Electron Paramagnetic Resonance and Mutational Analyses Revealed the Involvement of Photosystem II-L Subunit in the Oxidation Step of Tyr-Z by P_{680}^+ To Form the Tyr-$Z^+P_{680}Pheo^-$ State in Photosystem II[†,‡]

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Received May 9, 1997; Revised Manuscript Received July 7, 1997[⊗]

ABSTRACT: To reveal the molecular mechanism of involvement of photosystem II (PSII)-L protein in the electron transfer in PSII, effects of mutations in PSII-L on the photochemistry of PSII were investigated by means of electron paramagnetic resonance (EPR) and flash photolysis. Wild type and a series of mutant versions of PSII-L were overproduced in *Escherichia coli* and chromatographically purified. Plastoquinone 9 (PQ-9) depleted PSII reaction center core complex consisting of CP47/D1/D2/Cytb-559/PSII-I/PSII-W was prepared and reconstituted with the wild type and each mutant version of PSII-L together with or without PQ-9. EPR signal indicating the formation of Tyr-$Z^+P_{680}Pheo^-$ state upon room-temperature illumination disappeared in CP47/D1/D2/Cytb-559/PSII-I/PSII-W, and it was recovered when the complex was reconstituted with the wild-type PSII-L. Mutation of a few amino acid residues in the carboxyl-terminal region of PSII-L, such as substitution of a triad of Tyr34, Phe35, and Phe36 by Leu, selectively resulted in the loss of the capability of PSII-L to recover the light-induced formation of Tyr-$Z^+P_{680}Pheo^-$ state in the reconstituted complex. Hydropathy profile of PSII-L suggests that it spans the membrane once by a hydrophobic stretch of the carboxyl-terminal side as its carboxyl end to face to the lumen. If this is the case, the amino acid residues essential for PSII-L to function are expected to be located close to the donor side of P_{680}, suggesting the interaction of PSII-L with Tyr-Z (and/or Tyr-D) or P_{680} to facilitate the oxidation of Tyr-Z by P_{680}^+ to form Tyr-$Z^+P_{680}Pheo^-$ state in PSII. Evidence against PSII-L being involved in the electron transfer from $Pheo^-$ to Q_A was obtained by the flash photolysis experiments.

It is now well established that all of the redox components which participate in the light-induced primary charge separation at the photochemical reaction center of PSII[1] and the succeeding electron transfer at the donor and acceptor sides of the reaction center are located in a heterodimer of two polypeptides denoted as D1 and D2 proteins. A minimal size preparation of PSII which has photochemical activity to take place the primary charge separation between P_{680} and pheophytin (Pheo) was isolated from higher plants by the use of Triton X-100 (*1*). This complex is composed of D1/D2/Cytb-559(α/*β*)/PSII-I (denoted as D1/D2/b-559/I) and associated with 4−5 chlorophyll (Chl) *a*, 2 Pheo, and 2 *β*-carotene but is depleted of plastoquinone 9 (PQ-9), which is the redox component at the primary (Q_A) and secondary (Q_B) quinone sites; as the result, reduction of Q_A by $Pheo^-$ is not observed. In D1/D2/b-559/I, a spin-polarized triplet signal which is a result of charge recombination between P_{680}^+ and $Pheo^-$ was generated upon low-temperature illumination (*2*), while the EPR signal exhibiting the formation of Tyr-Z^+ was hardly observed upon room-temperature illumination (*3*). These results indicate that the activity of the primary charge separation between P_{680} and Pheo by light remains in D1/D2/b-559/I, but the succeeding electron transfer is substantially blocked not only at the acceptor side but also at the donor side. Reconstitution of D1/D2/b-559/I with exogenous quinones (*4*, *5*) as well as naturally occurring PQ-9 (*6*) were reported, but the quantum yields for the electron transfer reaction via Q_A quinone in the resulting complexes were extremely low in every case. An alternative complex composed of CP47/D1/D2/Cytb-559(α/*β*)/PSII-I (denoted as CP47/D1/D2/b-559/I) was prepared by the use of a milder detergent, dodecyl maltoside, followed by the treatment with a mixed detergent system [*n*-octyl *β*-D-glucopyranoside (OG)/*n*-octyl *β*-D-thioglucopyranoside-(OTG)] (*7*). This complex was also depleted of PQ-9 so that the $Pheo^-$ could not be oxidized by Q_A, and reconstitu-

[†] This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (07251209) and a Grant-in-Aid for Scientific Research (04453167) from the Ministry of Education, Science and Culture of Japan.

[‡] This work is part II of a series of works entitled "Role of PSII-L protein (psbL gene product) on the electron transfer in photosystem II complex". Part I is ref *10* (Ozawa et al., 1997).

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[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Chl, chlorophyll; Cytb-559, cytochrome *b*-559; DCPIP, 2,6-dichlorophenolindophenol; DGDG, digalactosyl diglyceride; DM, *n*-dodecyl *β*-D-maltopyranoside; DPC, diphenylcarbazide; EPR, electron paramagnetic resonance; IPTG, isopropyl *β*-D-thiogalactopyranoside; MBP, maltose-binding protein; MES, 2-(*n*-morpholino)ethanesulfonic acid; OG, *n*-octyl *β*-D-glucopyranoside; OTG, *n*-octyl *β*-D-thioglucopyranoside; PQ-9, plastoquinone 9; PSII, photosystem II; P_{680}, primary electron donor in photosystem II; Pheo, pheophytin; Q_A, primary quinone acceptor of photosystem II; Q_B, secondary quinone acceptor of photosystem II; RC, reaction center complex of photosystem II; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Tyr-D, a tyrosine electron donor in photosystem II; Tyr-Z, secondary electron donor in photosystem II.

