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The extrinsic proteins of an oxygen-evolving complex in marine diatom *Cylindrotheca fusiformis*

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ABSTRACT. Diatom *Cylindrotheca fusiformis's* oxygen-evolving complex (OEC) exhibited much lower 2,6-dichlorophenylindophenol (DCIP) photoreduction activity (the electron transfer rate during O_2 evolution) when compared to that of spinach. The protein composition of the OEC in *C. fusiformis* diatom thylakoids consist of only two extrinsic proteins with apparent molecular masses of 33 kDa and 15 kDa after Tris or NaCl washing. The 33 kDa protein was mainly responsible for O_2 evolving activity and for about 60% of the DCIP photoreduction activity, whereas the 15 kDa protein's photoreduction activity was approximately 40%. Beside the two extrinsic proteins, we found that Ca^{2+}, Cl^- and Mn ions also participate in the O_2 evolution of *C. fusiformis*. The concentrations of Ca^{2+} and Cl^- required for O_2 evolution in diatom thylakoids were higher than those for spinach, suggesting that the binding affinity of Ca^{2+} and Cl^- in diatom OEC is lower than that of spinach. The 33 kDa and 15 kDa proteins may coincidently function in Ca^{2+} trapping due to a 23% diminishment in content when diatom thylakoids were depleted of both proteins. These two proteins were also involved in the binding of Mn ions. Analysis of amino acid occurrence frequencies confirmed that both 33 kDa and 15 kDa proteins were extrinsic.

Keywords: Diatom thylakoids; Electron transfer; Extrinsic proteins; Oxygen-evolving complex; Photoreduction; Photosystem II.

Abbreviations: **BSA,** bovine serum albumin; **Chl,** chlorophyll; **DCIP,** 2,6-dichlorophenylindophenol; **EGTA,** ethyleneglycol-bis-(aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; **MES,** 2-(*N*-morpholino)ethanesulfonic acid; **OEC,** oxygen-evolving complex; **PS,** photosystem; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **Tris,** tris(hydroxymethyl)aminomethane.

INTRODUCTION

The involvement of an oxygen-evolving complex (OEC) with several extrinsic proteins has been clearly shown in studies of O_2 evolution in photosynthetic organisms (Seidler, 1996; Barber, 2002; Bricker and Ghanotakis, 2003; Ferreira et al., 2004; Roose et al., 2007; De Las Rivas et al., 2007; Barber, 2008). These extrinsic proteins of the OEC are located in the lumen of thylakoid membranes and differ greatly among species. In higher plants, there are at least three extrinsic proteins of 33 kDa (PsbO), 23 kDa (or 24 kDa) (PsbP) and 17 kDa (or 18 kDa) (PsbQ); in other organisms, there may be between two and five extrinsic proteins with different molecular weights (De Las Rivas and Barber, 2004; Enami et al., 2008). Along with these extrinsic polypeptides, tetranuclear manganese (Mn_4) cluster and cation calcium (Ca^{2+}) and anion chloride (Cl^-) cofactors also participate in O_2 evolution (Ono and Inoue, 1983; Andersson et al., 1984; Pstorius and Schmid, 1984; Coleman and Govindjee, 1987; Ono and Inoue, 1988; Debus, 1992; Loll et al., 2005; Yocum, 2008).

It has been demonstrated that washing PSII spinach particles in either Tris or NaCl inactivates O_2 evolution and the partial or complete release of three extrinsic proteins (Yamamoto et al., 1981; Ono and Inoue, 1983). We found that Tris washing completely removed 33 kDa, 23 kDa and 17 kDa proteins, while NaCl washing merely removed 23 kDa and 17 kDa proteins (Ono and Inoue, 1983). The 33 kDa component is involved in stabilizing Mn in the catalytic center of the OEC (Enami et al., 1994; Bricker and Frankel, 1998; De Las Rivas and Heredia, 1999). Mn is essential for water oxidation, and there is evidence that four Mn ions exist per O_2-evolving center in algae and higher plants (Hsu et al., 1987; Yamamoto, 1988; Ferreira et al., 2004; Barber and Murry, 2008; Pushkar et al., 2008). We found that two Mn ions of the Mn_4-cluster were released in the absence of the 33 kDa protein (Miyao and Murata,

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1984b). Upon Tris washing, a complete disassociation of the Mn_4-cluster occurred, while NaCl washing caused the release of a small amount of Mn ions from the Mn_4-cluster (Ghanotakis et al., 1984a; Enami et al., 1994). 23 kDa and 17 kDa proteins are required for Ca^{2+} and/or Cl^- binding, which is in turn necessary for optimal O_2 evolution (Andersson et al., 1984; Miyao and Murata, 1984a; Miyao and Murata, 1985). Exogenous Ca^{2+} was able to stimulate O_2 evolution of inactive PSII particles depleted of the 23 kDa and 17 kDa proteins (Ghanotakis et al., 1984b; Miqyass et al., 2007). Also, numerous functions have been attributed to the OEC anion Cl^- (Yocum, 2008), including its role in activating O_2 evolution (Coleman and Govindjee, 1987). Several lines of evidence also suggest that Cl^- functions as a ligand to the Mn_4-cluster and is required for the S-states transition (Popelkova and Yocum, 2007). Although OEC:Cl^- stoichiometry is believed to be 1:1, we found that the Cl^- binding site existed in both low and high affinity states (Lindberg and Andreasson, 1996; Tiwari et al., 2007; Yocum, 2008).

