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Functional analysis of site-directed mutants on the C-terminal Leu-343 and Ala-344 of D1 protein

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Introduction

One of the core proteins of the photosynthetic reaction center of photosystem II, D1, is synthesized as a precursor form. The carboxy terminal extension with 8-16 amino acids is posttranslationally cleaved at the carboxyl side of Ala residue at position 344 (Takahashi *et al*. 1990). The carboxy terminal processing is indispensable for the capability of O_2 evolution as shown by the studies on a low fluorescent mutant of green algae *Scenedesmus*. The LF-1 mutant is unable to process the C-terminal extension due to the lack of the enzyme activity for the processing, resulting in the absence of the functional Mn cluster for water oxidation (Taylor *et al*. 1988). The site-directed mutants with a stop codon introduced at position 345 (+1 position), generated in *Synechocystis* (Nixon *et al*. 1992) and *C. reinhardtii* (Schrader and Johanningmeier 1992), showed O_2 evolution activity. These results suggested that the presence of the free carboxy terminus of the mature D1 polypeptide might be required for the formation of the functional Mn cluster and/or ligation of Mn ions of the cluster.

Previous mutagenesis studies suggested that proximal amino acid residues to the C-terminal processing site on D1 protein could influence the assembly of the functional Mn cluster. The mutant S345P (Ser-345 to Pro) in *Synechocystis* accumulates only the precursor form of D1 and assembles non-oxygen-evolving photosystem II (Nixon *et al*. 1992). The same mutation in *C. reinhardtii* causes a 100-fold reduction of the processing rate, but allows to accumulate the functional oxygen-evolving complex eventually, indicating that nature of the amino acid residue at +1 position could affect the rate of the processing and that the functional Mn complex could be assembled once the precursor D1 is processed (Hatano-Iwasaki *et al*. 2000). When both of the residues at -1 and -2 position are mutated simultaneously to Ser and Phe, respectively, *C. reinhardtii* cells contain two functionally heterogeneous manganese clusters: One is equivalent to wild-type, while the other shows lower redox potential in the S_1/S_2 and is incapable of water oxidation. In contrast, when the mutation is introduced independently at -1 and -2 positions, the mutants show only the normal cluster (Hatano-Iwasaki *et al*. 2001).

Materials and methods

Strains – Site-directed mutagenesis was performed by LA PCR in vitro Mutagenesis Kit (Takara, Kyoto). LP-stop (Leu-343 to Pro, Ser-345 to stop) and AP-stop (Ala-344 to Pro, Ser-345 to stop) mutations (Table I) were constructed as described previously (Hatano-Iwasaki et al. 2000). S345-stop (Ser-345 to stop) mutation was designed to encode the mature wild-type D1 protein, which was used as a control. The mutated plasmids were introduced in wild-type *C. reinhardtii* strain 137c. For preparing the oxygen evolving photosystem II cores, the His-tagging technologies were employed. The newly generated B-His strain carrying a C-terminal His-tag on the *psbB* gene (Minagawa, J. unpublished) was used as a host.

Thermoluminescence – Thermoluminescence was measured essentially as described (Hatano-Iwasaki *et al*. 2001). Cells (~5 μg Chl) were resuspended in 1 mL of buffer containing 10 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 25 mM MES, pH 6.5. To measure glow curves due to $S_2Q_A^-$ charge recombination (Q-band), the cells were treated with 10 μM DCMU to block reduction of Q_B by reduced Q_A. To record glow curves due to $S_2Q_B^-$ charge recombination (B-band), the DCMU treatment was skipped.

Kinetics of Fluorescence – Decay of Chl a fluorescence yield after a single turnover actinic flash was measured with a pulsed kinetic fluorometer (Occam Technologies,Cincinnati,OH) as described previously (Hatano-Iwasaki *et al*. 2001). Dark-adapted cells in a fresh TAP medium (5μg Chl/mL) were treated with 10 μM DCMU, and given a blue-filtered Xenon flash. Fluorescence yield was monitored by weak red pulses from LEDs.