S0006-2960(97)01088-X CCC: $14.00

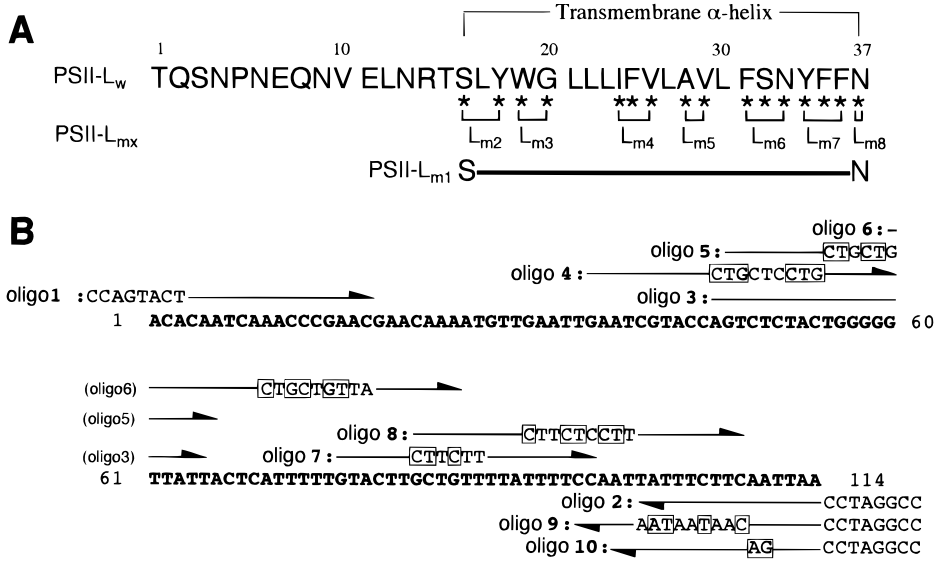

FIGURE 1: Mutations introduced in PSII-L. (A) Amino acid sequence of spinach PSII-L protein and the site substituted or deleted. The asterisks under the amino acid sequence of PSII-L_w show the residues substituted by Leu (PSII-L_{m2} through PSII-L_{m7}) or Ser (PSII-L_{m8}) in the mutants of PSII-L. (B) Nucleotide sequence of spinach *psbL* and the oligonucleotides used for PCR. Sense and antisense primers are indicated by right- and left-directed arrows, respectively. The boxed portions of oligonucleotides represent the bases mutated. A deletion mutant of the amino-terminal region of PSII-L (PSII-L_{m1}) was prepared by means of PCR. PSII-L_{mx} with amino acid substitutions in the putative transmembrane α-helix region were prepared by means of oligonucleotide site-directed mutagenesis (PSII-L_{m2} to PSII-L_{m6}) or PCR (PSII-L_{m7} and PSII-L_{m8}). The resulting DNA fragments were cloned in pMAL-c2 expression vector.

tion of the complex with PQ-9 resulted in a recovery of the electron transfer via Q_A to a quite low extent, similarly to the situation observed in D1/D2/b-559/I. It was found, however, that recovery of the electron transfer in CP47/D1/D2/b-559/I via the reinserted PQ-9 was significantly enhanced when a small subunit protein of PSII, PSII-L, encoded by the *psbL* gene in plastid DNA, was simultaneously reconstituted in the complex (*8*). From a different line of experiments, PSII-L was also found to be essential for the electron transfer in PSII. Destruction of the *psbL* gene in *Synechocystis* sp. PCC 6803 by site-directed mutagenesis resulted in loss of oxygen evolution activity in the resultant mutant strain, but normal PSI activity remained (*9*). Thus, PSII-L is certainly involved in the electron transfer reactions upon illumination in the PSII complex. However, the actual site in the electron transfer chain on which PSII-L participates has not been identified yet.

In this report, we first describe the EPR observation indicating that stable charge separation between $Pheo^-$ and $Tyr\text{-}Z^+$ upon room-temperature illumination, which is not detected in a PQ-9 depleted complex composed of CP47/D1/D2/Cytb-559(α/β)/PSII-I/PSII-W, is recovered in the complex after reconstitution with PSII-L. The dark EPR signal arising from $Tyr\text{-}D^+$ is also partially stabilized in the CP47/D1/D2/Cytb-559(α/β)/PSII-I/PSII-W complex after reconstitution with PSII-L. Second, it is shown that introduction of mutation of a few amino acid residues in the carboxyl-terminal region of PSII-L selectively resulted in the loss of the capabilities of PSII-L to recover the oxidation of Tyr-Z by the light-induced P_{680}^+ and to enhance the electron transfer from DPC to DCPIP in the reconstituted RC. From these results, it is proposed that PSII-L interacts with Tyr-Z (and/or Tyr-D) or P_{680} to facilitate the electron transfer at the donor side from Tyr-Z to P_{680}^+.

EXPERIMENTAL PROCEDURES (MATERIALS AND METHODS)

Construction of the Expression Plasmids to Overproduce Wild-Type and Mutant Versions of PSII-L. Plasmid to overproduce the wild-type PSII-L (PSII-L_w) in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP) was prepared as described in ref *10*. Briefly, a 125 bp DNA fragment containing the *psbL* gene was amplified by PCR using plasmid pTS-9 containing a 5.5 kbp *Sal*I fragment of tobacco chloroplast DNA that encodes the *psbE*−*psbF*−*psbL*−*psbJ* operon as a template and oligo 1 (5′-CC*AGTACT*CAATCAAACCCGAAC-3′) and oligo 2 (5′-CC*GGATCC*TTAATTGAAGAAATA-3′) as primers. After digestion at *Sca*I and *Bam*HI sites (underlined), the PCR-amplified fragment was cloned between the *Xmn*I and *Bam*HI sites of pMAL-c2 vector to give pMAL/psbL.

To prepare expression plasmids for mutant versions of PSII-L (PSII-L_{mx}; $x = 1-8$) with an N-terminal deletion mutation (PSII-L_{m1}) or a series of amino acid substitution mutations in the putative transmembrane α-helix region (PSII-L_{m2} through PSII-L_{m8}), various types of double-stranded DNA fragments were constructed. DNA fragments to prepare PSII-L_{m1}, PSII-L_{m7}, and PSII-L_{m8} were obtained by PCR using given couples of primers containing the mutations shown in Figure 1, while the remaining DNA fragments were constructed by the Kunkel method as follows according to ref 11. PCR-amplified *Sca*I and *Bam*HI fragment from pTS-9 was inserted between the *Sma*I and *Bam*HI sites of M13mp19. The resulting recombinant phage was transfected to *E. coli* CJ236 (*dut*$^-$ *ung*$^-$) and a single-stranded DNA in which thymine bases were converted to deoxyuracil (dU) was isolated. The single-stranded DNA thus obtained and each oligo 3−8 in Figure 1 were mixed, annealed at 75 °C for 15 min, gradually cooled to 37 °C and kept at 37 °C for a further 20 min. Primer extension and subsequent ligation were carried out in a 30 μL reaction

containing 1 unit of T4 DNA polymerase and 200 units of T4 DNA ligase for 2 h at 37 °C. The double stranded DNAs obtained were transfected to *E. coli* BMH71-18 (*ung*$^+$) and subsequently to *E. coli* MV1184. The mutated *psbL* fragments were amplified by PCR with oligos 1 and 2 using the mutated double-stranded DNAs as template and digested with *Sca*I and *Bam*HI to be inserted between the *Xmn*I and *Bam*HI sites of pMAL-c2 vector (pMAL/psbL$_1$ through pMAL/psbL$_8$). Introduction of given mutations in *psbL* was confirmed by DNA sequencing of all of the resulting plasmids (Pharmacia, ALF red DNA Sequencer).

