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Two functionally distinct manganese clusters formed by introducing a mutation in the carboxyl terminus of a photosystem II reaction center polypeptide, D1, of the green alga *Chlamydomonas reinhardtii*

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Abstract

To study the function of the carboxyl-terminal domain of a photosystem II (PSII) reaction center polypeptide, D1, chloroplast mutants of the green alga *Chlamydomonas reinhardtii* have been generated in which Leu-343 and Ala-344 have been simultaneously or individually replaced by Phe and Ser, respectively. The mutants carrying these replacements individually, L343F and A344S, showed a wild-type phenotype. In contrast, the double mutant, L343FA344S, evolved O_2 at only 20–30% of the wild-type rate and was unable to grow photosynthetically. In this mutant, PSII accumulated to 60% of the wild-type level, indicating that the O_2-evolving activity per PSII was reduced to approximately half that of the wild-type. However, the amount of Mn atom detected in the thylakoids suggested that a normal amount of Mn cluster was assembled. An investigation of the kinetics of flash-induced fluorescence yield decay revealed that the electron transfer from Q_A^- to Q_B was not affected. When a back electron transfer from Q_A^- to a donor component was measured in the presence of 3-(3,4-dichlorophenol)-1,1-dimethylurea, a significantly slower component of the Q_A^- oxidation was detected in addition to the normal component that corresponds to the back electron transfer from the Q_A^- to the S_2-state of the Mn cluster. Thermoluminescence measurements revealed that L343FA344S cells contained two functionally distinct Mn clusters. One was equivalent to that of the wild-type, while the other was incapable of water oxidation and was able to advance the transition from the S_1-state to the S_2-state. These results suggested that a fraction of the Mn cluster had been impaired by the L343FA344S mutation, leading to decreased O_2 evolution. We concluded that the structure of the C-terminus of D1 is critical for the formation of the Mn cluster that is capable of water oxidation, in particular, transition to higher S-states.

Keywords: Photosystem II; O_2 evolution; *psbA*; Manganese cluster; Chloroplast transformation; *Chlamydomonas reinhardtii*

1. Introduction

Photosystem II (PSII) forms an intrinsic multiprotein complex that consists of two homologous reaction center polypeptides, D1 and D2, two core antenna complexes, CP47 and CP43, and cytochrome b_{559}. In addition to these polypeptides that carry cofactors, the complex contains several small polypeptides that have a putative transmembrane helix, and

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the extrinsic polypeptides, such as OEE1, OEE2, and OEE3, which are encoded by the *psbO*, *psbP*, and *psbQ* genes, respectively [1]. The Mn cluster is bound to or in close interaction with reaction center polypeptides on the lumenal side of the thylakoid membranes. The three extrinsic polypeptides are present in the proximity of the Mn cluster and facilitate and stabilize the O_2-evolving complex (OEC).

PSII catalyzes the light-driven electron transfer from water to plastoquinone (for recent reviews, see [2,3]). The primary photochemical reaction takes place between the primary electron donor, P680, and the intermediate electron acceptor, I. The resulting charge separation is stabilized by electron transfer from I^- to the primary electron acceptor, Q_A, and subsequently to the secondary electron acceptor, Q_B, on the acceptor side. On the donor side, $P680^+$ is reduced by the secondary electron donor, Y_Z, which is in turn reduced by the OEC. To oxidize water to oxygen, four oxidizing equivalents are extracted from the OEC, whose core consists of a cluster of four manganese ions together with Ca^{2+} and Cl^- as necessary cofactors. The redox components involved in the photochemical reaction of PSII are mainly present on the heterodimer of the reaction center polypeptides, D1 and D2, that are encoded by the *psbA* and *psbD* genes, respectively.

Several lines of evidence suggested that the D1-D2 heterodimer plays a central role in the ligation of the Mn cluster. To elucidate the structure-function relationship of the OEC, it is important to identify amino acid residues that bind the Mn cluster as ligands and that form a microenvironment around the Mn cluster. In particular, it is proposed that possible ligands for the OEC are those that are conserved among photosynthetic organisms and are located in the lumenal interhelical domain. A systematic survey using site-directed mutants revealed that Asp-59, Asp-61, Glu-65, His-92, Asp-170, Glu-189, His-190, His-332, Glu-333, His-337, and Asp-342 on D1 [4–7] and Glu-70 on D2 [8] are essential for the assembly and/or stability of the Mn cluster. D1 is synthesized in precursor form with a C-terminal extension (pD1) and subsequently processed to a mature form [9,10]. The processing is indispensable for the assembly of the Mn cluster [11–14]. In addition, a truncation mutant of cyanobacterium *Synechocystis* 6803 that lacks Ala-344 accumulates non-O_2-evolving PSII [13]. Consequently, the C-terminal residue, Ala-344, of the mature D1 is essential for the assembly of the OEC and is a possible ligand of the Mn cluster. A number of amino acid residues, His-332, Glu-333, His-337, Asp-342, and Ala-344, which are involved in formation of the Mn cluster, are clustered in the C-terminal domain of D1. However, less is known about the protein microenvironment around the binding site for the Mn cluster, although the chemical characteristics of the amino acid residues in the proximity of the Mn cluster should significantly affect its redox properties.