In contrast to the numerous studies on higher plants, there is little information about OECs in lower eukaryotic organisms such as diatoms. Compared with common higher plants, marine diatoms can grow in the ocean at high salt concentration. The mechanism of O_2 evolution in marine diatoms may well be distinct from that of common higher plants (Martinson et al., 1998; Nagao et al., 2007; Okumura et al., 2008). There are, however, higher plants that grow at high salt concentrations: the vast group of halophytes (Flowers and Colmer, 2008). In some of these plants, the OEC is modified with respect to that of common higher plants OEC made of 33 kDa, 23 kDa and 17 kDa subunits (Pagliano et al., 2009). We found that although the PSII of the halophyte *Salicornia veneta* lacks the 17 kDa protein and contains a reduced amount of the 23 kDa protein, there was no loss of functional activity. Similarly, we detected some modifications in the OEC composition of diatoms living in the oceans. For this reason, we tested the composition and functionality of the OEC from diatom *Cylindrotheca fusiformis*. The protein composition of this OEC was clarified by Tris- and NaCl-washing, showing that the *C. fusiformis* OEC contained only 33 kDa and 15 kDa extrinsic proteins and not 23 kDa protein. This showed that both 33 kDa and 15 kDa proteins of OEC were involved in O_2 evolution, the 33 kDa proteins playing the main role. Both the 33 kDa and 15 kDa proteins were associated with Mn binding and Ca^{2+} trapping.

MATERIALS AND METHODS

Diatom culture

The *C. fusiformis* diatom was generously provided by Professor Chia-Wai Li (National Tsing Hua University). For continuous culture, diatoms were grown in artificial sea water at 25°C under an 8-h day/16-h night cycle with illumination of about 24 μE m^{-1} sec^{-1} (1,200 Lux). Spinach (*Spinacia oleracea* L.) was obtained fresh from the local market.

Thylakoids isolation

Diatom cells in late log phase were collected by centrifugation at 4,000 ×*g* for 6 min. Pelleted cells were washed twice with buffer I made of 20 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10 mM NaCl and 5 mM $MgCl_2$. The washed cells were resuspended in buffer II made of 25 mM MES (pH 6.6), 0.3 M sucrose, 50 mM NaCl and 10 mM $CaCl_2$. The cells were then sonicated with an ultrasonicator (Heatsystem-ultrasonics Inc. model W-225) at 4°C set at 7.5 pulses per 50% duty cycle for 20 sec. The unbroken cells were removed by centrifugation at 4,000 ×*g* for 10 min. The sonication procedure was repeated 5-7 times to obtain the maximum yield of thylakoids. After sonication, the thylakoids were spun down at 15,000 ×*g* for 30 min. The pelleted particles were resuspended in buffer II and applied to 35-55% (w/v) sucrose gradient and then centrifuged at 13,000 ×*g* for 1 h. The thylakoids, which are brown in color, were located at the band of about 45% (w/v) sucrose concentration. This band containing thylakoids was collected by a needle, washed twice with buffer II, then collected by centrifugation at 15,000 ×*g* for 30 min. Spinach thylakoids were prepared as previously described (Pan et al., 1987) and finally resuspended in storage medium [25 mM HEPES-NaOH (pH 7.5), 10 mM NaCl, 10% (v/v) glycerol, 2.5% (v/v) dimethyl sulfoxide, 2 mM dithiothreitol, 20 mg BSA mL^{-1}, and 2 mg chlorophyll (Chl) mL^{-1}]. After preparation, the Chl contents of the diatom and spinach thylakoids were measured using the respective methods of Jeffery (1986) and Arnon (1949).

Photoreduction of DCIP in thylakoids

The photoreduction of DCIP was measured spectrophotometrically at 590 nm as the electron transport rate during O_2 evolution in thylakoids (Pan et al., 1987; Eastman et al., 1997). Thylakoids (8 μg Chl mL^{-1}) and DCIP (33.3 μM) were applied into an assay medium containing 20 mM MES (pH 6.6) and 0.3 M sucrose with addition of different ion concentrations under an illumination of 150 μE m^{-1} sec^{-1}. If required, thylakoids were washed twice with buffer II containing 100 μM EGTA to remove Ca^{2+} or buffer III made of 25 mM MES (pH 6.6) and 0.3 M sucrose to remove Cl^-.

Tris and NaCl thylakoids washing

For salt washing, thylakoids (200-300 μg Chl mL^{-1}) were incubated with 1.2 M Tris-HCl (pH 8.3) or 0.8 M NaCl for 30 min at 4°C under room light of 10-20 μE m^{-1} sec^{-1} (Yamamoto et al., 1981). Thylakoids were then collected at 15,000×g for 30 min and then resuspended in buffer II. The Tris- or NaCl-washed supernatants were dialyzed against 20 mM sodium phosphate buffer (pH 7.0) and then concentrated by ultrafiltration (Amicon model 8010). For SDS-PAGE analysis, the thylakoid proteins of the supernatants were precipitated with 80% (v/v) acetone.

Gel electrophoresis

Protein components were analyzed with 13.5% SDS-

PAGE. The polypeptidic bands were visualized either by coomassie blue or silver staining. The relative amount of the protein component on SDS-PAGE was determined by a densitometric analysis (Lee et al., 2006).

Purification of 33 kDa and 15 kDa proteins from diatom thylakoids

The 33 kDa protein was extracted from diatom thylakoids with 1.2 M Tris-HCl (pH 8.3) buffer after 0.8 M NaCl treatment, and the 15 kDa protein was extracted with 0.8 M NaCl. The crude extracts were concentrated by ultrafiltration. The concentrations of 33 kDa and 15 kDa proteins were determined by a modified Lowry method (Larson et al., 1980).

Measurement of Ca and Mn content in diatom thylakoids

The contents of Ca and Mn in diatom thylakoids were determined with an Inductively Coupled Plasma Emission Spectrometer (ICPAES). To remove salts, the Tris- or NaCl-washed diatom thylakoids were dialyzed with distilled deionized water overnight. Thylakoids were collected at 15,000 ×*g* for 30 min and then dissolved with pure HNO_3 and H_2SO_4 (4:1) just prior to measurement.