Overproduction and Purification of the Wild-Type and Mutant Versions of PSII-L Protein. Wild-type and a series of mutant versions of PSII-L were overproduced as fusion proteins with MBP in *E. coli* JM109 transformed with given plasmids and isolated as described in ref *10*. Briefly, *E. coli* JM109 transformed with plasmid pMAL/psbL was cultured in 100 mL of LB medium. After addition of IPTG to give a final concentration of 1 mM, the culture was incubated at 37 °C for an additional 4 h. Cells were harvested by centrifugation, suspended in 12.5 mL of solution A [20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM NaN_3], sonicated on ice for 1.5 min in total with 30 time intervals of 7 s each and subjected to a centrifugation at 24000*g* for 10 min at 4 °C. The pellet was solubilized in solution A additionally containing 17.5 mM OG and 10 mM OTG and centrifuged at 24000*g* for 20 min at 4 °C. The supernatant was diluted 2.5-fold with solution A and applied to an amylose resin (New England Biolabs) column. After being washed with solution A, the column was subjected to elution with solution A additionally containing the mixed detergents and 20 mM maltose to recover the fusion protein. The obtained solution containing the fusion protein was stored at −80 °C before being subjected to further treatments.

Cleavage of PSII-L from the corresponding fusion protein was performed by trypsin digestion on the Arg residue at the carboxyl end of the linker sequence connecting MBP and PSII-L as described in ref *10*, since spinach PSII-L had been found to be completely protected against trypsin. After trypsin digestion at 37 °C for 24 h, the solution was dialyzed against a buffer solution of 5 mM Tris-HCl (pH 7.5) for 3 h at 4 °C and loaded on a DEAE-Sephacel (Pharmacia Biotech) column equilibrated with solution B [17.5 mM OG, 10 mM OTG, and 5 mM Tris-HCl (pH 7.5)]. The column was extensively washed with solution B and then subjected to elution with 10 mM NaCl. The fractions that showed a single band at 5.0 kDa on silver-stained SDS−PAGE were collected and finally passed through an SP-Sepharose (Pharmacia Biotech) column equilibrated with solution B to remove a trace of trypsin from the solution. Amino-terminal amino acid sequencing of the 5.0 kDa protein was performed by an amino acid sequencer (Shimadzu, PPSQ-10). SDS−PAGE was done as described by Ikeuchi and Inoue (*12*) on a 15% or 22% polyacrylamide gel with 7.5 M urea.

Disintegration and Reconstitution of PSII Reaction Center Complex. PSII reaction center complex (designated as original RC), which consists of CP47/CP43/D1/D2/Cytb-559/PSII-H/PSII-W/PSII-I/PSII-L/PSII-T/PSII-X/PSII-K and retains high activity of the electron transfer from diphenylcarbazide (DPC) to dichlorophenolindophenol (DCPIP) upon illumination, was isolated from spinach chloroplast according to the method of Dekker et al. (*13*) with some modification as described in ref *8*. Plastoquinone 9 (PQ-9) and naturally occurring PSII-L were also purified from spinach chloroplast as in ref *8*. PQ-9-depleted PSII reaction center complex, which consists of CP47/D1/D2/Cytb-559/PSII-I/PSII-W (designated as depleted RC), was prepared from original RC as described in ref *10*. Briefly, original RC dispersed in solution C [0.03% DM, 20 mM Bis-Tris (pH 6.5), 20 mM NaCl, 10 mM $MgCl_2$, and 1.5% taurine] was loaded on a Q-Sepharose (Pharmacia Biotech) anion-exchange column (bed volume 6 mL) and washed by cycling 20 mL of a solution [17.5 mM OG, 10 mM OTG, 20 mM Bis-Tris (pH 6.5), 20 mM NaCl, and 10 mM $MgCl_2$] at a flow rate of 1 mL/min and at 10 °C in darkness for 1 h. After washing, the column was subjected to a high-salt elution with solution C containing 100 mM $MgSO_4$ to recover depleted RC.

Reconstitution of depleted RC with PQ-9 together with PSII-L (PSII-L$_w$ or each of PSII-L$_{mx}$) was carried out changing the amount of PSII-L as described in refs *7* and *8* and the resulting RC (reconstituted RC) was immediately submitted to further experiments. Reconstitution was also carried out with PSII-L, but without PQ-9 for the EPR experiments. Besides PQ-9 and/or PSII-L, digalactosyl diglyceride (DGDG) purchased from Serdary Research Laboratories Inc. was always added in the reconstitution mixture to give 0.3 μg of DGDG/nmol of reaction center in every reconstitution experiment.

Concentration of Cytb-559 in the original RC, depleted RC, and reconstituted RC preparations was measured as described in ref *14*, from which the concentration of reaction center was determined under an assumption of Cytb-559/reaction center = 1 (*15*). Chlorophyll was quantitated using 80% acetone by the method of Arnon (*16*). Concentration of purified PSII-L was determined by using a protein assay kit (Pierce BCA protein assay reagent kit) against albumin.

Electron Transfer Measurements. The PSII-mediated electron transfer from exogenous electron donor DPC to acceptor DCPIP was measured by following the absorbance change at 605 nm upon illumination in a spectrophotometer (Hitachi U-3500) equipped with sideways illumination with continuous red light provided by a tungsten lamp of 1 kW through a pair of glass filters (Toshiba IRA-25 and R-65) and a 10 cm water layer (*8, 10*). The assay medium consisted of 10 mM NaCl, 5 mM $MgCl_2$, 40 mM MES (pH 6.5), and 30 μM DCPIP as electron acceptor. Each of the RC samples (original RC, depleted RC, or reconstituted RC) and DPC was added in the assay medium to final concentrations of 0.3 μM and 0.5 mM, respectively.

Flash Photolysis. Reduction and reoxidation of Q_A in original RC, depleted RC, and reconstituted RC after flash illumination were measured by monitoring the absorption change at 325 nm with a single-beam flash spectrophotometer basically similar to that described in ref *7*. The measuring light was provided by a xenon lamp of 150 W through a band-pass (250−400 nm) filter (Toshiba, UV-D33S). The light beam which passed through the sample solution in a cuvette with 1 cm path length was focused on the entrance slit of a monochromator (JASCO, CT-25C) and detected by a photomultiplier, digitized and accumulated by a transient recorder (LeCroy 9310A), and processed by using an NEC 9801 personal computer. Each RC sample was added in solution C containing 0.2 mM ferricyanide to a final concentration of 0.8 μM. Before the flash experiment, each sample solution was kept in darkness on ice. After 5 min

of additional dark adaptation in the cuvette at 25 °C, the sample was illuminated by a single laser flash or a train of flashes (pulse width, 5 ns; intensity, 300 mJ/pulse; interval, 2 s) from a Q-switched frequency-doubled Nd−YAG laser (Continuum Powerlite 7000), which was set rectangularly against the measuring beam, and the absorbance change at 325 nm was recorded. The experiments were repeated under the same condition for at least 10 times changing the samples and the obtained data were averaged.