In the present study, we have mutated two C-terminal amino acid residues to elucidate their functional significance for assembly of the functional Mn cluster. Leu-343 and Ala-344 were replaced simultaneously or individually by Phe and Ser, respectively. The two individual mutants, L343F and A344S, showed wild-type phenotypes. However, two distinct Mn clusters were formed in the double mutant, L343FA344S. These results indicate that the C-terminal residues of the mature D1 are critical for forming the functional Mn cluster that is capable of water oxidation.

2. Materials and methods

2.1. Strain and growth medium

Chlamydomonas reinhardtii wild-type strain 137C and the chloroplast *psbA* deletion mutant, Fud7, were used. The control strain is a transformant containing wild-type *psbA* gene and an *aadA* cassette, which grows photosynthetically and shows the same PSII activity as a wild-type strain [15]. Cells were grown in Tris-acetate-phosphate (TAP) medium for heterotrophic growth or in high-salt minimal (HSM) medium for autotrophic growth at 25°C.

2.2. Site-directed mutagenesis and chloroplast transformation

Oligonucleotide-mediated mutagenesis was performed as described in [14] using the following oligonucleotides: L343FA344S: 5′-CTTCCCTCTAGACTTCTCTTCAACTAACTCTA-3′, L343F: 5′-CTTCCCTCTAGACTTCGCTTCAACTAACTCT-

3′, A344S: 5′-TTCCCTCTAGACTTATCTTCAAC-TAACTCTA-3′ (underlines indicate the mutated nucleotides). These mutations eliminate an *Alu*I site.

For chloroplast transformation, the plasmid pEX-50-AAD was used, which contains the 5.0 kb *Eco*RI-*Xho*I fragment cloned into pBluescript KS$^-$ (Stratagene) and an *aadA* cassette inserted at the *Bam*HI site as described in [15]. The *Eco*RI-*Xho*I fragment contains the *psbA* exon 5 and its 5′ region. The pEX-50-AAD containing the mutation was transformed into wild-type cells of *C. reinhardtii* using a helium-driven particle inflow gun [14]. The putative mutants were selected on TAP plates containing 150 μg/ml spectinomycin and recloned three times to obtain homoplasmic lines.

Total DNA of wild-type and transformants was isolated as described in [16]. The DNA fragment of *psbA* exon 5 was amplified by polymerase chain reaction using the oligonucleotide primers 5′-GGTTACTTTGGTCGTCTAAT-3′ and 5′-TTAGTTGTTTGAGCTAGAGT-3′. The resulting 303 bp fragments of wild-type and transformants were digested with *Alu*I and separated by agarose gel electrophoresis to confirm that the mutants were homoplasmic. In addition, the mutations were verified by sequencing the *psbA* exon 5, which had been amplified by polymerase chain reaction. Southern blot analysis using total DNA indicated that the *aadA* cassette had as expected been integrated into the downstream region of the *psbA* gene by homologous recombination.

2.3. Chlorophyll and Mn content

Chlorophylls were extracted from cells with absolute methanol and quantitatively measured as described in [17]. Mn bound to the thylakoid membranes was determined using internal standards with an atomic absorption spectrophotometer AA-640-13 equipped with a GFA-3 graphite furnace atomizer (Shimadzu, Kyoto) [18].

2.4. Western blot analysis

Western blot analysis using total cell proteins was performed as described previously [14]. Total cell proteins were solubilized with 2% SDS and 0.1 M dithiothreitol at 100°C for 1 min, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to [19], except that the resolving gel contained 6 M urea as described in [15]. The separated total cell proteins were subsequently electrotransferred to a nitrocellulose filter and immunodetected by an enhanced chemiluminescence method. Antibodies raised against D1 protein and OEE1 were provided by Dr. M. Ikeuchi (University of Tokyo) and Dr. Y. Yamamoto (Okayama University), respectively. Antibodies raised against OEE2 and OEE3 were provided by Dr. O. Vallon (Institut de Biologie Physico-Chimique).

2.5. Measurements of PSII activities

Light-induced O_2 evolution was measured with a Clark-type electrode at 25°C using intact cells (20 μg chlorophyll/ml) in the presence of 0.2 mM phenyl-*p*-benzoquinone as an artificial electron acceptor, as described in [15].