Reconstitution of Tris- and NaCl-washed diatom thylakoids

The Tris- or NaCl-washed diatom thylakoids were incubated with 33 kDa and/or 15 kDa proteins with or without addition of 40 mM $MnCl_2$ in buffer II at 4°C stirring for 1 h under room light of 10-20 μE m^{-1} sec^{-1}. The protein to Chl content ratio was 2:1 (μg proteins versus μg Chl) in the reaction solution. The reconstituted thylakoids were then collected by centrifugation at 15,000 ×*g* for 30 min.

Amino acid composition analysis

To prepare the 33 kDa and 15 kDa proteins for amino acid analysis, polypeptide bands on SDS-PAGE were visualized with 4 M sodium acetate in the dark (Higgins and Dahmus, 1979). The 33 kDa and 15 kDa bands were cut, chopped into pieces, homogenized, and then immersed into distilled water and stirred overnight. The eluted proteins were precipitated with 80% (v/v) acetone and dissolved with 6 M HCl in new tubes at 110°C for 24 h in the presence of 1% (v/v) phenol. The hydrolytes were subjected to analysis by an LKB4150 Alpha Acid analyzer (Taipei Regional Analytical Instrumentation Center).

RESULTS

Effect of Tris and NaCl washing on electron transfer in the OEC of diatom thylakoids

The polypeptidic components in the OEC of diatom thylakoids were analyzed by SDS-PAGE and compared with those of spinach. In contrast to spinach thylakoids (Figure 1A, lane 1), diatom thylakoids showed two proteins of 33 kDa and15 kDa (molecular weights estimated in accordance with protein markers) but no 23 kDa protein (Figure

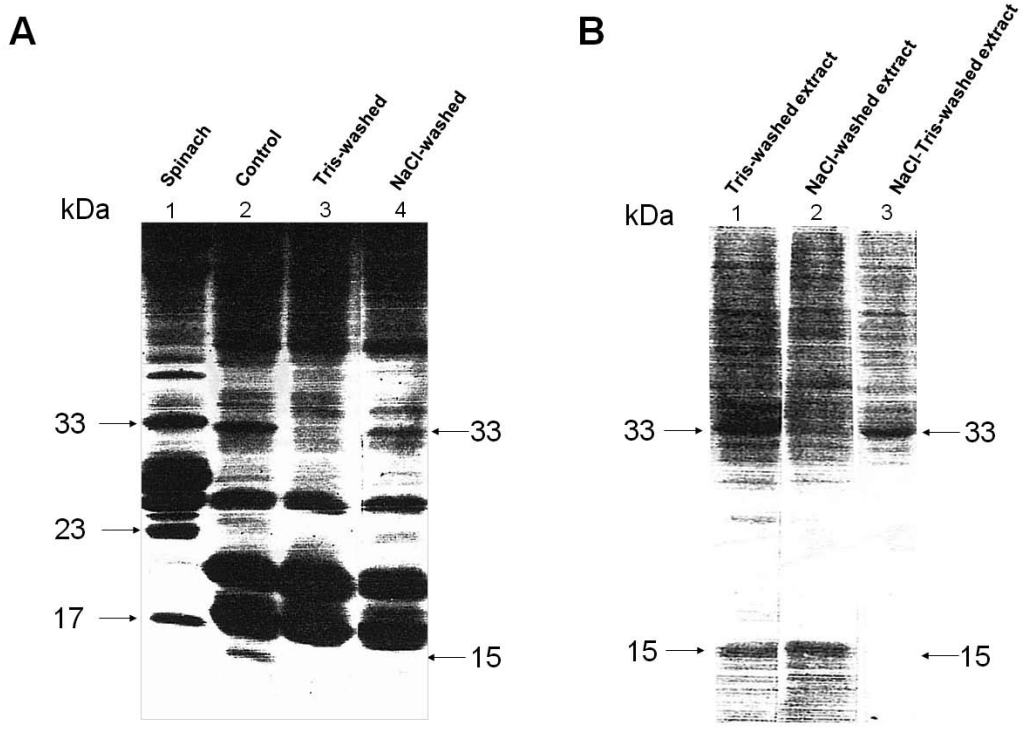

Figure 1. SDS-PAGE analysis of thylakoid membranes. (A) Lane 1, spinach control membranes; lane 2, diatom control membranes; lane 3, Tris-washed diatom membranes; lane 4, NaCl-washed diatom membranes. (B) Lane 1, Tris-washed diatom extract; lane 2, NaCl-washed diatom extract; lane 3, Tris-washed diatom extract after NaCl treatment.

1A, lane 2). After washing with 1.2 M Tris (Figure 1A, lane 3) or 0.8 M NaCl (Figure 1A, lane 4), some proteins were depleted from the diatom thylakoids, in particular the 33 kDa and 15 kDa proteins. Subsequently, the 33 kDa and 15 kDa proteins were found in the crude extract of the Tris-washed supernatant (Figure 1B, lane 1). The 15 kDa protein was found in the NaCl-washed extract (Figure 1B, lane 2) and the 33 kDa protein in the NaCl-Tris-washed extract (Figure 1B, lane 3). These results suggested that the diatom O_2 evolving center contained 33 kDa and 15 kDa proteins but lacked 23 kDa protein.

In order to study the functionality of electron transfer during O_2 evolution in the diatom OEC, the photoreduction of DCIP was measured (Table 1). The results showed that the diatom thylakoids exhibited a much lower DCIP photoreduction rate than spinach thylakoids. The photoreduction activity of the Tris-washed (i.e., 33 kDa- and 15 kDa-depleted) diatom thylakoids was completely inhibited; as it was in Tris-washed (i.e., 33 kDa-, 23 kDa- and 17 kDa-depleted) spinach thylakoids. The photoreduction activity of the NaCl-washed (i.e., 15 kDa-depleted) diatom thylakoids was reduced to 60% of the control; however, that of the NaCl-washed (i.e., 17 kDa- and 23 kDa-depleted) spinach thylakoids was reduced to 36% of the control.