EPR Spectroscopy. X-band EPR measurements were carried out with a Jeol JES-FE2XG spectrometer for original RC, depleted RC, and reconstituted RC with various combination of PQ-9 and PSII-L. Each of the RC samples was suspended in 0.4 M sucrose and 50 mM MES (pH 6.0) containing glycerol of spectroscopic grade (solution D) at 12.5∼50 μM Cytb-559, with or without 5 mM ferricyanide. For comparison, the EPR measurements were done with Tris-washed BBY-type PSII membranes (*17*). The sample solution shielded in a quartz tube of 1.2 mm inner diameter was inserted in a bigger size quartz tube mounted in an EPR cavity. After 10 min of dark adaptation at 25 °C, the EPR spectrum was recorded in the dark and then a saturating, heat-filtered red light provided by a 1 kW tungsten lamp through a pair of glass filters (Toshiba IRA-25 and R-65) was directed into the cavity with a light guide to obtain the spectrum under illumination. The spectrum was also measured 2 min after termination of the light. These measurements were done for every sample in the presence or absence of ferricyanide. Spectrometer conditions are indicated in the corresponding figure legends. The spectrum produced under illumination in the presence of 5 mM ferricyanide was assigned as signal II from total tyrosine radicals. Due to the rather fast decay of Tyr-D^+ in the complexes studied in this work, the signal attributed to Tyr-D^+ (signal II_{slow}) was determined from the spectrum obtained 2 min after termination of the light and that of Tyr-Z^+ (signal II_{fast}) was calculated as total signal II minus signal II_{slow}. To be used for spin standard, BBY-PSII membranes (*17*), which contain 225 chlorophyll molecules/Cytb-559, were prepared and their EPR spectra were recorded with the same spectrometer settings as those used for the RC preparations. We estimated the spin count per reaction center in each RC preparation on the basis of Cytb-559, using the signal II intensity obtained with the BBY-PSII membranes after dark adaptation and a well-established value of Tyr-D^+ spin concentration of 1/235 chlorophyll molecules in BBY-PSII membranes (*17*−*19*). Dynamics of signal II was measured by monitoring the time variation of the signal intensity at $H = 3350$ G, which corresponds to the field strength giving the low-field peak of signal II (indicated by a vertical arrow in Figure 3) responding to turning on/off of light.

RESULTS

Preparation of Wild-Type and Mutant Versions of PSII-L. Figure 2 shows overproduction and purification of the wild-type and mutant versions of PSII-L on an SDS−polyacrylamide gel. As illustrated in lane 1, IPTG induced one product, which migrated at the position of 53 kDa on SDS−PAGE, in *E. coli* JM109 transformed with pMAL/psbL_w. Lane 2 shows the fusion protein of PSII-L_w with MBP purified from the *E. coli* cells by an amylose resin column.

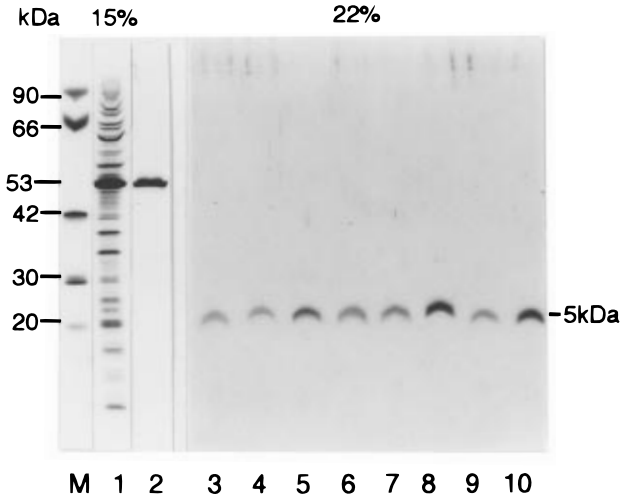

FIGURE 2: Expression and purification of the wild-type and mutant versions of PSII-L. SDS−PAGE was carried out on 15% (lanes 1 and 2) and 22% (lanes 3−10) polyacrylamide gels containing 7.5 M urea. Lane 1, total proteins of *E. coli* cells transformed with pMAL-psbL after addition of IPTG; lane 2, 53 kDa fusion protein purified by an amylose resin column. Lanes 3−10 are the wild-type and mutant versions of PSII-L purified by a DEAE-Sephacel and an SP-Sepharose column. Lane 3, PSII-L_w; lane 4, PSII-L_{m2}; lane 5, PSII-L_{m3}; lane 6, PSII-L_{m4}; lane 7, PSII-L_{m5}; lane 8, PSII-L_{m6}; lane 9, PSII-L_{m7}; lane 10, PSII-L_{m8}; M, molecular weight marker for 15% polyacrylamide gel.

Table 1: Mutation in each PSII-L_{mx} ($x = 1-8$)

mutant	amino acid substitution
PSII-L_{m1}	deletion of Thr 1−Thr 15
PSII-L_{m2}	Ser 16 / Leu 17 / Tyr 18 → Leu / Leu / Leu
PSII-L_{m3}	Trp 19 / Gly 20 / Leu 21 → Leu / Leu / Leu
PSII-L_{m4}	Ile 24 / Phe 25 / Val 26 → Leu / Leu / Leu
PSII-L_{m5}	Ala 28 / Val 29 / Leu 30 → Leu / Leu / Leu
PSII-L_{m6}	Phe 31 / Ser 32 / Asn 33 → Leu / Leu / Leu
PSII-L_{m7}	Tyr 34 / Phe 35 /Phe 36 → Leu / Leu / Leu
PSII-L_{m8}	Asn 37 → Ser

Each PSII-L except for PSII-L_{m1} was cleaved from the corresponding fusion protein by trypsin digestion. After trypsin digestion, they were chromatographically purified on a DEAE-Sephacel column followed by an SP-Sepharose column as described in ref *10*. As shown in lanes 3−10, all of the purified PSII-L gave a single band at the position around 5.0 kDa on SDS−PAGE, although the exact positions slightly deviated from each other, reflecting the change in the amino acid sequence by mutation. Production of the PSII-L with the expected amino acid sequence was confirmed by the amino acid sequence analysis. Table 1 shows the mutations introduced in each mutant version of PSII-L.