Thermoluminescence was measured essentially as described [20] from cells (92 μg chl) suspended in 0.2 M sucrose, 10 mM NaCl, 20 mM $MgCl_2$, 25 mM MES, pH 6.5, and 20% glycerol. The cells were first illuminated with moderate white light for 20 s at room temperature. After 10 min dark adaptation at room temperature, a saturating flash was given from a xenon lamp at 5°C in the absence of DCMU (3-(3,4-dichlorophenol)-1,1-dimethylurea) or at −10°C in the presence of 10 μM DCMU. To detect period-four oscillation of the B-band, cells were illuminated with a series of xenon flashes at 5°C. The illuminated samples were rapidly cooled to 77 K which was followed by slow heating (0.7°C/s) to record glow curves. The relative peak height of each thermoluminescence band was determined as described previously [21] by simulating glow curves based on a published method [22].

2.6. Flash-induced fluorescence decay

Decay of chlorophyll *a* fluorescence yield after a single turnover actinic flash was measured with a pulsed kinetic fluorometer (Occam Technologies, Cincinnati, OH) based on the method described in [23]. Short-duration, high-intensity flashes from a xenon flash lamp (FX201, EG&G) filtered with a cyan filter, Schott BG18, served to drive photochemistry,

while a series of low-intensity, short-duration (2–3 μs) pulses from a bank of seven light-emitting diodes, filtered with a 635 nm interference filter, were used to probe the fluorescence yield. Fluorescence was measured by a photodiode filtered with an appropriate long-wavelength pass filter (Schott RG695). To ensure reproducible distribution of Q_B/Q_B^- and S_1/S_0 for all measurements, cells at mid-logarithmic phase (approx. 2×10^6 cells/ml) in a fresh TAP medium were preilluminated with moderate light for 30 s and dark-adapted at room temperature for 10 min. Samples were then treated with 100 μM *p*-benzoquinone and incubated for 10 min in the dark at room temperature to oxidize the residual Q_B^-. DCMU was then added to block reduction of Q_B by Q_A^- where indicated.

The nonlinear relationship between fluorescence level and the fraction of the total Q_A that remains reduced after a flash [24] was compensated by the probability of intersystem energy transfer as in [25]. Maximum fluorescence level was determined after five saturating flashes in the presence of 10 mM DCMU.

3. Results

3.1. Replacements at C-terminal residues of D1 significantly affect PSII activity

We generated chloroplast *psbA* mutants of the green alga *C. reinhardtii* by particle gun-mediated transformation to test the function of amino acid residues at the C-terminal domain of one of the PSII reaction center subunits, D1. Both or each of the two C-terminal residues, Leu-343 and Ala-344, have been mutated to Phe and Ser, respectively. These replacements of the amino acid residues were expected to modify the functional characteristics of the C-terminal domain of D1 that is responsible for the assembly and/or the optimal functioning of the O_2-evolving Mn cluster [7,13].

Two individual mutants, L343F and A344S, in which Leu-343 or Ala-344 has been replaced by Phe or Ser, respectively, evolved O_2 at nearly the wild-type rate and grew normally under photoautotrophic conditions, as shown in Table 1. In contrast, the double mutant, L343FA344S, in which both Leu-343 and Ala-344 have been replaced by Phe and Ser, respectively, evolved O_2 at 20% of the wild-type level and was unable to grow photosynthetically on HSM plates in weak and moderate light. Consequently, the single amino acid replacements at the C-terminus of D1 barely affected PSII activity, whereas the double mutation impaired PSII activity to a large extent.

We used quantitative Western blotting analysis to investigate the effects of these amino acid replacements on the amount of PSII complex. Total cell proteins of the control, L343FA344S, L343F, and A344S cells were solubilized and fractionated by SDS-PAGE, and subsequently electroblotted onto a nitrocellulose membrane and probed with an antibody raised against D1. A series of dilutions of the control cells that were mixed with Fud7 mutant lack-

Table 1
Phenotype of the *psbA* mutants of *Chlamydomonas*

Strains	Cell growth[a]				% PSII content[b]	% Mn content[c]	% O_2 evolution[d]
	TAP			HSM			
	dark	30	200	light			
Control	+	+++	+++	++	100	100	100
L343FA344S	+	+++	+	−	60	72	20–30
L343F	+	+++	+++	++	90	95	89
A344S	+	+++	+++	++	80–90	83	91

+, growth; −, no growth.
[a]Cells were grown on TAP plates in the dark or under light (30 and 200 μE/m^2/s) and on HSM plates under various light intensities (10, 18, 30, and 45 μE/m^2/s).
[b]PSII content was estimated by Western blotting using an antibody against D1, as shown in Fig. 1.
[c]Thylakoids of the control contained 4.0 Mn atoms per 460 chlorophylls.
[d]Rate of O_2 evolution in the control was 180 μmol O_2/mg chl/h.

