DOI: 10.1007/s11099-018-0778-8 PHOTOSYNTHETICA 56 (1): 185-191, 2018

The PsbQ′ protein affects the redox potential of the Q_A in photosystem II

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Abstract

Red alga contains four extrinsic proteins in photosystem II (PSII), which are PsbO, PsbV, PsbU, and PsbQ′. Except for the PsbQ′, the composition is the same in cyanobacterial PSII. Reconstitution analysis of cyanobacterial PSII has shown that oxygen-evolving activity does not depend on the presence of PsbQ′. Recently, the structure of red algal PSII was elucidated. However, the role of PsbQ′ remains unknown. In this study, the function of the acceptor side of PSII was analyzed in PsbQ′-reconstituted PSII by redox titration of Q_A and thermoluminescence. The redox potential of Q_A was positively shifted when PsbQ′ was attached to the PSII. The positive shift of Q_A is thought to cause a decrease in the amount of triplet chlorophyll in PSII. On the basis of these results, we propose that PsbQ′ has a photoprotective function when irradiated with strong light.

Additional key words: diversity; evolution; photoinhibition; photosynthesis.

Introduction

The overall processes of photosynthesis are quite similar among photosynthetic organisms. However, photosynthetic organisms show a high degree of diversity, such as light-harvesting systems, photosynthetic pigments, peptide compositions, and primary reactions. Oxygenic photosynthetic organisms are thought to have evolved from cyanobacteria (Shevela and Govindjee 2011). Photosynthetic eukaryotes originated following a primary endosymbiotic event between an ancestral cyanobacterium and an early eukaryotic phagotroph (Björn 2015). This primary endosymbiosis event gave rise to two autotrophic clades: green algae (and green plants) and red algae. Therefore, red algae are one of the oldest groups of eukaryotic algae.

The big difference between red algae and green plants in primary photosynthesis is in the light-harvesting systems. Red algae use phycobilisomes, which are soluble protein complexes, but green plants use light-harvesting membrane proteins. In addition, the composition of the extrinsic protein of PSII is quite diverse. Green plant PSII binds PsbO, PsbP, and PsbQ. On the other hand, cyanobacterial PSII binds PsbO, PsbV, and PsbU (Shen and Inoue 1993, Eaton-Rye 2005, Enami *et al*. 2005, Enami *et al*. 2008, Bricker *et al*. 2012). PsbQ′ is the 4th extrinsic protein of PSII in red algae and diatoms (Ohta *et al*. 2003, Nagao *et al*. 2010). Moreover, Psb31 was identified as the 5th extrinsic protein in diatoms (Nagao *et al*. 2010, Nagao *et al*. 2013). In the evolutionary process, red algae bind the PsbQ′ protein in addition to the cyanobacterial extrinsic proteins of PSII. PsbQ′ shows low homology with PsbQ of green algal and higher plant PSII (Ohta *et al*. 2003). The gene homolog of PsbQ is present in cyanobacteria as CyanoQ (De Las Rivas *et al*. 2004, Fagerlund *et al*. 2011). However, this protein is missing in the crystal structure of PSII. The homology of CyanoQ and PsbQ′ is also low.

The structures of PsbQ, CyanoQ, and PsbQ′ have been elucidated by X-ray crystallographic analysis (Calderone *et al*. 2003, Balsera *et al*. 2005, Jackson *et al*. 2010, Ago *et al*. 2016). Despite low homology, the overall structures,

Received 8 May 2017, *accepted* 16 August 2017 *published as online-first* 10 January 2018.
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Abbreviations: Chl – chlorophyll; CP43 – chlorophyll protein 43 kDa; CP47 – chlorophyll protein 47 kDa; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; IPTG – isopropyl β-D-1-thiogalactopyranoside; Ni-NTA – nickel-nitrilotriacetic acid.
Acknowledgments: This study was supported by the Grants-in-Aids for Scientific Research from JSPS (No. 24370025, 26220801, 17K07453 to T.T.) and a grant from JST PRESTO (T.T.). The authors thank Professor Kintake Sonoike for his kind support. We express our heartfelt gratitude to Professor Govindjee for his numerous invaluable contributions to the research field of photosynthesis.