Requirement of Ca^{2+} and Cl^- for electron transfer in the OEC of diatom thylakoids

It has been reported that Ca^{2+} and Cl^- binding to OEC is related to the 23 kDa protein in higher plants (Ifuku et al., 2005; Miqyass et al., 2007); we decided to assess the impact of Ca^{2+} and Cl^- on the diatom OEC lacking the 23 kDa protein.

To clarify the effect of Ca^{2+} on electron transfer during O_2 evolution in the OEC of diatom and spinach thylakoids, the DCIP photoreduction was examined at different $CaCl_2$ concentrations (Figure 2). Before measuring, diatom and spinach thylakoids were washed with buffer II containing the chelator EGTA to remove endogenous Ca^{2+} (Stevens and Lukin, 2003; Semin et al., 2008). The data showed that the photoreduction activity of EGTA-washed diatom thylakoids was reduced to 42% of the control, whereas that of EGTA-washed spinach thylakoids was not significantly affected. This suggested that the Ca^{2+} binding in the diatoms' OEC may be not as tight as it is in spinach. Re-addition of

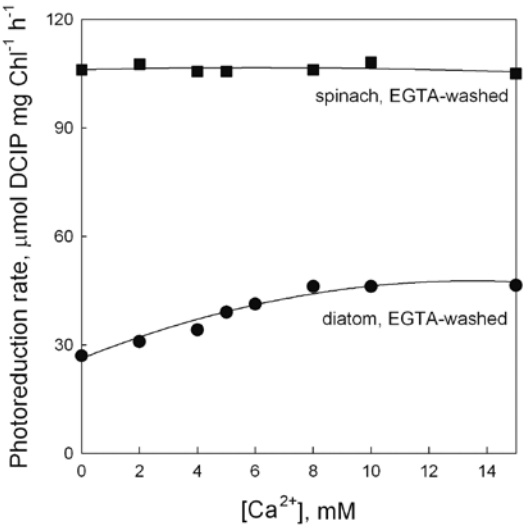

Figure 2. Effect of Ca^{2+} on photoreduction rate of EGTA-washed thylakoid membranes. DCIP photoreduction rates of 100 μM EGTA washed thylakoid membranes were measured at different designated $CaCl_2$ concentrations in the presence of 40 mM NaCl. (●), diatom; (■), spinach.

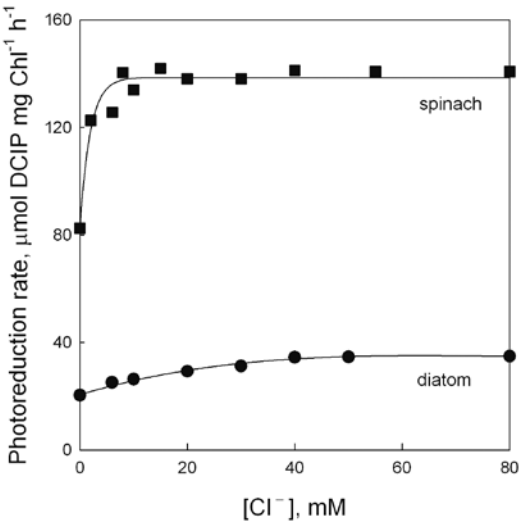

Figure 3. Effect of Cl^- on photoreduction rate of thylakoid membranes. DCIP photoreduction rates of diatom or spinach thylakoid membranes were measured at different designated NaCl concentrations. (●), diatom; (■), spinach.

Table 1. Effect of Tris or NaCl washing on DCIP photoreduction rate of diatom and spinach thylakoids.

Thylakoids	Diatom			Spinach			
	DCIP photoreduction rate[a] (%)	Protein released[b] (%)		DCIP photoreduction rate[a] (%)	Protein released[b] (%)		
		33 kDa	15 kDa		33 kDa	23 kDa	17 kDa
Control	100	100	100	100	100	100	100
Tris-washed	0	95	90	0	100	100	100
NaCl-washed	60	10	90	36	1	91	100

[a]100% represented the photoreduction rate of 42.7 μmole DCIP mg Chl^{-1} h^{-1} in diatom or 160 μmole DCIP mg Chl^{-1} h^{-1} in spinach.
[b]The protein released was estimated from SDS-PAGE of Tris- or NaCl-washed diatom (Figure 1A) and spinach (data not shown) thylakoids after analyzed by a densitometer.

more than 10 mM exogenous Ca^{2+} into EGTA-washed diatom thylakoids restored the photoreduction activity.

We also tested the effect of Cl^- on electron transfer during O_2 evolution in the OEC of diatom and spinach thylakoids (Figure 3). Before measuring, the diatom and spinach thylakoids were washed with buffer III to remove Cl^-. We found that the DCIP photoreduction activity of diatom thylakoids increased with increasing Cl^- concentrations, reaching an optimal rate at 40 mM Cl^-, whereas spinach thylakoid activity reached an optimal rate at 15 mM Cl^-. These results showed that the electron transfer rate during O_2 evolution of diatom thylakoids was about three times slower than for spinach. The double reciprocal plots revealed that the Mechelis-Menton constant for the Cl^- requirement of diatom ($K_{m,d}$) was about 5 mM, while that of spinach ($K_{m,s}$) was 0.8 mM (Figure 4). This indicated that the binding affinity of Cl^- for diatom thylakoids was lower than that for spinach thylakoids.