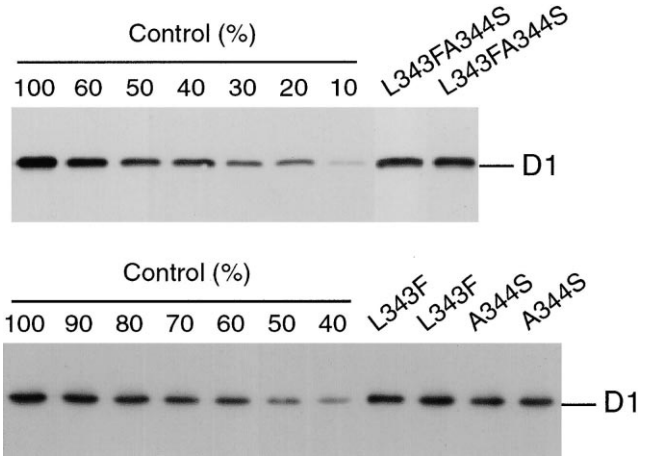

Fig. 1. Accumulation of PSII in the mutants. Total cell proteins (2.5 μg chlorophyll) of the control and three mutants were separated by SDS-PAGE and detected by Western blotting using an antibody raised against D1 protein. A series of dilutions of the control cells was made by mixing a *psbA* deletion mutant, Fud7, to ensure that a constant amount of protein was loaded on the gel. This series of dilutions was used as a standard to estimate quantitatively the amounts of D1 in the mutants. The signal intensities of two independent clones of L343FA344S mutant (upper panel) and L343F and A344S mutants (lower panel) were compared with those of the series of dilutions.

ing D1 was loaded on a gel and the signal intensities of these diluted samples compared to those of the mutants. Fig. 1 shows that the double mutant, L343FA344S, contained D1 at approx. 60% of the wild-type level whereas the two individual mutants, L343F and A344S, contained D1 at approx. 80–90% of the wild-type level. The migration pattern of D1 in these mutants was identical to that of wild-type D1, indicating that their pD1 is processed to mature D1. Pulse-chase labeling experiments of chloroplast-encoded proteins revealed that the processing of pD1 occurred normally in these mutants (data not shown).

The effects of the amino acid replacements on the activity of O_2 evolution are summarized in Table 1. A slight decrease in the O_2-evolving activity was observed in the L343F and A344S mutants, which could be ascribed to the small decrease in the amount of D1 on the thylakoid membranes. However, L343FA344S showed a more drastic decrease in the activity of O_2 evolution than in the amount of D1. The decrease in O_2-evolving activity was approx. 2-fold larger than the decrease in the amount of PSII.

3.2. L343FA344S mutant retains normal electron transfer from Q_A^- to Q_B

Replacements of amino acid residues on the lumenal side of the thylakoid membranes could modify the entire structure of the PSII complex. To investigate the effects of the amino acid replacements on the activity of the acceptor side of PSII, we examined the electron transfer reaction from Q_A^- to Q_B by measuring the relaxation kinetics of flash-induced fluorescence yield change. When control cells were excited with a saturating single turnover flash, the primary acceptor, Q_A, was rapidly reduced. Fig. 2 shows that the reduced Q_A was subsequently oxidized with exponential and constant phases. The decay half-time of the major exponential phase was 120–140 μs (Table 2), which corresponds to the electron transfer rate from Q_A^- to Q_B [26]. The decay kinetics of L343FA344S were identical to those of the control, indicating that the electron transfer reaction in L343FA344S is not affected by the amino acid replacements. We therefore concluded that the reduced activity of the O_2 evolution in L343FA344S is not due to an impaired electron transfer reaction on the acceptor side of PSII.

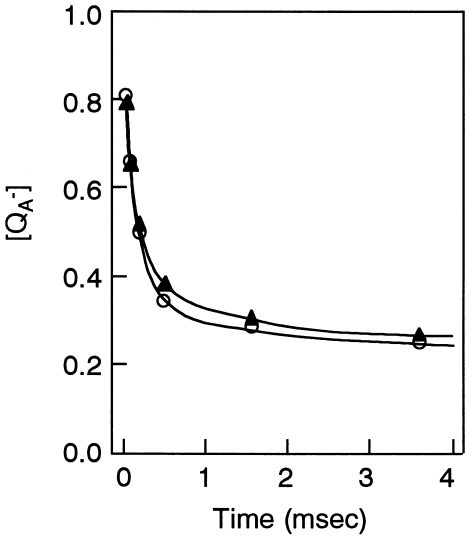

Fig. 2. Electron transfer kinetics from Q_A^- to Q_B. Dark-adapted control cells (○) and L343FA344S cells (▲) were excited with a single flash. The decay of high fluorescence state after the single flash excitation was measured and expressed as the concentration of Q_A^- according to the method described in Section 2.

Table 2
Decay half-times and amplitudes of the oxidation of Q_A^- reduced by a flash excitation in the absence of DCMU

Strain	Fast phase τ_1 (a_1)	Slow phase τ_2 (a_2)	Constant phase (a_0)
Control	135 μs (60.6%)	1.25 ms (13.8%)	(25.6%)
L343FA344S	122 μs (52.0%)	1.12 ms (19.9%)	(28.1%)

The decay kinetics of the normalized fluorescence emission were fitted to the equation: a_0+a_1 exp.$(\tau/\tau_1)+a_2$ exp.(τ/τ_2), where a_n and τ_n are amplitudes and life times, respectively.