which consist of four bundle helix-type proteins, are similar to each other. The structure of cyanobacterial and red algal PSII has been also revealed by X-ray crystallographic analysis (Ferreira *et al*. 2004, Loll *et al*. 2005, Umena *et al*. 2011, Suga *et al*. 2015, Ago *et al*. 2016). From the structure of red algal PSII, PsbQ′ has been shown to be located under CP43 and in close vicinity to PsbV (Ago *et al*. 2016). In reconstitution experiments of PsbQ′ to the cyanobacterium PSII, PsbQ′ was found to be attached to PSII in stoichiometrical proportions. However, oxygen-evolving activity has been shown to not depend on the presence of PsbQ′ (Enami *et al*. 1998). Fourier transform infrared analysis has also demonstrated that binding of PsbQ′ has no effect on the structural changes of protein conformation around the Mn_4CaO_5 cluster in the S_1 to S_2 transition (Uno *et al*. 2013). Therefore, the function of PsbQ′ remains unknown.

The PSII reaction initiates the charge separation at the primary donor chlorophyll (Chl). Then, the electron transfers to the primary electron acceptor, pheophytin *a*, in the D1 protein. The second and third electron acceptors of PSII are the plastoquinone molecules, Q_A and Q_B, in the D2 and D1 proteins, respectively. There have been many studies of the potential of Q_A (Krieger *et al*. 1995, Shibamoto *et al*. 2009, Allakhverdiev *et al*. 2011). However, the reported potential values vary greatly. Shibamoto *et al*. (2010) used a spectro-electrochemical method in order to show the species dependence of the redox potential of Q_A in an isolated PSII complex. The redox potentials of the Q_A of *Chlamydomonas reinhardtii* (green alga) and spinach (green plant) were approximately −170 and −160 mV, respectively. The redox potential of the Q_A of *Thermosynechococcus elongatus* (cyanobacterium) was approximately –140 mV. The redox potential of the Q_A in red alga, *Cyanidioschyzon merolae*, was 40 mV higher than that of *T. elongatus* in that report. The amino acids surrounding the Q_A are known to be entirely conserved between *C. merolae* and *T. elongatus.* In this case, the major difference between red algal and cyanobacterial PSII is the presence of the PsbQ′ protein.

In this study, we reconstituted the PsbQ′ protein to cyanobacterial PSII. The redox potential of Q_A was positively shifted relative to that of cyanobacteria. On the basis of our new findings, we discuss the role of PsbQ′ protein in red algae and its evolutionary mechanism.

Materials and methods

The PSII complexes were isolated from the thermophilic cyanobacteria, *T. elongatus,* by using a 6 × histidine tag, which was introduced at the C-terminus of CP47 (Iwai *et al*. 2004). Briefly, cells were suspended in buffer [50 mM MES-NaOH buffer (pH 6.0) containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 25% (w/v) glycerol] and broken by using glass beads at 0°C. The thylakoid membranes were recovered by preparative centrifugation (2,000 × *g* for 5 min at 4°C) followed by centrifugation (40,000 × *g* for 20 min at 4°C) and resuspended in the same buffer with a Chl concentration of 1 mg mL^{-1}. PSII complexes were solubilized from thylakoid membranes with 0.8% *n*-dodecyl-β-D-maltoside for 20 min at 4°C in the dark and then purified by Ni^{2+}-NTA affinity column chromatography (*ProBond resin*, *Invitrogen*). The purified PSII complexes were concentrated by using *Amicon Ultra 100K* and dissolved in the same buffer.

The gene encoding PsbQ′ was obtained from a red alga, *Cyanidium caldarium*. The degree of identity of the amino acid sequences between *C. merolae* and *C. caldarium* was 70.2%. The cloned gene was designed and expressed in *Escherichia coli* as fusion proteins with a 6 × histidine tag, thioredoxin, and a factor Xa-site at the N-terminus of PsbQ′. The expressed protein was purified by Ni^{2+}-NTA affinity chromatography, and the 6 × histidine tag and thioredoxin were proteolytically removed by using factor Xa. This purified PsbQ′ protein was used for the following analyses. The detailed method was described by Ohta *et al*. (2003).