EPR – EPR measurements were performed using a Bruker ESP-580E X-band spectrometer, equipped with a ER4116 DM X-band (TE102) resonator. The oxygen-evolving photosystem II cores were prepared according to the procedure published previously (Sugiura *et al*. 1998).

Results

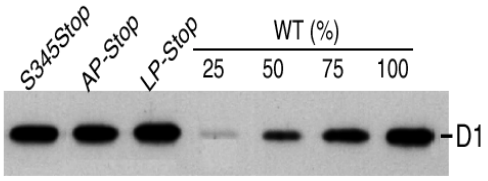

Fig. 1. Effects of mutations on the accumulation of D1 protein. Total cell proteins (2.5μg Chl) were solubilized, separated by SDS-PAGE, and electro-blotted onto a nitrocellulose filter, where D1 was probed with an anti-D1 antibody

In order to further investigate the significance of -1 and -2 positions for the assembly of the functional Mn complex, we introduced Pro at Leu-343 or Ala-344 of D1 protein in *C. reinhardtii*. Replacement by Pro could cause the most significant structural perturbation in point mutagenesis. The C-terminal extension was genetically removed to screen the mutational effects on the C-terminal processing from those on the structure of the mature C-terminus. Firstly, we examined biosynthesis of D1 proteins (Fig. 1). There was no considerable effect of either mutation on the accumulation of D1 proteins on the thylakoids. The expression levels of the two mutants were around 90% of wild-type level.

To examine the mutational effects on the overall function, we checked the photoautotrophic growth. Only LP-stop mutant grew normally (Table I). When we examined steady-state oxygen evolution, LP-stop mutant showed 65% activity of the control while AP-stop mutant showed only 23%. However, Mn content in either of

Table I. Characteristics of the site-directed mutants of *C. reinhardtii*

Strain	amino acid sequence[a]			photoautotrophic	% oxygen	% Mn
	343(-2)	344(-1)	345(+1)	growth[b]	evol.	content[c]
WT	Leu	Ala	Ser	++	N.D.	N.D.
S345-stop	Leu	Ala	**(stop)**	++	100	100
LP-stop	**Pro**	Ala	**(stop)**	++	65	87
AP-stop	Leu	**Pro**	**(stop)**	–	23	86

+, growth; –, no growth.

[a]The mutated residues are in *bold*.

[b]Cells were grown on HSM minimal plates under illumination at 200 μE/m^2/s.

[c]Mn content was measured with atomic absorption spectra.

the mutants was only marginally affected. These results suggested that the presence of Pro in the C-terminal sequence did not interfere with ligating Mn, but when placed at the very C-terminus, namely -1 position, the ligated Mn ions do not form the normal complex.

Thermoluminescence was measured to gather information on the physical state of the redox components. In Fig.2, thermoluminescence Q-band is shown, which originates from $S_2Q_A^-$ charge recombination as cells were treated with DCMU. Compared to the control peaked at +5 ˚C, the two mutants show upshifted peaks at around +20 ˚C. Since the mutants also show the similar upshifts of B-bands (data not shown), we concluded that the redox potential of the S_1/S_2 in the Mn clusters in AP-stop and LP-stop mutants was lower than the control.

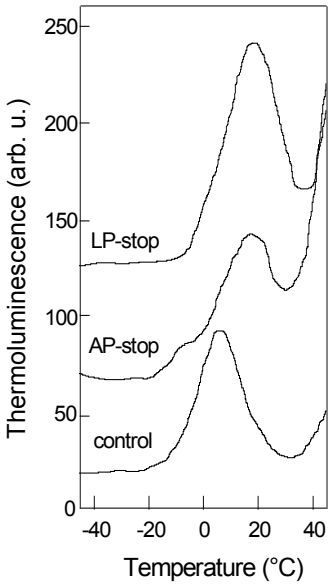

Fig. 2. Thermoluminescence Q-band. Cells were treated with 10 μM DCMU.