Effect of Ca^{2+} and Cl^- on electron transfer in the OEC of 15 kDa-depleted diatom thylakoids

To understand whether the 15 kDa protein of the diatom OEC could compensate for the 23 kDa protein of the higher plant OEC for Ca^{2+} binding, the electron transfer activity was examined by re-adding Ca^{2+} into 15 kDa-depleted (NaCl-washed) diatom thylakoids (Boussac et al., 1985; Ifuku et al., 2005). The data showed that the DCIP photoreduction rates of the 15 kDa-depleted diatom thylakoids were not changed by re-adding exogenous Ca^{2+}, whereas those of the 23 kDa- and 17 kDa-depleted (NaCl-washed) spinach thylakoids could be stimulated by re-adding Ca^{2+} above 5 mM (Figure 5).

Furthermore, addition of 10 mM Ca^{2+} to diatom thylakoids stimulated DCIP photoreduction activity and reduced the Cl^- requirement from 40 mM to 20 mM (Figure 6, curve a, b). Addition of 10 mM Ca^{2+} to 15 kDa-depleted (NaCl-washed) diatom thylakoids (Figure 6, curve c) did not stimulate DCIP photoreduction activity; however, it prevented a further reduction (Figure 6, curve d). This suggested that the effect of Ca^{2+} on O_2 evolution of diatom thylakoids was dependent on the presence of the 15 kDa protein. The requirement of Cl^- for O_2 evolution in the 15 kDa-depleted diatom thylakoids was diminished by adding exogenous Ca^{2+}, implying that the function of the 15 kDa protein may be partially replaced by Ca^{2+}.

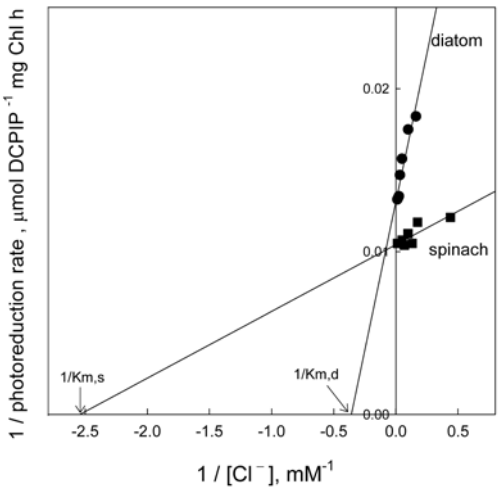

Figure 4. Double reciprocal plots of the dependence of the photoreduction rate on the concentrations of Cl^-. (●), diatom; (■), spinach. $K_{m,d}$, Mechelis-Menton constant for Cl^- requirement of diatom; $K_{m,s}$, Mechelis-Menton constant for Cl^- requirement of spinach.

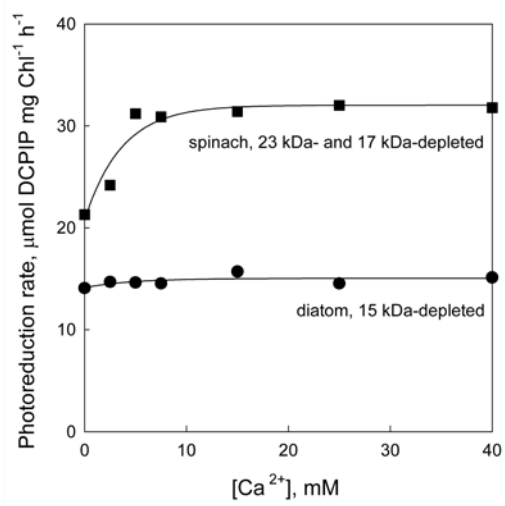

Figure 5. Effect of Ca^{2+} on photoreduction rate of 15 kDa-depleted diatom thylakoid. DCIP photoreduction rates of 0.8 M NaCl washed diatom thylakoid membranes were measured at different designated $CaCl_2$ concentration in the presence of 40 mM NaCl. (■), NaCl-washed, 23 kDa- and 17 kDa-depleted, spinach thylakoids; (●), NaCl-washed, 15 kDa-depleted, diatom thylakoids.

Table 2. Ca and Mn abundance and DCIP photoreduction rate in 33 kDa-depleted and/or 15 kDa-depleted diatom thylakoids.

Diatom thylakoids	DCIP photoreduction rate[a] (%)	Ca abundance[b] (atoms/400 Chl)	Mn abundance (atoms/400 Chl)
Control	100	1	3.8
33 kDa-depleted, 15 kDa-depleted (Tris-washed)	0	0.23	2.1
15 kDa-depleted (NaCl-washed)	60	0.29	3.1

[a]100% represented the photoreduction rate of 36.4 µmole DCIP mg Chl^{-1} h^{-1} in diatom.
[b]The Ca abundance of the control was set to 1 atom per 400 Chl molecules in accordance with Pushkar et al. (2008) so that all the original data were divided by a factor of 436 which was obtained in the background.

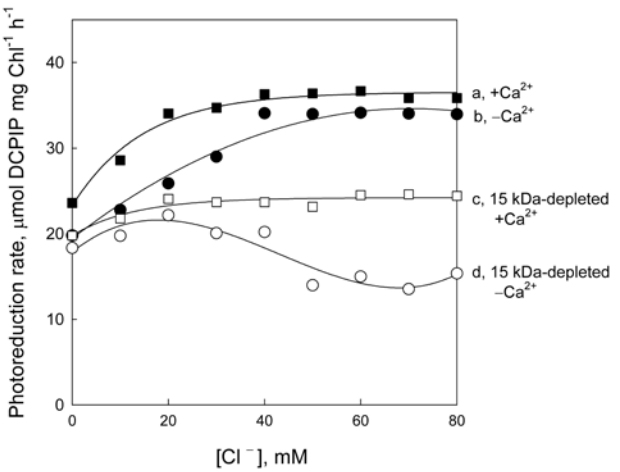

Figure 6. Effect of Cl^- on photoreduction rate of 15 kDa-depleted diatom thylakoids in the absence and presence of Ca^{2+}. DCIP photoreduction rates of diatom thylakoids were measured in the absence (curve a, ●) and presence (curve b, ■) of 10 mM $CaCl_2$, or, those of 15 kDa-depleted (NaCl-washed) diatom thylakoids were measured in the absence (curve c, ○) and presence (curve d, □) of 10 mM $CaCl_2$ at different designated NaCl concentrations.