In order to reconstitute the PsbQ′ protein, three-fold higher amounts of PsbQ′ were added to the PSII complexes, then unbound PsbQ′ was removed from the PSII complexes by addition of polyethylene glycol 6000 to give a final concentration of 10% and centrifuged at 40,000 × *g* for 30 min at 4°C. The unbound PsbQ′ protein was in the supernatant fraction.

The Q_A potential was measured by using a potentiostat (*VersaSTAT 3*, *Princeton Applied Research*, USA) and a thin-layer electrode cell comprised of a gold mesh working electrode, a platinum wire counter electrode, and an Ag–AgCl reference electrode to monitor the redox-induced fluorescence change at 681 nm under argon gas. This system was essentially based on that described in a previous report (Tomo *et al*. 2008, Shibamoto *et al*. 2010). For measurement of the redox potential, the samples were suspended in 50 mM MES-NaOH, 0.04% *n*-dodecyl-β-D-maltoside, 10 mM NaCl, 5 mM $CaCl_2$, 1 M betaine, 50 μM anthraquinone-2-sulfonic acid, 50 μM 2-hydroxy-1,4-naphtoquinone, and 50 μM 1-methoxy-5-methylphenazinium methylsulfate. Values of redox potential are shown as mean ± S. D., with $n = 5$ (independent experiments).

Thermoluminescence was measured by using a custom-made apparatus with control PSII and PsbQ′-reconstituted PSII in the presence or absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Endo *et al*. 2015). The thermoluminescence glow curves obtained after single turnover flash at 0°C provided the so-called B-band in the absence of DCMU, which originates from Q_B^- and S_2 state of the Mn_4CaO_5 cluster recombination, and the Q-band in the presence of DCMU, which originates from Q_A^- and S_2 state recombination (Vass and Govindjee 1996). Samples were incubated in the dark for 10 min

before excitation with a single-turnover saturating flash. Thermoluminescence glow curves were recorded at a heating rate of 60°C min^{-1}.

Oxygen-evolving activity was measured by using a Clark-type oxygen electrode at 25°C with 0.4 mM phenyl-*p*-benzoquinone as the electron acceptor. The measurements were performed in a buffer solution (50 mM MES-NaOH, pH 6.0) containing 0.4 M sucrose under saturating light conditions (Nagao *et al*. 2016). Chl concentrations were determined in 80% acetone using the equation of Porra *et al*. (1989).

Results and discussion

We expressed the PsbQ′ fusion protein with thioredoxin and 6 × histidine tag by using *E. coli*. Initially, this PsbQ′ fusion protein was purified by Ni^{2+}-NTA column chromatography. Then, to remove the 6 × histidine tag and thioredoxin, we performed Xa digestion, the site of which was the N-terminus of the PsbQ′ protein. In Fig. 1, *lane 1* contains the total protein of *E. coli* before IPTG induction. *Lane 2* clearly shows that an approximate 50-kDa protein was increased by IPTG induction, forming the fusion protein of PsbQ′. *Lane 3* contained the purified PsbQ′ fusion protein obtained by Ni^{2+}-NTA column chromatography. *Lane 4* contained the purified mature PsbQ′ protein after Xa digestion. Next, we performed reconstitution experiments of the PsbQ′ protein to cyanobacterial PSII. *Lane 5* showed the control polypeptides profile of cyanobacterial PSII (hereafter referred to as control PSII). For reconstitution of the PsbQ′ protein to control PSII, the PsbQ′ protein was attached stoichiometrically (Fig. 1, *lane 6*). We also performed reconstitution experiments with each of the four extrinsic proteins separately after $CaCl_2$ treatment, which removed all extrinsic proteins from the control PSII. We obtained the same results