3.3. L343FA344S mutation does not affect assembly of the OEC but impairs its function

Fig. 3 shows the amount of Q_A^- estimated by measuring the change in flash-induced fluorescence yield when the electron flow from Q_A^- to Q_B was blocked by DCMU. Under these conditions, the flash-induced Q_A^- is oxidized by a donor-side component. Control cells showed a single exponential phase ($t_{1/2}=3.6$ s) and a residual constant phase after flash excitation (Table 3), which was within the same range as findings in a previous report on *C. reinhardtii* [27]. The amplitude of the exponential phase significantly decreased in L343FA344S cells, whereas the level of the constant phase increased. If the Mn cluster was absent or totally inactive in this mutant, the charge recombination between Q_A^- and the oxidized secondary electron donor, Y_Z^+, would occur, which could be much more rapid ($t_{1/2}\approx 350$ ms) [14,28]. The absence of this rapid phase indicates that the back reaction between Q_A^- and Y_Z^+ competes with a rapid rereduction of Y_Z^+. It is not clear from the fluorescence kinetics whether Y_Z^+ is reduced by some secondary component present in close proximity to Y_Z or by the Mn cluster that is partially impaired by the mutation. However, these results strongly suggest that the L343FA344S mutation significantly impaired electron transfer reactions on the donor side of PSII.

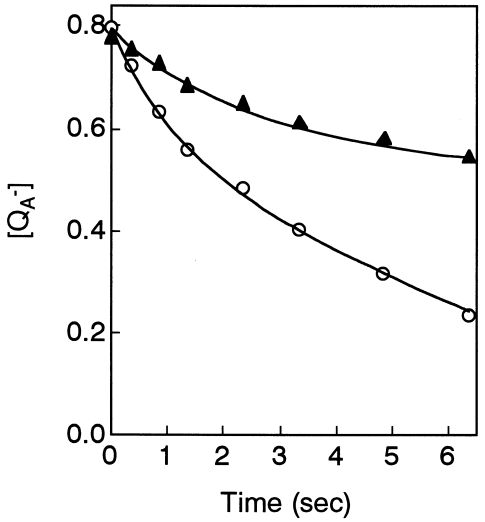

Fig. 3. Kinetics of charge recombination between Q_A^- and the donor side of PSII. Dark-adapted control cells (○) and L343FA344S mutant (▲) were excited with a single flash in the presence of DCMU. The decay of high fluorescence state after the single flash excitation was measured and expressed as the concentration of Q_A^- according to the method described in Section 2.

To address the question of whether an O_2-evolving Mn cluster is assembled in the double mutant L343FA344S, the thylakoid membranes were isolated and the content of Mn atom was assayed by atomic absorption spectroscopy. As shown in Table 1, the amount of Mn atom in the thylakoid membranes of L343FA344S was reduced to 72% of that in the control thylakoid membranes. Since the decrease in the amount of Mn atom is approximately proportional to that in the content of PSII, it can be concluded that a Mn cluster is assembled in the PSII complex of L343FA344S. Thus, Y_Z^+ is rapidly rereduced by the Mn cluster in the L343FA344S mutant. Two possible causes account for the lower activity of the O_2 evolution per PSII in L343FA344S: (1) the

Table 3
Decay life times and amplitudes of kinetic components of flash-induced fluorescence decay in the presence of DCMU

Strain	Fast phase τ_1 (a_1)	Very slow phase (a_0)
Control	3.55 s (82.3%)	(17.7%)
L343FA344S	3.62 s (35.2%)	(64.8%)

The decay kinetics of the normalized fluorescence emission were fitted to the equation: a_0+a_1 exp.$(\tau/\tau_1)+a_2$ exp.(τ/τ_2), where a_n and τ_n are amplitudes and life times, respectively.

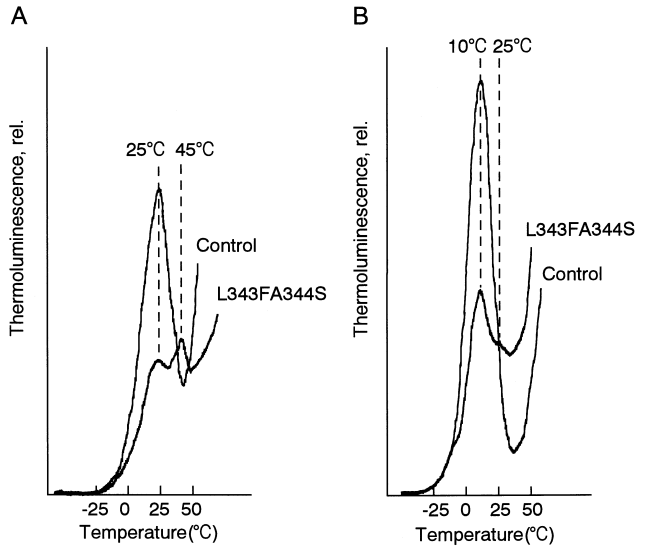

Fig. 4. Thermoluminescence glow curves of control and L343FA344S mutant cells. The cells were excited with a single flash in the absence (B-band; A) or presence (Q-band; B) of DCMU.

entire Mn cluster in L343FA344S is active but inefficient in O_2 evolution, or (2) only a fraction of the Mn cluster is fully functional in O_2 evolution, while the remainder of the cluster is inactive.