Effect of PSII-L_w on the Photochemistry of PSII: PSII-Mediated Electron Transfer from DPC to DCPIP. We previously revealed that restoration of the electron transfer (ET) activity in depleted RC upon reconstitution with PQ-9 was greatly enhanced when it was carried out together with the spinach PSII-L (*10*). Recovery of the electron transfer from DPC to DCPIP upon continuous illumination was detectable in the reconstituted RC with PQ-9 alone, but it did not exceed 11% of the value observed in original RC [7.6 mol of DCPIP reduction (mol of RC)$^{-1}$ s^{-1}; 673 μmol of DCPIP reduction (mg of Chl)$^{-1}$ h^{-1}]. When the reconstitution was carried out together with the recombinant PSII-L_w, the ET activity in the resulting RC increased with increasing amounts of PSII-L_w added in the reconstitution

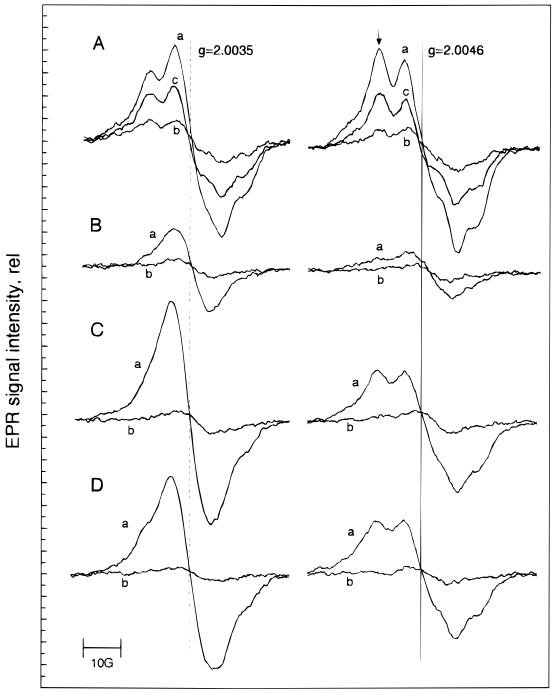

FIGURE 3: Room-temperature EPR spectra of original RC (A), depleted RC (B), reconstituted RC with PSII-L_w (C), and reconstituted RC with PSII-L_w and PQ-9 (D), in the absence (left) or presence (right) of ferricyanide (5 mM). Samples were suspended in solution D at concentration of 50 μM Cytb-559. After 10 min of dark adaptation at 25 °C, EPR spectra were recorded in the dark (b), during continuous illumination (a), and 2 min after terminating the light (c). The solid and dotted lines indicate the positions corresponding to $g = 2.0046$ and $g = 2.0035$, respectively. The arrow indicates $H = 3350$ G. EPR measurement conditions were as follows: microwave frequency, 9.43 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 3.2 G.

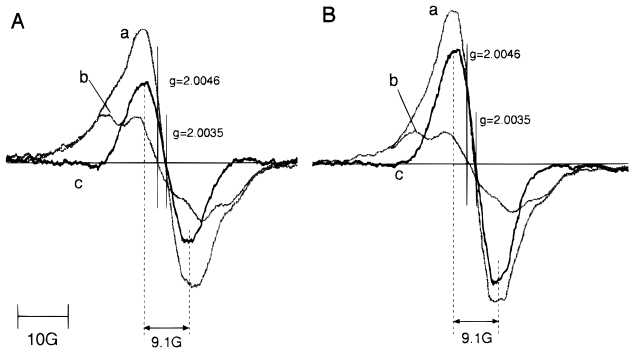

FIGURE 4: Effect of PQ-9 simultaneously reconstituted with PSII-L_w on the photoinduced formation of tyrosine radicals and $Pheo^-$ in the resulting RC. EPR spectra (a) of the reconstituted RC with PSII-L_w together with (A) and without PQ-9 (B) recorded during continuous illumination in the absence of ferricyanide are decomposed into two components arising from tyrosine radicals (b) and $Pheo^-$ (c). EPR measurements were carried out as in Figure 3.

medium and saturated at about 35% [2.7 mol of DCPIP reduction (mol of RC)$^{-1}$ s^{-1}] of that of original RC (Figure 4 in ref *10*). Any difference with statistical meanings was not recognized between the recombinant PSII-L_w and naturally occurring spinach PSII-L in the effectiveness to enhance the ET activity in the resulting complexes, suggesting that recombinant PSII-L_w is functionally equivalent to the spinach protein. The reconstitution was also carried out with a fusion protein, MBP/PSII-L_w, to find that MBP existing at the amino-terminal end of PSII-L_w causes no interference in the function of PSII-L_w.

Electron Transfer to Form the Tyr-$Z^+P_{680}Pheo^-$ State in PSII. To examine the effect of PSII-L on the photoinduced electron transfer at the donor side of P_{680}, EPR experiments were carried out. Figure 3 compares EPR signals in the free radical region observed at room temperature (25 °C) with original RC, depleted RC, and reconstituted RC under same spectrometer settings. Original RC shows signals similar to those observed in Tris-washed PSII membranes. Both in the presence and in the absence of ferricyanide, a weak signal with the characteristic hyperfine structure and g value of a tyrosine radical arising from dark-stable Tyr-D^+ (*20, 21*) was observed. Continuous illumination greatly enhanced the tyrosine radical signal, indicating the photooxidation of tyrosine (Tyr-Z and/or Tyr-D) to take place in this complex. In the presence of ferricyanide, the signal observed under illumination was reversibly reduced by a factor of about 2 by terminating the light and this reversible variation in the signal intensity occurred with a rise time less than 10 ms (instrument-limited) and decay time of 2 s, responding to repeated on/off switching of light. We attribute this light-responding component of the signal to signal II_{fast} (Tyr-Z^+) and the remaining one to signal II_{slow} (Tyr-D^+). In the absence of ferricyanide, a distortion which is supposedly due to a contribution from $Pheo^-$ radical was observed in the spectrum produced by illumination.

In depleted RC (CP47/D1/D2/Cytb-559/PSII-I/PSII-W), no significant EPR signal due to the tyrosine radicals was observed either in the light or in the dark; alternatively, a 10-G wide Gaussian signal with $g \doteqdot 2.003$ was induced upon illumination in the absence of ferricyanide. Thus, Tyr-D is in the reduced form in the dark and neither Tyr-D nor Tyr-Z can be photooxidized in depleted RC.

In the reconstituted RC with PSII-L_w, the signal due to the dark-stable radical was not observed irrespective of the presence or absence of ferricyanide. However, illumination induced the tyrosine radical signal in the presence of ferricyanide, although the signal intensity was about 45% that observed in original RC. The tyrosine radical is a dominant photoaccumulated species in the $g = 2$ region in the presence of ferricyanide and its rise time was less than 10 ms (instrument-limited) and its decay by termination of light was biphasic with half-times of about 2 s and 5 min. While, in the absence of ferricyanide, a light-induced 9.5-G wide signal at $g = 2.003$ dominated the spectrum, augmented by a weak signal in the low-field region which is due to a small contribution from tyrosine radicals. Figure 4 shows that the signal observed in the absence of ferricyanide is a composite of signals from the reduced primary electron acceptor $Pheo^-$ ($g = 2.0032$) detected by Petersen et al. (*3*) in the quinone-depleted complex and tyrosine radicals. Although PQ-9 had little effect by itself on either the dark or light EPR spectra, irrespective of the presence or absence of ferricyanide (data not shown), additional insertion of PQ-9 to the PSII-L_w reconstituted RC partly reduced the intensity of the $Pheo^-$ signal observed in the reconstituted RC with PSII-L_w alone in the absence of ferricyanide and increased the intensity of the tyrosine radical signal. In the presence of ferricyanide, PQ-9 did not affect the intensity of the tyrosine radical signal at all.