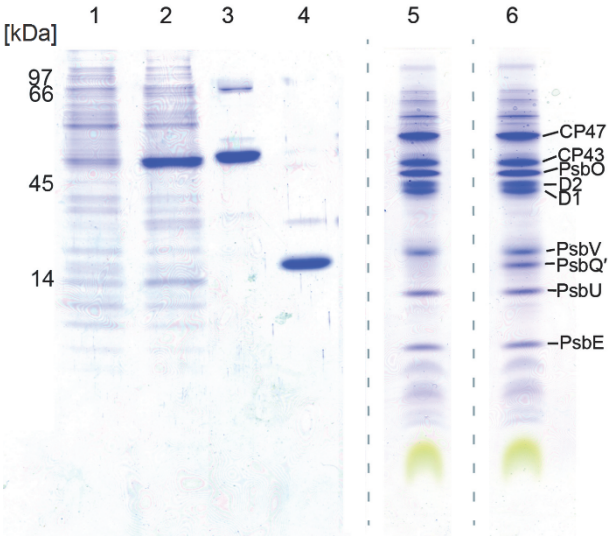

Fig. 1. Purification of the PsbQ protein from *Escherichia coli* (*lanes 1–4*). *Lane 1* – total protein of *E. coli* before IPTG induction; *lane 2* – total protein of *E. coli* after IPTG induction; *lane 3* – purified PsbQ′ fusion protein that contains thioredoxin and 6 × histidine tag; *lane 4* – purified mature PsbQ′ protein; *lane 5* – polypeptides profile of cyanobacterial PSII; *lane 6* – PsbQ′-reconstituted PSII.

Table 1. Oxygen evolving activity in PsbQ′ reconstituted PSII and control PSII Values are shown as mean ± SD, $n = 3$.

	O_2-evolving activity [μmol(O_2) mg(Chl)$^{-1}$ h^{-1}]
PSII (−PsbQ′)	1504.5 ± 85.7 [100%]
PSII (+PsbQ′)	1598.6 ± 83.1 [106%]

in these two experiments (data not shown). In the structures of red algal and cyanobacterial PSII, the PsbQ′ protein is bound to the membrane protein directly and the position of cyanobacterial PSII is vacant (Loll *et al*. 2005, Umena *et al*. 2011). Therefore, it is reasonable to suggest that PsbQ′ binds to cyanobacterial PSII directly and stoichiometrically.

The oxygen-evolving activity was measured in PsbQ′-reconstituted cyanobacterial PSII and control PSII (Table 1). The activities of PsbQ′-reconstituted PSII and control PSII were similar. This result was consistent with that of a previous report (Enami *et al*. 1998), and also reconfirmed that PsbQ′ did not affect oxygen evolution directly.

Shibamoto *et al*. (2010) reported the species-dependent redox potential change of the Q_A molecule. That report showed a positive shift of the redox potential of the Q_A molecule in red algal PSII relative to that of the cyanobacterial PSII. The main difference between red algal and cyanobacterial PSII is the existence of the PsbQ′ protein. However, the primary structures of the reaction center proteins of both do not fully match, so it is difficult to conclude that the difference in the potentials is because of the PsbQ′ protein in this stage. In the reconstitution experiment of extrinsic proteins in red algae, the effective binding of PsbV and PsbU required the PsbQ' protein (Enami *et al*. 1998). Therefore, we performed reconstitution experiments of the PsbQ′ protein to the cyanobacterial PSII. In this case, it is possible to conclude that the only difference is the presence or absence of the PsbQ′ protein. For the above reasons, we monitored the redox potential of the Q_A molecule in PsbQ′-reconstituted PSII and control PSII. The fluorescence intensity of PSII is determined by the redox state of Q_A, so it has been used as an indicator for the redox measurement of Q_A. In this study, the redox potential of Q_A was determined by using an electrochemical titration method with a potentiostat to monitor fluorescence changes at 681 nm. We monitored the value of the redox potential against that of the standard hydrogen electrode. First, we measured the redox potential