Ca and Mn abundance in the diatom OEC

In an attempt to characterize more precisely the relationship between Ca^{2+} and the 15 kDa protein, the Ca abundance was measured (Table 2). In the 15 kDa-depleted (NaCl-washed) diatom thylakoids, the Ca content was reduced to about 0.29 atoms per 400 Chl molecules, suggesting that the 15 kDa protein mediated the binding of 0.71 Ca atoms. In the 33 kDa- and 15 kDa-depleted (Tris-washed) diatom thylakoids, the Ca content was reduced to about 0.23 atoms, suggesting that 33 kDa protein mediated the binding of 0.06 Ca atoms. Although the 33 kDa protein has been demonstrated to have a Ca^{2+} binding site, the 23 kDa and 17 kDa proteins of higher plants were also reported to be involved in Ca^{2+} binding (Ghanotakis et al., 1984b; Kruk et al., 2003; Murray and Barber, 2006; Miqyass et al., 2007). Our Tris treatment results implied that the binding of Ca^{2+} in the diatom *C. fusiformis* OEC was more related to the 15 kDa protein than to the 33 kDa protein (Table 2). This was consistent with the results in Figure 5, showing that the reduced O_2 evolving activity of the 15 kDa-depleted diatom thylakoids could not be restored by re-adding exogenous Ca^{2+}.

The number of Mn atoms in the two types of diatom thylakoids was also determined (Table 2). This analysis showed that 33 kDa- and 15 kDa-depleted (Tris-washed) diatom thylakoids contained 2.1 Mn atoms per 400 Chl molecules, while 15 kDa-depleted (NaCl-washed) diatom thylakoids contained 3.1 Mn atoms. This demonstrated that both 33 and 15 kDa proteins were involved in Mn binding in the diatom OEC.

Further characterization of the 33 kDa and 15 kDa proteins

To study further how the 33 kDa and 15 kDa proteins cooperated in the diatom OEC, reconstitution experiments were carried out (Ono and Inoue, 1984). We showed earlier that Tris washing released 33 kDa and 15 kDa proteins, extracted two out of four Mn atoms, and reduced the O_2 evolving activity to zero (Table 2). By reconstituting Tris-washed thylakoids with the 33 and 15 kDa proteins, the reduced O_2 evolving activity could be restored to 33% of the control (Table 3). Moreover, NaCl washing released the 15 kDa protein, extracted one out of four Mn atoms, and reduced the DCIP photoreduction activity to 48% of the control (Table 2). By reconstituting NaCl-washed thylakoids with the 15 kDa protein, the reduced DCIP photoreduction activity could be restored to 66% of the control (Table 3). There was no increase in activity by further addition of Mn^{2+}.

We analyzed the amino acid compositions of the 33 kDa and 15 kDa proteins of the diatom OEC and the 33 kDa and 17 kDa proteins of the spinach OEC (Table 4). The nonpolar R group composition of the diatom 33 kDa protein (40.1%) was larger than that of spinach (35.5%). The total number of amino acid residues of the 33 kDa protein in the diatom OEC was 249 residues per protein, as in spinach. The non-polar composition of the diatom 15 kDa protein (41.4%) was larger than that of the corresponding spinach 17 kDa protein (39.4%). The total num-

Table 3. Restoration of DCIP photoreduction rate by reconstituting 33 kDa and/or 15 kDa proteins into diatom thylakoids with or without Mn^{2+}.

Diatom thylakoids	Addition[a]	DCIP photoreduction rate[b] (%)
Control	None	100
33 kDa-depleted	None	0
15 kDa-depleted (Tris-washed)	+ 33 kDa + 15 kDa	0
	+ 33 kDa + 15 kDa + Mn^{2+}	33
15 kDa-depleted (NaCl-washed)	None	48
	+ 15 kDa	66
	+ 15 kDa + Mn^{2+}	66

[a]Under the conditions: proteins Chl^{-1} = 0.8 mol mol^{-1} and Mn^{2+} = 40 mM.
[b]100% represented the photoreduction rate of 30.0 μmole DCIP mg Chl^{-1} h^{-1} in diatom.

Table 4. Amino acid compositions of 33 kDa and 15 kDa proteins in diatom and those of 33 kDa and 17 kDa polypeptides in spinach.

Amino acid	Diatom (residues[a]/protein)		Spinach (residues[a]/protein)	
	33 kDa	15 kDa	33 kDa	17 kDa
Lys	7.6	7.9	9.2	9.2
His	0.7	1.2	0.0	0.9
Arg	4.6	5.4	2.7	5.6
Asp	10.9	12.8	8.8	11.2
Glx	13.0	9.0	13.2	11.1
Thr	5.3	5.3	7.7	5.3
Ser	3.7	4.7	7.4	8.6
Pro	5.6	5.7	6.1	8.8
Gly	11.4	9.3	12.3	5.1
Ala	8.4	10.4	6.0	9.0
Val	7.0	6.4	7.2	3.7
Met	1.4	1.1	0.4	0.0
Ile	5.1	5.3	3.0	3.8
Leu	8.1	8.6	7.1	11.4
Tyr	2.8	3.4	3.2	3.6
Phe	4.1	3.2	5.3	2.1
Trp	0.4	0.7	0.4	0.6
Cys	ND[b]	ND[b]	ND[b]	ND[b]
Total residues	249	122	249	139
Percentage				
Nonpolar R group	40.1%	41.4%	35.5%	39.4%
Polar R group				
Uncharged and negative	46.9%	44.1%	52.6%	44.9%
Positive	13.0%	14.5%	11.9%	15.7%