These results indicate that the reconstitution of depleted RC with PSII-L_w resulted in the recovery of the capability

Table 2: Spin Numbers Attributed to Tyrosine Radicals

sample	total Tyr radical[a] (signal II)	Tyr-Z^{+} [b] (signal II_{fast})
original RC	1.6	0.74
depleted RC	0.09	0.02
reconstituted RC with PSII-L_w	0.72	0.37

[a] Spin number attributed to total tyrosine radicals produced under continuous illumination. [b] Spin number attributed to Tyr-Z^{+} calculated from the difference between the signal II intensities observed under continuous illumination and after 2 min of dark adaptation after terminating the light.

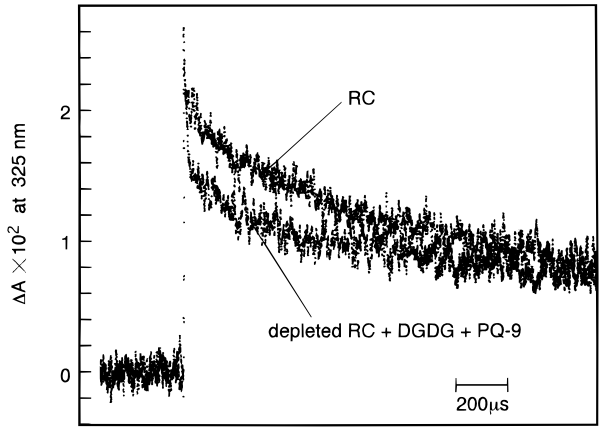

FIGURE 5: Traces of transient absorbance change at 325 nm induced by a single flash illumination in original RC and the reconstituted RC with PQ-9. Samples were suspended in solution C containing 0.2 mM ferricyanide at a concentration of 0.8 μM Cytb-559 and submitted to the flash photolysis experiments at 25 ± 1 °C. The sample was changed after a single laser flash excitation (pulse width, 5 ns; intensity, 300 mJ/pulse) and the data recorded with 10 samples were accumulated.

to form the Tyr-$Z^{+}P_{680}$Pheo^{-} state in the resultant complex and that the electron transfer from Pheo^{-} to the reinserted PQ-9 can take place at least partially. Table 2 summaries the total spin numbers and the portions attributed to Tyr-Z^{+} produced under continuous illumination in original RC, depleted RC, and reconstituted RC with PSII-L_w in the presence of ferricyanide. As shown in Table 2, reconstitution of depleted RC with PSII-L_w recovers the photoproduction of 0.72 spin/reaction center attributed to tyrosine radicals, which is 45% that in original RC (1.6/reaction center).

Electron Transfer at Q_A Site. The effect of PSII-L on the electron transfer at Q_A site was investigated by measuring the transient absorbance change arising from the reduction and reoxidation of Q_A upon flash illumination at 25 °C with original RC, depleted RC, and reconstituted RC with PQ-9 with or without PSII-L_w. Figure 5 shows typical traces of the absorbance change at 325 nm induced by a single flash after dark adaptation in the presence of 0.2 mM ferricyanide. In original RC, a rapid increase in the absorbance within 1 μs followed by decay consisting of at least three components was detected. The decay component with a relaxation time of about 150 μs was 15% ± 3% of the initial change. The component comprising 55% ± 5% of the total amplitude decayed with a relaxation time of 1.5 ms and the remaining 30% ± 5% decayed more slowly than 3 ms, indicating that the back reaction between P_{680}^{+} and Q_A^{-} (150 μs component) is insignificant and the reoxidation of Q_A^{-} is done by ferricyanide mainly via Q_B. As expected, the transient absorbance change at 325 nm upon flash illumination was

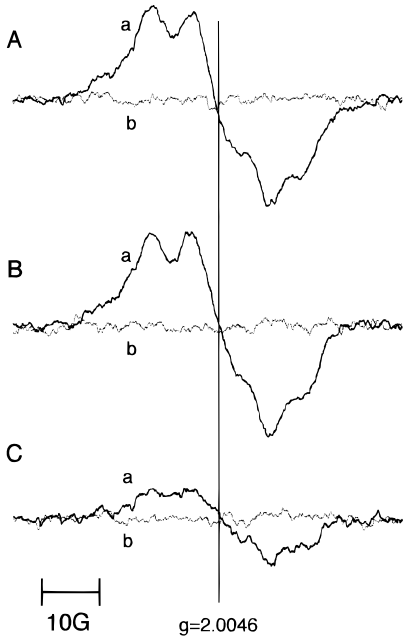

FIGURE 6: Typical examples showing difference in the effect of different mutations of PSII-L on the photoproduction of tyrosine radicals in the PSII-L reconstituted RC. EPR spectra were recorded with the reconstituted RC with PSII-L_w (A), PSII-L_{m1} (B), and PSII-L_{m7} (C) during illumination in the presence of 5 mM ferricyanide at 25 °C. EPR measurements were carried out as in Figure 3.

almost undetectable in depleted RC as the initial amplitude of the signal was less than 5% in original RC (data not shown), but the signal was recovered in the reconstituted RC with PQ-9. The initial amplitude of the signal was 65% ± 5% of the original level, but a significant difference in the reoxidation process of Q_A^{-} was observed between original RC and reconstituted RC with PQ-9. In the PQ-9-reconstituted RC, the fast decay component of 150 μs increased to 50% ± 5% of the initial amplitude and the remaining 50% ± 5% decayed more slowly than 3 ms. Addition of PSII-L_w in the reconstitution medium containing PQ-9 did not make any difference in the initial amplitude of the signal from the resulting RC, but the amplitude of the 1.5∼3 ms decay components increased to 70% ± 5% at the expense of the 150 μs component (data not shown), suggesting reduction of P_{680}^{+} by Tyr-Z takes place, competing with the charge recombination between Q_A^{-} and P_{680}^{+} in the simultaneously reconstituted RC with PQ-9 and PSII-L. These results also suggest that PSII-L is not involved in the electron transfer step from Pheo^{-} to Q_A but in the step from Tyr-Z to P_{680}^{+}.