To test these possibilities, we investigated the redox properties of the Mn cluster by measuring thermoluminescence glow curves of control and L343FA344S cells. Fig. 4A shows the thermoluminescence glow curves obtained from cells excited with a single flash in the absence of DCMU to detect the B-band. Control cells gave rise to the B-band peaking at 25°C, while L343FA344S showed two distinct peaks at 25 and 45°C. When the thermoluminescence was measured in the presence of DCMU, the control cells gave rise to the Q-band at 10°C (Fig. 4B). In contrast, L343FA344S showed a peak and a shoulder at 10 and 25°C, respectively. Since a shift in thermoluminescence peak temperature could similarly result from the influences on the donor side and/or the acceptor side [29], thermoluminescence measurements alone do not always allow unambiguous separation of the two effects. However, we noted that modified peaks in the presence and absence of DCMU were upshifted to a similar extent (15–20°C). As presented above, the function of the acceptor side, specifically the electron transfer from Q_A^- to Q_B, was not affected, but the O_2-evolving activity was significantly impaired in L343FA344S. Thus, it is likely that the primary site of the modification in PSII is on the donor side.

The intensity of the thermoluminescence B-band depends on the number of actinic flashes and shows a period-four oscillation [29]. As shown in Fig. 5A, the intensity of the B-band from the control cells oscillated periodically, dependent on the number of flashes used to excite PSII. Fig. 6 shows that the intensity of the B-band was maximal after the second and sixth flashes and was minimal after the fourth flash. The period-four oscillation indicates that the OEC in the control cells is normal, as expected. Fig. 5B shows that the normal B-band at 25°C in L343FA344S exhibited period-four oscillation while that at 45°C did not. Fig. 6 shows that the intensity of the peak at 45°C varied little; a small increase in

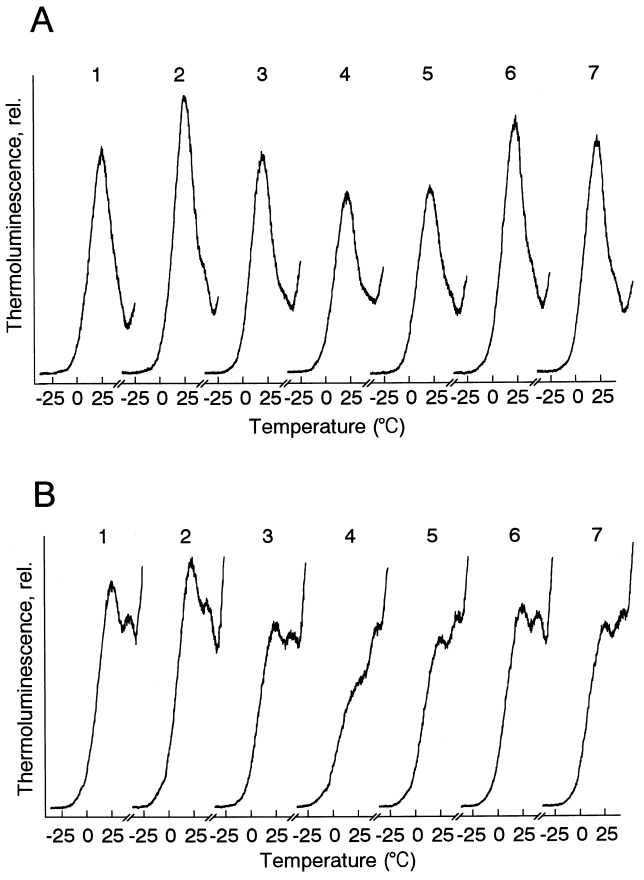

Fig. 5. Dependence of B-band thermoluminescence glow curves on the number of flash excitations in the control (A) and L343FA344S mutant (B) cells. The cells were excited with a series of xenon flashes at 5°C and the thermoluminescence glow curves were recorded as described in Section 2.