[a]The amino acid composition was calculated from the moles of residues divided by the moles of total proteins.
[b]Not determined.

ber of amino acid residues of the diatom 15 kDa protein was 122 while that of the spinach 17 kDa protein was 139. The location of a protein with respect to the membrane can be predicted from its amino acid composition (Cantor and Schimmel, 1980). To obtain information about the internal or external membrane location of the proteins, the following parameters were assessed: R_3, the mole fraction ratio of the two amino acid sets, $H_{\Phi ave}$, average of total hydrophobicity, Z, discriminate function value, and NPS, the fraction of non-polar side chains (Table 5). Comparing these values, we confirmed that diatom 33 kDa and 15 kDa proteins, which were released from thylakoids by Tris or NaCl washing, were extrinsic proteins.

DISCUSSION

The extrinsic proteins of OEC, located in the lumen of thylakoid membranes, show significant differences among species. These extrinsic proteins have been examined in several organisms and classified into cyanobacterial type (PsbO, PsbV and PsbU), red algal type (PsbO, PsbQ', PsbP and PsbU), and green algal and higher plant type (PsbO, PsbQ and PsbP) (De Las Rivas et al., 2007; Roose et al., 2007; Enami et al., 2008). The OEC of the diatom *Chaetoceros garacilis* has very recently been shown to include five extrinsic proteins with molecular weights of 33 kDa (PsbO), 20 kDa (PsbQ'), 17 kDa (PsbV), 13.5 kDa and 12 kDa (PsbU) (Nagao et al., 2007; Enami et al., 2008). Unlike that of *C. gracilis*, the OEC of diatom *C. fusiformis* contained only two extrinsic proteins 33 kDa and 15 kDa. Undoubtedly, the 33 kDa protein in *C. fusiformis* acted in accordance with the highly-conserved PsbO protein in all O_2 evolving photosynthetic organisms (Tohri et al., 2002; De Las Rivas et al., 2007). Interestingly, the 23 kDa (PsbP) protein did not appear in either *C. fusiformis* or *C. gracilis* (Nagao et al., 2007). The 15 kDa protein in *C. fusiformis* could function independently and may be a 20 kDa (PsbQ')-like protein like that in *C. gracilis* (Nagao et al., 2007; Enami et al., 2008).

In our current study, 95% of the 33 kDa protein of the

Table 5. The parameters of R3, $H_{\Phi ave}$, Z and NPS for 33 kDa and 15 kDa proteins in diatom and spinach.

Protein	R_3[a]	$H_{\Phi ave}$[b]	Z[c]	NPS[d]	Categorization
Membrane protein					
Believed internal	0.57±0.18	1.197±0.097	0.52±0.11		Internal
Believed external	1.37±0.35	0.986±0.075	0.16±0.17		External
Diatom					
33 kDa	1.69	1.062	0.05	0.22	External
15 kDa	1.76	1.067	0.05	0.29	External
Spinach					
33 kDa	1.62	0.921	-0.01	0.18	External
23 kDa	1.61	0.934	0.01	0.18	External
17 kDa	1.30	1.121	0.20	0.25	External

[a]R_3 was the mole fraction ratio of the two amino acid sets. ($R_3 = \frac{jXj}{kXk}$; j, k, the number of residues; X_j= the composition of Lys, Arg, His, Asp, Glx, or Glu; X_k= the composition of Ile, Leu, Try, Phe, Met, or Val).
[b]$H_{\Phi ave}$ was the total hydrophobicity divided by the number of residues.
[c] Z was the discriminate functions of linear combinations of R_3 and $H_{\Phi ave}$. ($Z=-0.345R_3+0.0006\ H_{\Phi ave}$).
[d]NPS (non-polar side chains) was calculated as the fraction of the nonpolar amino acid residues such as Trp, Ile, Try, Phe, Pro, Leu and Val to the total number of residues.

diatom OEC can be released by washing with 1.2 M Tris (Table 1), while that of spinach could be completely released using the lower concentration of 0.9 M Tris (data not shown). This implied that the diatom 33 kDa protein may be located in a more hydrophobic environment than the spinach one. We further confirmed this observation by estimating the parameters of R_3, $H_{\Phi ave}$, Z and NPS, which provide information about the location of internal or external membrane proteins (Table 5). The $H_{\Phi ave}$ of the diatom 33 kDa protein (1.062) was larger than that of spinach (0.921), suggesting that the diatom 33 kDa polypeptide was located in a more hydrophobic environment. A similar result was obtained earlier using cyanobacteria (Steward et al., 1985). Here, when compared to spinach, only a minimal amount of 33 kDa protein was released via alkaline Tris treatment. The $H_{\Phi ave}$ of the diatom 15 kDa protein (1.067) was smaller than that of spinach (1.121). Meanwhile, the amount of the 15 kDa protein (100%) released from diatom thylakoids by NaCl treatment was greater than that of the 17 kDa (90%) from spinach. We therefore believe that the 15 kDa protein of the diatom OEC was located in a more hydrophilic environment than the corresponding 17 kDa protein of spinach.