Effect of Various Mutations in PSII-L on the Photochemistry of PSII. In order to reveal the amino acid residues in PSII-L that actually influence the photochemistry of PSII, effect of introduction of mutations at various spots of PSII-L on the ET activity and EPR spectrum in reconstituted RC was examined. Figure 6 shows typical examples of the effects of two different mutations on the EPR spectrum. Complete substitution of the successive 15 amino acid residues from the amino-terminal end, which are possibly exposed to the aqueous phase by the sequence of MBP (PSII-L_{m1}), did not affect the capability of PSII-L to recover the tyrosine radical formation upon illumination in the reconstituted RC. On the other hand, if the three amino acid residues (Tyr34, Phe35, and Phe36) in the putative transmembrane α-helix in PSII-L were substituted by Leu, the

resultant PSII-L (PSII-L_{m7}) almost completely lost the capability to produce tyrosine radical upon illumination in the reconstituted RC, even when PSII-L_{m7} was added in the reconstitution medium to give a molar ratio of PSII-L_{m7}: reaction center = 3:1.

Figure 7 summarizes the effect of mutation in the different spots of PSII-L on the ET activity and the formation of tyrosine radicals upon illumination in the reconstituted RC with each of PSII-L_{mx} together with PQ-9. The amount of Tyr-Z^+ induced by light was estimated from the difference between the signal II intensities observed during illumination and 2 min after termination of the light in the presence of ferricyanide. The effect of Leu scanning mutagenesis to give Leu triads in the putative transmembrane α-helix exhibits a strong regional specificity both on the ET activity and on the photooxidation of Tyr-Z. At a glance, the parallel relationship in the effect of the mutations is found between the ET activity and the photooxidation of Tyr-Z. Substitution by Leu of a pair of Trp19/Gly20 (PSII-L_{m3}), a triad of Ile24/Phe25/Val26 (PSII-L_{m4}), and a pair of Ala28/Val29 (PSII-L_{m5}), which are located at the amino-terminal side and middle part of the α-helix, hardly affected the function of PSII-L, although Leu substitution of a pair of Ser16 and Tyr18 at the amino-terminal end of the α-helix (PSII-L_{m2}) seemed to reduce the activity of the PSII-L to some extent. In contrast, Leu substitution of triads of Phe31/Ser32/Asn33 (PSII-L_{m6}) and of Tyr34/Phe35/Phe36 (PSII-L_{m7}) located at the carboxyl-terminal side of the α-helix resulted in a drastic suppression of the abilities of PSII-L to enhance the ET activity and to recover the oxidation of Tyr-Z in reconstituted RC. These results indicate that PSII-L participates in the electron transfer step from Tyr-Z to P_{680}^+ through its carboxyl-terminal region. Substitution of Asn37 located at the carboxyl-terminal end of PSII-L by Ser (PSII-L_{m8}) slightly reduced the activity of PSII-L.

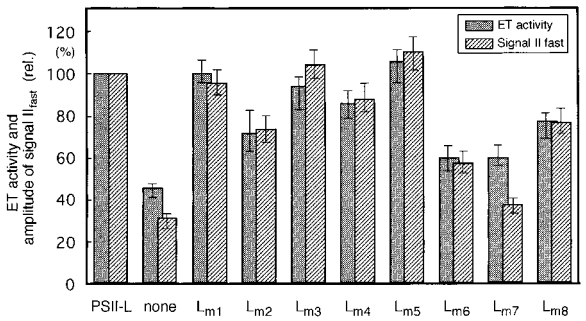

FIGURE 7: Effect of mutation in the different spots of PSII-L on the ET activity and the photooxidation of Tyr-Z in the reconstituted RC. Depleted RC was reconstituted with PSII-L_w or each of PSII-L_{mx} ($x = 1-8$) together with PQ-9 and submitted to the measurements of the ET activity and EPR signal. The ET activity was determined by measuring the rate of electron transfer from DPC to DCPIP under illumination. The amplitude of Tyr-Z^+ signal (signal II_{fast}) was estimated from the difference between the signal II intensities observed under light and 2 min dark adaptation after terminating the light. None shows the activity of the RC reconstituted with DGDG and PQ-9. The ordinate shows the relative values of the ET activity (dotted column) and the amplitude of Tyr-Z^+ signal (hatched column) observed with each of the reconstituted RCs to those with the PSII-L_w reconstituted RC together with PQ-9 and DGDG. All data are given as mean values of at least three independent experiments with indication of deviations.

DISCUSSION

This study provides the first evidence for the functional significance of PSII-L on the electron transfer at the donor side of P_{680} in PSII. Extraction of CP43, PSII-H, PSII-L, PSII-X, PSII-T, and PSII-K concomitantly with PQ-9 from the CP47/CP43/D1/D2/Cytb-559/PSII-H/PSII-I/PSII-W/PSII-L/PSII-T/PSII-X/PSII-K complex (original RC) by the treatment with an mixture of OG and OTG resulted in the loss of the electron transfer activities at both of the acceptor ($Pheo^- \rightarrow Q_A$) and donor (Tyr-Z $\rightarrow$ P_{680}^+) sides of the reaction center in the resulting complex (D1/D2/CP47/Cytb-559/PSII-I/PSII-W). Although the electron transfer at the acceptor side was recovered when it was reconstituted with PQ-9, without any help of the subunit proteins liberated from original RC, the recovery of the electron transfer at the donor side selectively required PSII-L. Although illumination of depleted RC produced a weak 10-G wide Gaussian signal with $g \doteqdot 2.003$, it may be mainly due to oxidation of Chl by P_{680}^+, which was observed by Paula et al. (*22*) in Tris-washed PSII upon illumination at 77 K where the electron donation from Tyr-Z to P_{680}^+ is inhibited.

Petersen at al. (*3*) isolated a PSII reaction center complex which was largely depleted of PQ-9 by solubilizing a Tris-treated reaction center preparation with DM (DM complex). In the presence of ferricyanide, an EPR signal with the characteristic line shape and *g* value of the tyrosine radicals associated with PSII was found to be photoaccumulated in the DM complex. In the absence of ferricyanide, a 9.5−11-G wide signal was observed at $g = 2.003$. These signals are essentially the same as those observed in the present works with the PSII-L_w reconstituted RC under the same EPR conditions. The protein composition of the DM complex examined by SDS−PAGE analysis was reported to be CP47/D1/D2/Cytb-559 as it was named. Since the SDS−PAGE was carried out on a gel containing 13.5% acrylamide, focusing on the proteins with molecular weight larger than 9 kDa (α-subunit of Cytb-559), information on the subunits with lower molecular weight, such as PSII-I, PSII-L, and PSII-K, was lacking. We prepared the DM complex according to the procedure reported and examined the protein composition by SDS−PAGE on a gel containing 22% acrylamide and found that the complex retains PSII-F (β-subunit of Cytb-559), PSII-I, and PSII- L. This complex exhibited the essentially same room-temperature EPR behavior as that previously reported by Petersen et al. (*3*) with their DM complex. Furthermore, the number of spins generated by the photoproduced tyrosine radicals in the DM complex had been estimated to be 0.65−0.70/reaction center (*3*), which well agrees with the value of 0.72 obtained for the reconstituted RC with PSII-L_w in the present work. As one explanation why tyrosine radicals can be observed in the DM complex but not in D1/D2/b-559/I, the presence of the Chl-binding CP47 protein in the DM complex was invoked (*3*). The present works, however, clearly demonstrated that the presence of CP47 is not sufficient for photoaccumulation of the tyrosine radicals in the complex, because a signal indicating photoproduction of the tyrosine radicals was not observed in depleted RC, which retains CP47, but it was observed in the complex after reconstitution with PSII-L_w. However, it should be mentioned that the possibility of the involvement of CP47 in the Tyr-Z $\rightarrow$ P_{680}^+ step has not been ruled out by the present results, because we did not succeed in observation of the tyrosine radical formation in D1/D2/b-559/I reconstituted with PSII-L_w (data