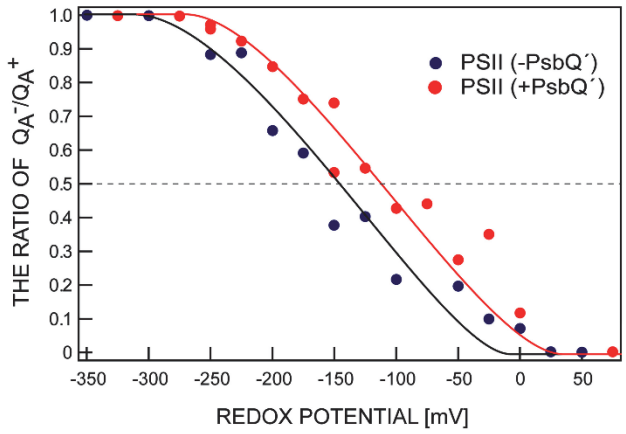

Fig. 2. Redox titration of the fluorescence at 681 nm of Q_A molecule. *Blue plot* – control PSII; *red plot* – PsbQ′-reconstituted PSII.

of Q_A in control PSII. The redox potentials are calculated by the 50% oxidized and 50% reduced forms of Q_A (Fig. 2) and intercepts of the formulas in Nernst curves (Fig. 3). The obtained value of the redox potential was –142 ± 8 mV in the control PSII. This value was similar to that in a previous report (–140 mV) (Shibamoto *et al*. 2009). Next, we measured the PsbQ′-reconstituted PSII. The obtained value was –110 ± 6 mV (Figs. 2, 3), which was positively shifted approximately 32 mV relative to that of the control PSII of *T. elongatus*. This positive shift of the cyanobacterial PSII was also observed in red algal PSII, which binds the PsbQ′ protein (Shibamoto *et al*. 2010). The reversibility of the redox reaction was maintained over several hours during the experiments. The molar ratio of Q_A was plotted against the applied voltage, and the curve was simulated by a one-electron Nernst curve (Fig. 3). When the potential value was plotted against the logarithmic fraction of Q_A^-, slopes of 62.5 mV per decade

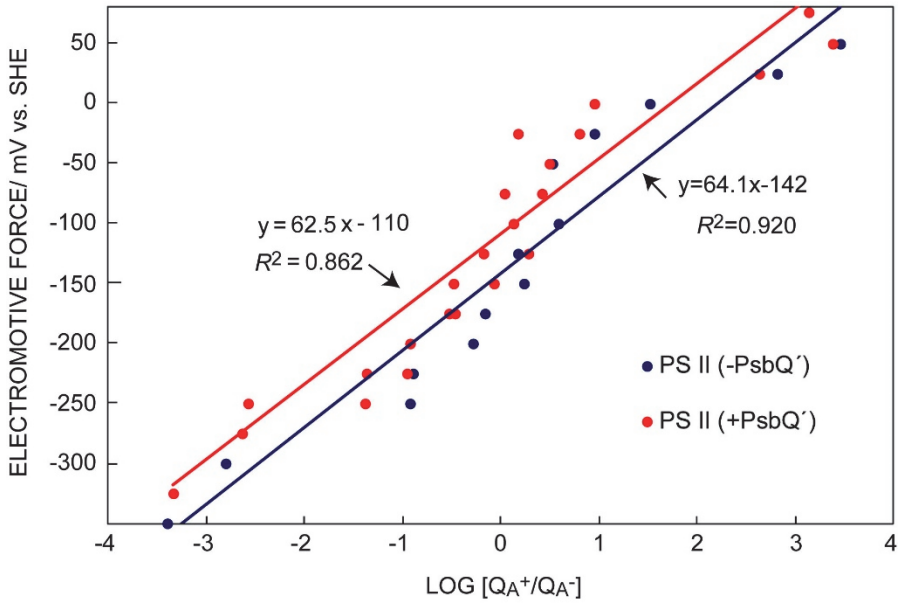

Fig. 3. Nernst plot of the Q_A molecule. Nernst plot of the Q_A molecule data from Fig. 2. The lines drawn by least-squares fits to the plots have slopes of 64.1 mV (*blue*) and 62.5 mV (*red*) for the control PSII and PsbQ′-reconstituted PSII, respectively. Intercept of the formula represents the redox potential.

and 64.1 mV per decade were obtained for PsbQ′-reconstituted PSII and control PSII, respectively. An ideal one-electron reaction on a Nernst plot has a value of 59 mV per decade; therefore, our measurement was sufficiently accurate to estimate the redox potential.

