinus Nijhoff, Dordrecht.
10 Dismukes, D.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278.
11 Kuwabara, T., Miyao, M., Murata, T. and Murata, N. (1985) Biochim. Biophys. Acta 806, 283–289.
12 Sager, R. and Granick, S. (1953) Ann. NY Acad. Sci. 56, 831–838.
13 Yamamoto, Y., Hermodson, M.A. and Krogmann, D.W. (1986) FEBS Lett. 195, 155–158.
14 Yamamoto, Y., Nakayama, S., Cohn, C.L. and Krogmann, D.W. (1987) Arch. Biochem. Biophys. 255, 156–161.
15 Yamamoto, Y. and Nishimura, M. (1985) Biochim. Biophys. Acta 724, 294–297.
16 Yamamoto, Y., Shimada, S. and Nishimura, M. (1983) FEBS Lett. 15, 49–53.
17 Cammarata, K., Tamura, N., Sayre, R. and Cheniae, G. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 311–320, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
18 Brudvig, G.W. and Crabtree, R.H. (1986) Proc. Natl. Acad. Sci. USA 83, 4586–4588.
19 Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–289.
20 Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260.