not shown). Alternatively, it is possible that preparation of D1/D2/b-559/I using Triton X-100 induces changes that inhibit reassembling of PSII-L in the complex.

The formation of photoaccumulated EPR signal in the DM complex in the absence of ferricyanide had been explained by a kinetic model (*3*) and the explanation is applicable to the reconstituted RC with PSII-L_w in the present work. Briefly, under strong light illumination, the Tyr-$Z^+P_{680}Pheo^-$ state would accumulate if its formation is faster than its decay. The formation rate was estimated from the result obtained in Tris-inhibited PSII membranes (*23*) as about 5 μs at pH 6.5. Although the kinetics of the decay have not been reported, a reasonable estimation was given. The recombination of radical pair $P_{680}^+Pheo^-$ was found to occur with a half-time of 50 ns in D1/D2/b-559/I complex (*24*). The recombination of Tyr-$Z^+Q_A^-$ was reported to be about 500 times slower than that of $P_{680}^+Q_A^-$ (*20*); if the recombination process of Tyr-Z^+Pheo^- is also 500 times slower than that of $P_{680}^+Pheo^-$, then decay kinetics of about 25 μs would be expected, which is sufficiently slow to permit an accumulation of the state $Tyr^+P_{680}Pheo^-$.

The enhancement of the ET activity in the PQ-9 reconstituted RC by simultaneous insertion of PSII-L is supposed to be a result of recovery of the electron transfer at the donor side rather than the acceptor side. When the electron transfer step from Tyr-Z to P_{680}^+ is blocked, P_{680}^+ is more rapidly reduced through the back reaction from $Pheo^-$ or Q_A^- than by an exogenous electron donor DPC, since DPC acts as an efficient electron donor to Tyr-Z^+ but not to P_{680}^+. Judging from the results of the flash photolysis experiments, PSII-L may not be involved in the electron transfer step from $Pheo^-$ to Q_A, because the transient absorbance change at 325 nm attributed to the accumulation of Q_A^- upon a single laser flash was recovered in the PQ-9 reconstituted RC as to be 65% ± 5% the original level, independent of the presence or absence of PSII-L (Figure 5). Absence of PSII-L in the complex, however, affected the decay process of Q_A^- to increase the amplitude of the 150 μs decay component at the expense of the 1.5 ms component. This result indicates that the charge recombination between P_{680}^+ and Q_A^- (150 μs) frequently takes place in the PSII-L-lacking complex by the inhibition of the normal electron flow from Tyr-Z to P_{680}^+ and is consistent with the enhancement of the ET activity in the PQ-9 reconstituted RC by PSII-L. It should be mentioned here that these results were obtained under a single flash excitation. When the signals induced by a train of laser flashes without changing the sample of the reconstituted RC with PQ-9 alone were accumulated, the averaged intensity decreased with increasing number of flashes. Such a decrease in the signal intensity with flash number was not observed in original RC up to 30 flashes as we previously reported (*8*). When the reduction of P_{680}^+ by Tyr-Z is blocked in the reconstituted RC with PQ-9 alone, the oxidation of Chl *a* in the core complex by P_{680}^+ occurs in competition with the charge recombination between Q_A^- and P_{680}^+ (*22*), and Chl a^+ radical produced by the repeated flash excitations may lead to destruction of the complex (*25*).

PSII-L consists of 37 amino acid residues and has a sufficient hydrophobic stretch indicating a single transmembrane α-helix in the carboxyl-terminal region (*26*, *27*) and a domain composed of hydrophilic amino acids in the amino-terminal side. It is a consensus understanding that intrinsic subunit proteins of PSII complex encoded by the plastid DNA orient in thylakoid membrane such that their amino-terminal ends are exposed or faced to the stroma in chloroplast (*28*−*32*). If this is the case for PSII-L, the most likely organization of PSII-L in the PSII complex suggested from the primary sequence is with the hydrophilic domain in the amino-terminal side exposed to the stroma and a hydrophobic stretch of the carboxyl-terminal side spanning the membrane once toward the lumen side. Therefore, the carboxyl-terminal region containing the amino acid residues essential for PSII-L to function (from Phe31 to Phe36) is expected to be located at the lumen side in the membrane, close to the donor side rather than the acceptor side of the reaction center. Thus, the results of the Leu scanning mutagenesis on a putative α-helix region of PSII-L strongly support our proposal that PSII-L participates to the step of electron transfer to oxidize Tyr-Z by the photoinduced P_{680}^+. Substitution by Leu of a pair of amino acid residues (Ser16 and Tyr18) at the amino-terminal end of the transmembrane α-helix slightly but significantly reduced the capability of the PSII-L to produce tyrosine radicals upon illumination in the reconstituted RC. Since these residues are expected to be located at the position corresponding to the membrane surface or the hydrophilic head groups of the lipid bilayer, it is likely that substitution of Ser residue to hydrophobic Leu residue may push PSII-L into the membrane to hinder normal interaction of PSII-L with the core part of PSII.

The EPR experiments with the reconstituted RC seem to indicate that PSII-L recovers the signals from both Tyr-Z^+ and Tyr-D^+, which suggests that PSII-L participates in the electron donation from Tyr-Z and Tyr-D to P_{680}^+. One explanation is that PSII-L may alter the redox potential of P_{680}^+ to facilitate the oxidation of both Tyr-Z and Tyr-D. Alternatively, it is possible that the stoichiometry of PSII-L to reaction center (Cytb-559) is 2 rather than 1 and each PSII-L interacts independently with Tyr-Z on D1 and Tyr-D on D2 proteins. Detailed *in vitro* and *in vivo* mutational analysis of amino acid residues of PSII-L will appear elsewhere to give further information on the molecular mechanism of the role of PSII-L in the electron transfer at the donor side of PSII.

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BI9710885