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

1,2J Minagawa, 3D Ibara, 3A Hatano-Iwasaki, 3Y Takahashi, 2A Ishii, 2,4H Mino, 2T Ono

1Inst. of Low Tem. Sci., Hokkaido Univ., Kita, Sapporo 060-0819, Japan, fax: +81-11-706-5493, email: minagawa@pop.lowtem.hokudai.ac.jp,
2Lab. for Photo-biology, RIKEN-PDC, Aoba, Sendai 980-0845, Japan,
3Dept. of Biol., Okayama Univ., Tsushima-naka, Okayama 700-8530, Japan,
4Fac. of Sci., Nagoya Univ., Chikusa, Nagoya 464-8602, Japan.

Keywords: Chlamydomonas reinhardtii, photosystem II, site-directed mutagenesis, oxygen evolution

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 O2 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 O2 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 S1/S2 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 into 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$_2$·6H$_2$O, 25 mM MES, pH 6.5. To measure glow curves due to S$_2$Q$_A^-$ charge recombination (Q-band), the cells were treated with 10 µM DCMU to block reduction of QB by reduced QA. To record glow curves due to S$_2$QB$^-$ 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

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*

<table>
<thead>
<tr>
<th>Strain</th>
<th>amino acid sequence</th>
<th>photoautotrophic growth</th>
<th>% oxygen evolution</th>
<th>% Mn content</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Leu</td>
<td>Ser</td>
<td>++</td>
<td>N.D.</td>
</tr>
<tr>
<td>S345-stop</td>
<td>Leu</td>
<td>Ala</td>
<td>(stop)</td>
<td>100</td>
</tr>
<tr>
<td>LP-stop</td>
<td>Pro</td>
<td>Ala</td>
<td>(stop)</td>
<td>65</td>
</tr>
<tr>
<td>AP-stop</td>
<td>Leu</td>
<td>Pro</td>
<td>(stop)</td>
<td>23</td>
</tr>
</tbody>
</table>

+, growth; –, no growth.

*The mutated residues are in bold.*

*Cells were grown on HSM minimal plates under illumination at 200 µE/m²/s.*

*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 S₂Q̄⁻ 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 S₁/S₂ in the Mn clusters in AP-stop and LP-stop mutants was lower than the control.

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 S₁ via YZ, while the decay phase reflects the charge recombination of S₂Q̄⁻. The LP-stop mutant showed a two-fold lower rate in the S₂Q̄⁻ 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 YZ⁺ and/or a smaller equilibrium constant of the rereduction reaction of P₆₈₀⁻ by YZ. The fast decay indicates that the charge recombination between a donor-side component and Q̄⁻ is accelerated.

The upshifts of thermoluminescence Q- and B-bands presumably reflect that the redox potential of S₁/S₂ are lowered in the AP-stop and LP-stop mutants, which readily indicate the increases in the equilibrium constants of electron sharing between S₁P₆₈₀⁻ and S₂P₆₈₀⁻. Since the rate of charge recombination of S₂P₆₈₀Q̄⁻ state is proportional to [S₁P₆₈₀⁻], 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 YZQ̄⁻ 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).

**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.

**Acknowledgments**

We wish to thank Dr. M. Ikeuchi for antibody against D1. This study was supported by Grants-in Aid for Encouragement of Young Scientists (A) to J. M., Grants-in Aid for General Research (C) (2) and for Scientific Research on Priority Area (A) to Y. T. from the Ministry of Education. This study was also carried out as a part of “Ground Research Announcement for Space Utilization” promoted by Japan Space Forum.

**References**