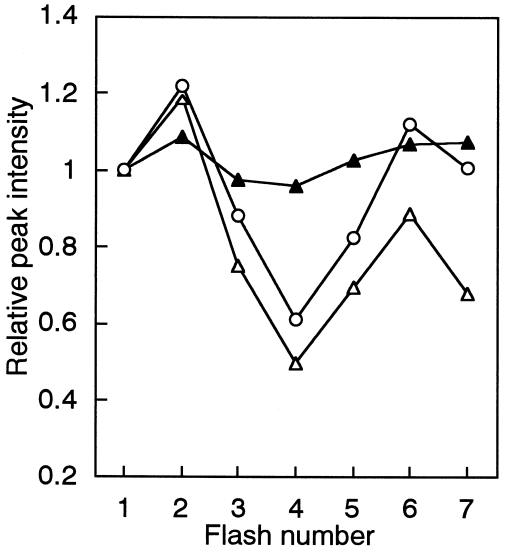

Fig. 6. Oscillation pattern of B-band intensity observed in Fig. 5. The intensity of the peak at 25°C in the control (○) and L343FA344S mutant (△) cells, and that at 45°C in L343FA344S mutant cells (▲) were plotted against the number of flash excitations. The relative peak heights were determined as described in Section 2.

the intensity was observed at the second flash, but the subsequent flash excitation did not make a significant difference. These results indicate that the peak observed at 25°C in L343FA344S arises from the fully functional Mn cluster that is able to split water into O_2 and H^+. In contrast, the peak detected at 45°C may arise from an impaired Mn cluster, which was able to proceed the transition at least from the S_1-state to the S_2-state, if the Mn cluster remains in the S_1-state in the dark. However, the impaired Mn cluster was unable to proceed the transitions from the S_3-state to the S_0-state, which are coupled to O_2 evolution.

4. Discussion

4.1. L343FA344S mutation affected O_2-evolving activity

The L343FA344S mutant generated in the present study contained PSII and Mn atom at 60% and 72% of wild-type levels, respectively, suggesting that this mutant fully assembles the Mn cluster on PSII. However, the apparent O_2-evolving activity was only 20–30% of the wild-type level, so that the specific activity based on PSII content was estimated to be approximately half of that in the wild-type cells. L343F and A344S mutants, in which the mutations were introduced individually, showed the wild-type phenotype, as summarized in Table 1. It is therefore unlikely that these two C-terminal residues are essential for PSII activities. We concluded that the double mutation, L343FA344S, exerted a synergetic effect, which leads to a functional alteration of the PSII complex.

Although Leu-343 and Ala-344 are present on the lumenal side of the thylakoids, the mutations at these positions may affect the function of PSII acceptors located on the stromal side [21]. However, the electron transfer from Q_A^- to Q_B in L343FA344S was not affected, as shown in Fig. 2 and Table 2, indicating that the functional structure of PSII on the acceptor side was not impaired by the mutations. In addition, electron transfer activity from an artificial electron donor, diphenylcarbazide, to an artificial electron acceptor, dichlorophenolindophenol, based on PSII content, was normal in the L343FA344S mutant, indicating that the photochemical reaction was not affected by the mutation (data not shown). In contrast, the charge recombination between Q_A^- and the Mn cluster in the presence of DCMU was significantly affected; approximately half of the population of normal charge recombination components ($t_{1/2} = 3.6$ s) was replaced by a very slow component (Fig. 3 and Table 3). These results strongly suggest that a fraction of the charge separated state between Q_A^- and the donor-side component was stabilized, most probably by functional modification of the corresponding donor component, which is presumably the Mn cluster. If the functionally modified Mn cluster had been completely inactive in reducing Y_Z^+, a very fast charge recombination between Q_A^- and the oxidized secondary electron donor, Y_Z^+, would have been observed. The absence of this fast decay kinetics indicates that the modified Mn cluster is capable of efficiently reducing Y_Z^+ in the L343FA344S mutant.

4.2. Two functionally distinct Mn clusters in L343FA344S

Thermoluminescence is emitted as the result of a charge recombination between a positive and a negative charge trapped at the donor and the acceptor

side of PSII, respectively. Measurements of this signal provide information on the physical state of the redox components on the acceptor and donor sides of PSII. The Q- and B-bands observed in the presence and absence of DCMU, respectively, revealed that the L343FA344S cells showed an abnormal component at higher temperatures in addition to the normal component under both conditions (Fig. 4). The emission temperatures of the altered signals were higher by 15–20°C as compared with those of normal signals. Since the upshift in emission temperature of the abnormal peaks was similar in either the presence or absence of DCMU, we may conclude that the donor-side component was specifically affected by the mutation. This view is consistent with the fact that the acceptor-side electron transfer reaction between Q_A^- and Q_B was not affected in this mutant. Upshifts of thermoluminescence emission temperature imply that the charge separated state between Q_B^- or Q_A^- and the modified Mn cluster was more stable, which is in accordance with the slower charge recombination kinetics between Q_A^- and the Mn cluster shown in Fig. 3. If the upshifts were solely caused by the donor-side modification(s) induced by the mutation, we may interpret that the redox potential of the S_1/S_2 in the modified Mn cluster was lowered in the mutant. Modified redox properties of the S-state(s) should result in a new equilibrium of the S-states in the dark. The new equilibrium might be responsible for the absence of period-four oscillation of the modified B-band. The modified Mn cluster is able to drive transition from the S_1-state to the S_2-state as the modified B-band was observed after a single flash excitation, but it may be unable to drive transitions from the S_3-state to the S_0-state. It is thus inferred that the modified Mn cluster is incapable of water oxidation, which leads the low specific O_2-evolving activity in L343FA344S.