Subsequently, we monitored the thermoluminescence of PsbQ′-reconstituted PSII and control PSII. Thermoluminescence reflects the redox properties of the electron acceptor of PSII (Q_A, Q_B) and the Mn_4CaO_5 cluster. In the presence of DCMU, electron transfer after charge separation from Q_A to Q_B was blocked. In this case, the so-called Q-band, which originates from charge recombination between the Q_A^- and S_2 states of the Mn_4CaO_5 cluster, was observed at 10–20°C (Vass *et al*. 1981). In the absence of DCMU, the so-called B-band, which originates from charge recombination between the Q_B^- and S_2 states of the Mn_4CaO_5 cluster, was observed at 20–40°C. These values change with different heating rates (Noguchi *et al*. 2002). Fig. 4*A* shows the Q-band of thermoluminescence glow curves after a single flash. A peak was observed at 20°C in the control PSII (−PsbQ′). In the case of the PsbQ′-reconstituted PSII, this peak shifted to a higher temperature near 30°C (Fig. 4*A*). The oxygen-evolving activities were similar between PsbQ′-reconstituted PSII and control PSII (Table 1). Therefore, this peak shift was attributed to the Q_A molecule. The upper shift of the Q-band corresponds to the positive shift in the Q_A potential (Demeter 1989, Vass and Govindjee 1996). On the other hand, the peak temperature of the B-band did not change between the PsbQ′-reconstituted PSII and control PSII (Fig. 4*B*). This result suggested that the redox potential of Q_B did not change between the PsbQ′ reconstituted PSII and control PSII.

The redox titration (Figs. 2 and 3) and thermolumi

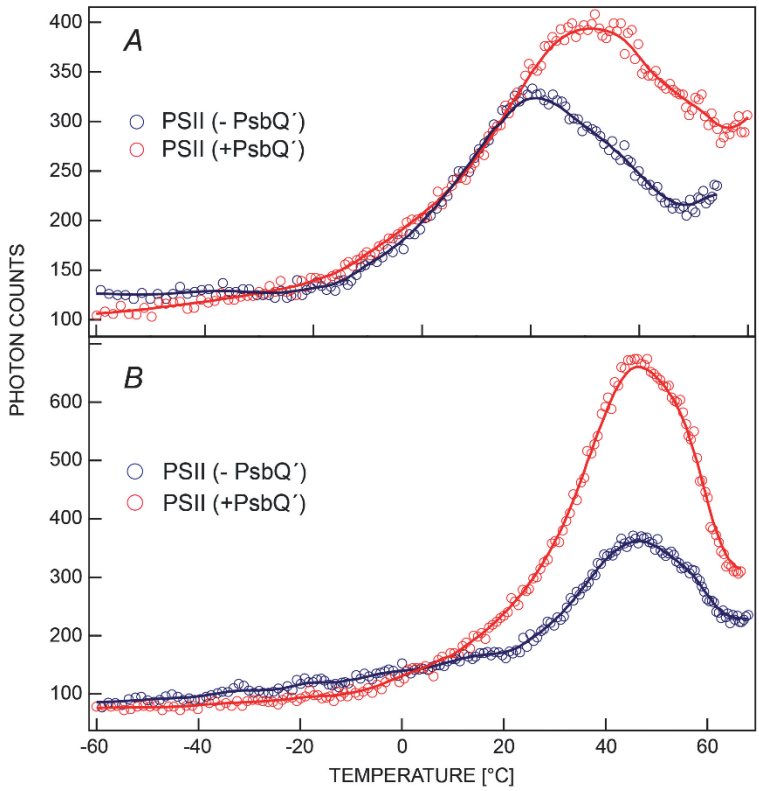

Fig. 4. Thermoluminescence glow curve. The thermoluminescence glow curve was measured by using control PSII and PsbQ′-reconstituted PSII in the presence (*A*) and absence (*B*) of 10 μM DCMU. Control PSII (*blue curve*); PsbQ′-reconstituted PSII (*red curve*). Thermoluminescence glow curves were recorded at a heating rate of 60°C min^{-1}.