The concentrations of Ca^{2+} and Cl^- required for diatom thylakoids was higher than those observed for spinach thylakoids (Figures 3 and 4), suggesting that the binding affinity of Ca^{2+} and Cl^- in the diatom OEC was lower than for spinach. Tris treatment reduced the amount of Mn ions in diatom thylakoids from 3.8 to 2.1 atoms and in spinach thylakoids it was reduced from 4 to 0.65 atoms, leading to complete inhibition of DCIP photoreduction (Table 2) (Hsu et al., 1987). A previous study showed that spinach PSII with 1.4 Mn upon NH_2OH treatment still retained 30% O_2 evolving activity (determined by the reduction of DCIP) and 30% multiline EPR signal intensity of the control (Miller and Brudvig, 1989). However, upon Tris washing, diatom PSII still retained 2 Mn ions and did not exhibit any DCIP photoreduction activity (Table 2). PSII should be able to reduce DCIP if an OEC center is intact. Thus, the loss of the full Mn cluster from only a fraction of the center, leaving the remaining center intact, should not occur. Moreover, a number of studies indicated that the Mn_4-cluster is coordinated by certain residues of the D1 and CP43 polypeptides (Barber and Murray, 2008; Stull et al., 2010). This implies that the Mn_4-cluster may be disrupted by Tris or NaCl washing, but that the Mn ions may not be completely depleted when 33 kDa protein is removed (Williamson, 2008). Subsequently, DCIP photoreduction activity was restored only by re-adding Mn^{2+} plus the 33 and 15 kDa proteins into the 33 kDa- and 15 kDa-depleted (Tris-washed) diatom thylakoids (Table 3). This suggested that these depleted Mn atoms played a crucial role in O_2 evolution. Their different requirements for the essential inorganic components of the Mn^{2+}, Ca^{2+} and Cl^- ions indicated that the diatom and spinach OECs have different characteristics.

Moreover, we found that the 15 kDa protein is required for O_2 evolution of the diatom OEC. The specific function of the 15 kDa protein was studied in the NaCl-washed diatom thylakoids. NaCl washing released the 15 kDa protein from diatom thylakoids and reduced their O_2 evolving activity to 63% of the control (Table 1). The 15 kDa-depleted (NaCl-washed) diatom thylakoids were more sensitive to high Cl^- concentration above 40 mM (Figure 6, curve d). This result suggested a possible role for the 15 kDa in preserving the O_2 evolving activity in diatoms exposed to the high concentrations of NaCl in the ocean.

From the results of Figure 5, we proposed that the function of the 15 kDa protein in the diatom OEC was related to Ca^{2+} binding, though it has been shown that the 23 kDa

protein in the higher plant OEC acted in Ca^{2+} trapping (Ghanotokis et al., 1984a; Miyao and Murata, 1984a). NaCl treatment depleted thylakoids of the 15 kDa protein and reduced their Ca^{2+} content to about 30% of the control (Table 2). The reduced O_2 evolving activity of diatom thylakoids without 15 kDa protein was not recovered by adding external Ca^{2+} (Figure 5 and Figure 6, curve c), whereas the O_2 evolving activity of diatom thylakoids containing the 15 kDa protein were restored to the optimal rate in the presence of different Cl^- concentrations (Figure 6, curve a). This suggested that the 15 kDa protein was related to the binding of Ca^{2+} in diatom thylakoids and that Ca^{2+} could participate in O_2 evolution only in the presence of the 15 kDa protein.

Reconstitution of Tris-washed thylakoids with 33 kDa and 15 kDa proteins restored O_2 evolving activity to 33% of the control (Table 3). The reason why the O_2 evolving activity was only partially restored may be due to the absence of Ca^{2+}, which was intentionally diminished in this experiment to avoid its interference. The reconstitution of the extrinsic proteins into salt washed PSII with addition of Ca^{2+} may increase the restored O_2-evolving activity (Ghanotakis et al., 1984b; Enami, 1994; Seidler et al., 1996; Suzuki et al., 2005). It is believed that the binding of Ca^{2+} could either provide the appropriate environment for a stable Mn complex or affect its redox properties (Bricker and Ghanotokis, 2003).

Based on these results, we concluded that two extrinsic proteins, 33 kDa and 15 kDa proteins, and three cofactors, Ca^{2+}, Cl^- and Mn ions, participated in O_2 evolution in the diatom *C. fusiformis*. The composition and functionality of the diatom *C. fusiformis* OEC is thus somewhat distinct from that of the spinach OEC.

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矽藻放氧複合體的膜外蛋白研究

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矽藻 (*Cylindrotheca fusiformis*) 放氧複合體 (oxygen-evolving complex; OEC) 的 2,6-dichlorophenylindophenol (DCIP) 光還原活性 (氧氣釋放時的電子傳遞速率) 較菠菜低。利用 Tris 或 NaCl 沖洗法，得知 *C. fusiformis* 的 OEC 是由位於類囊體表面的兩個分子量為 33 kDa 及 15 kDa 膜外蛋白所組成。33 kDa 蛋白是放氧活性的主要蛋白，因其負責約 60% DCIP 光還原活性，而 15 kDa 蛋白約 40%。除了 *C. fusiformis* 兩個膜外蛋白之外，發現 Ca^{2+}、Cl^- 及 Mn 離子也參與氧氣的釋放。矽藻類囊體氧氣釋放時之 Ca^{2+} 及 Cl^- 需求濃度較菠菜高，推測可能是矽藻 OEC 與 Ca^{2+} 及 Cl^- 的結合力較菠菜低。再者，33 kDa 及 15 kDa 兩個膜外蛋白與 Ca^{2+} 之吸附有關，因為當矽藻的類囊體中此兩蛋白被去除時，Ca^{2+} 含量下降至 23%。這兩個蛋白也證實與 Mn 離子的結合有關。經過胺基酸出現頻率分析之後，確認 33 kDa 及 15 kDa 蛋白皆為膜外蛋白。

關健詞：矽藻類囊體；電子傳遞；膜外蛋白；放氧複合體；光還原；光系統 II。