4.3. The C-terminal domain of D1 is important for functional Mn cluster

A similar functional modification of the Mn cluster has also been observed in PSII particles that are depleted of three extrinsic proteins, OEE1, OEE2, and OEE3 [20,30]. The depleted PSII particles retained a Mn cluster but showed a significantly impaired O_2-evolving activity. Measurements of flash number dependence on the thermoluminescence B-band intensity and oxygen evolution demonstrated that the transitions from the S_0-state to the S_3-state proceed normally, while the transition from the S_3-state to the S_4-state is blocked [31,32]. In addition to this in vitro experiment, *Synechocystis* mutants that lack the *psbO* gene showed a significantly perturbed Mn cluster and a reduced O_2-evolving activity at 30–70% of the wild-type level [33–35]. Measurements of thermoluminescence and flash-induced fluorescence kinetics of the *Synechocystis* mutants revealed that the charge separation between Q_A^- and the S_2-state was stabilized [35–38]. Although the flash-induced transitions from the S_2-state to the S_3-state occurred normally, the transitions from the S_3-state to the S_0-state were significantly retarded in these mutants.

Much greater effects of deletion of the extrinsic proteins on PSII activity were observed in *Chlamydomonas* cells than in *Synechocystis* cells. A nuclear mutant of *Chlamydomonas* that lacks the OEE1 showed a substantial deficiency in the PSII complex [39], while a nuclear mutant of *Chlamydomonas* that is deficient in OEE2 accumulates a normal amount of PSII complex but evolved O_2 at only 5% of the wild-type rate [40]. In the present study, OEE1, OEE2, and OEE3 remained associated with the thylakoid membranes isolated from L343FA344S, as seen in the wild-type (data not shown). Although the absence of OEE1 requires higher Ca^{2+} concentration for optimal water oxidation [31], the addition of $CaCl_2$ to the reaction medium in the present study barely enhanced O_2 evolution of the thylakoid membranes isolated from the L343FA344S mutant (data not shown). Thus, our data suggest that the functional modification of the Mn cluster in L343FA344S is ascribed to a modification of the Mn cluster binding domain rather than to an alteration in the association of the extrinsic polypeptide to PSII.

We propose two interpretations to explain the formation of the two functionally distinct Mn clusters by L343FA344S mutation. One is that a microenvironment, which affects the redox properties of the Mn cluster, has been altered by the L343FA344S mutation. It is very likely that the Leu-343 and Ala-344 are located near the Mn cluster, because several lines of evidence have indicated that His-332, Glu-333, His-337, Asp-342, and Ala-344 in the C-terminal do-

main are possible ligands for the Mn cluster [7,13]. The replacements of Leu-343 and Ala-344 by Phe and Ser introduced a hydrophilic environment and a steric constraint around the Mn cluster, respectively. This implies that at least some of the C-terminal amino acid residues of D1 form a binding domain for the Mn cluster. However, it remains to be elucidated why only a fraction of the Mn cluster was functionally modified, since we expected the amino acid replacements to change the microenvironment uniformly.

An alternative interpretation is that the L343FA344S mutation primarily induced a structural modification of D1 around the binding region of the Mn cluster, which resulted in the functional modification of the Mn cluster. The structural modification of D1 appears to be small, since the assembly of the Mn cluster was not affected. This suggests that a delicate structural optimization of the C-terminal domain of D1 is crucial for the functioning of the O_2-evolving Mn cluster, in particular, for transitions between higher S-states. The difference in energetic stability between the normal and modified D1 structures is most likely small because the two distinct Mn clusters were estimated to be formed at nearly equal levels. It is still possible, however, that modification of the microenvironment and protein structure around the Mn cluster occurred simultaneously.

A similar, but smaller upshift in peak emission temperature of the B-band and the Q-band was detected in mutant strains of *Synechocystis* PCC 6803 carrying short deletions in the large, lumen-exposed loop (loop E) of CP47 [41]. In addition, the charge separation between the Q_A^- and the S_2-state was stabilized. These results revealed that mutations of the lumenal hydrophilic region of CP47 in *Synechocystis* PCC 6803 affect the properties of the S_2-state of the Mn cluster in a similar manner to the mutation observed in the present study at the C-terminal domain of D1. This indicates that a number of amino acid residues in the lumenal domain of PSII core complex are involved in the structural optimization required for water oxidation by the Mn cluster. Further characterization of mutants with site-directed mutations on the lumenal regions of the PSII core complex would provide more insight into the microenvironment and its structural significance around the Mn cluster.

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