nescence (Fig. 4) showed a positive shift in the redox potential of the Q_A molecule. In general, the positive shift of the Q_A molecule is thought to reflect a decrease in the harmful singlet oxygen (Vass *et al*. 2009, Rutherford *et al*. 2012). In PSII, the triplet Chl was generated by charge recombination between pheophytin$^-$ and P680$^+$ under strong light irradiation. Then, triplet Chl reacts with triplet oxygen, and then triplet oxygen becomes singlet oxygen. Regarding the positive shift of the Q_A molecule, the rate of direct recombination between Q_A^- and P680$^+$ was thought to have increased. Consequently, the rate of triplet Chl is expected to decrease, because of the decrease in charge recombination between pheophytin$^-$ and P680$^+$. As a result, the positive shift of Q_A suggests that it has a photoprotective role under strong light irradiation. It was recently reported that he redox potential of the Q_B molecule was determined by using Fourier transform infrared spectroscopy (Kato *et al*. 2016). That result indicated that the energy difference between Q_A and Q_B was also important for photoprotection. In this study, the B-band of thermoluminescence did not change between the PsbQ′-reconstituted PSII and control PSII. Therefore, the energy gap between Q_A and Q_B became small in PsbQ′-reconstituted PSII. This energy gap is also expected to increase the photoprotective role of PsbQ′ protein. In addition, we performed the Q_A re-oxidation analysis, which revealed the electron transfer rate from Q_A to Q_B (Vass *et al*. 1999). However, the reduction rate of Q_B was almost identical between PsbQ′-reconstituted PSII and control PSII (data not shown). Currently, the reasons for this result are unknown.

PsbQ′ protein is an extrinsic protein and is located on the donor side of PSII. It is interesting that the donor side of PSII affected the potential of Q_A on the acceptor side. Some reports have shown that the donor side affects the acceptor side of PSII. Following removal of the Mn_4CaO_5-cluster, the redox potential of Q_A has been shown to be positively shifted in cyanobacterial and spinach PSII (Johnson *et al*. 1995, Krieger *et al*. 1995, Allakhverdiev *et al*. 2011, Ido *et al*. 2011, Kato *et al*. 2016). In spinach PSII, it was shown that not only the large loop E of CP43 exposed to the lumenal side but also the C-terminal region of CP43 exposed to stromal side was digested by trypsin treatment in the absence of the extrinsic PsbO protein but not in the presence of the protein. This indicates that the removal of the PsbO protein at the lumenal side induced a conformational change of CP43 at the stromal side (Enami *et al*. 1997). To account for this mechanism, more precise structures and theoretical analyses are necessary.

Recently, the structure of spinach PSII was reported at 3.2 Å resolution (Wei *et al*. 2016). Compared to the structures of spinach and red algal PSII, PsbQ, and PsbQ′ had similar positions below CP43, although the angles with the membrane were slightly different. The localizations of PsbP and PsbV were partly overlapped in both structures. However, the position of PsbU in cyanobacteria appeared to be vacant in spinach PSII. Therefore, the surrounding environment of red algal PsbQ′ was slightly different from that in green plant PSII. The role of PsbQ in green plants has not been fully understood yet. Some

reports have shown that the function of PsbQ is to produce the correct ionic environment during water oxidation (Ghanotakis *et al*. 1984, Miyao and Murata 1989). The RNA interference technique showed that PsbQ was dispensable under normal growth conditions (Ifuku *et al*. 2011). The results of the present study suggest that PsbQ′ has a photoprotective function. However, further analysis will be necessary to confirm this possibility in PsbQ and PsbQ′. The extrinsic proteins are important for stabilization of water oxidation. The roles of several extrinsic proteins of PSII have not yet been demonstrated. Further studies are necessary to define the roles of extrinsic proteins.

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