Electron transfer events on the donor-side of PS II were examined by measuring kinetics of fluorescence decay in the presence of DCMU (Fig. 3). The rise phase reflects the mixed kinetics of the re-reduction of $P680^+$ by S_1 via Y_Z, while the decay phase reflects the charge recombination of $S_2Q_A^-$. The LP-stop mutant showed a two-fold lower rate in the $S_2Q_A^-$ charge recombination. The AP-stop mutant showed a slower rise and a two-fold faster decay with much smaller amplitude. The slow rise indicates slow re-reduction of $P680^+$, which could be caused by a decreased rate constant for the electron transfer between S-state and Y_Z^+ and/or a smaller equilibrium constant of the rereduction reaction of $P680^+$ by Y_Z. The fast decay indicates that the charge recombination between a donor-side component and Q_A^- is accelerated.

The upshifts of thermoluminescence Q- and B-bands presumably reflect that the redox potential of S_1/S_2 are lowered in the AP-stop and LP-stop mutants, which readily indicate the increases in the equilibrium constants of electron sharing between $S_1P_{680}^+$ and S_2P_{680}. Since the rate of charge recombination of $S_2P_{680}Q_A^-$ state is proportional to $[S_1P_{680}^+]$, the fluorescence decay in these mutants could be slower. This is the case for the LP-stop mutant. However, the decay in the AP-stop mutant was nearly 2-fold more rapid. Therefore, it is more likely that the decay phase in this mutant represents a slowed $Y_ZQ_A^-$ charge recombination. Unidentified auxiliary donor might compete

with Y_Z in the rereduction of P_{680}^+, which could account for the presence of the large constant phase.

The magnetic structure of the Mn complex was studied by EPR using the oxygen-evolving photosystem II cores purified from the mutants. While the LP-stop mutant showed a normal multiline signal, the AP-stop mutant showed no multiline signal but instead an 'S_3-type' split signal (data not shown).

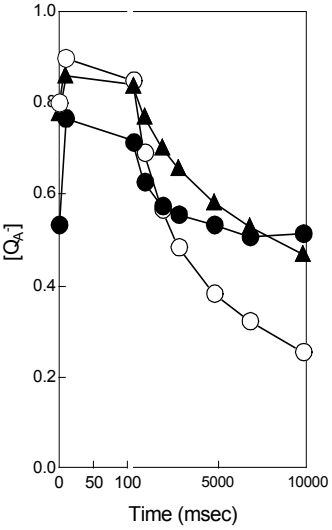

Fig. 3. Kinetics of Chl a fluorescence yield upon single turnover actinic flash. Cells were treated with 10 μM DCMU. Control (*Open circles*), AP-stop (*Closed circles*), LP-stop (*triangles*).

Discussion

Structural perturbation of -2 position of the D1 processing site by substitution with Pro (LP-stop) caused only slight effects on the oxygen evolution. The normal multiline signal supports that the hyperfine coupling in this mutant is not modified. The lowered redox potential of S_1/S_2 leads to a slower back reaction of $S_2Q_A^-$. The decreased oxygen evolution rate could indicate that the energy gap in this reaction in the mutant is suboptimal. On the other hand, the structural perturbation of -1 position (AP-stop) induced more deteriorative effects on the donor-side, which was reflected in the presence of the split signal in EPR spectra. The redox potential of S_1/S_2 was lowered as observed in the mutant at -2 position. However, the fast back reaction is probably accounted for by a slowed $Y_ZQ_A^-$ charge recombination that is competed with a secondary donor. This competition is reflected in the large constant phase of the decay kinetics, which in turn severely inhibits the overall oxygen evolution reaction and probably leads to non-photoautotrophic growth.

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