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## **Three-dimensional structure of photosystem II studied by pulsed EPR**

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### **Introduction**

Photosystem II (PS II) is a membrane-bound protein complex and responsible for the photochemical oxidation of water to di-oxygen and the reduction of the plastoquinone [Ort et al. 1996].

Recently, the three dimensional structure of the PSII core particle was resolved at 3.8 Å by X ray crystallography, and the number and geometry of chlorophyll molecules were mostly identified [Zouni et al. 2001]. The reaction center of P680 is composed of two chlorophylls separated by 10 Å between Mg atoms. Furthermore, the Mn<sub>4</sub> complex was assigned to a Y-shape cluster with a large size of about 7 Å. The cytochrome b<sub>559</sub> is located nearby the D2 protein and is, thereby, close to Chl<sub>D2</sub>.

Pulsed EPR techniques, PELDOR (Pulsed Electron eLectron DOuble Resonance) and spin polarized ESEEM (Electron Spin Echo Envelope Modulation) have provided powerful methods for the determination of the distances between photosynthetic electron transfer components. In ESEEM, the distance between P680 and Q<sub>A</sub> was also determined to be 27.4 Å [Zech et al. 1997, Hara et al. 1997]. Though these distances represent the separation between the spin centers of a radical pair, the accuracy of the distance resolution of 0.3 Å is higher than that of present X-ray analysis. We have applied PELDOR and '2+1' pulse sequence methods to PS II and determined distances between radicals of electron transfer components in PS II; 27 Å for Mn-cluster in the S<sub>2</sub>-state and Y<sub>D</sub> [Kodera et al. 1995], 30 Å for Y<sub>Z</sub>-Y<sub>D</sub> [Astashkin et al. 1994], 38.5 Å for Y<sub>D</sub>-Q<sub>A</sub> and 29.4 Å for Y<sub>D</sub>-Chl<sub>Z</sub> [Shigemori et al. 1998], respectively. However, these distances were measured from Y<sub>D</sub><sup>•</sup>, which is the most stable radical in PS II in the dark. In order to elucidate the relative arrangement of the radicals in PS II, reliable distances between respective radicals and Y<sub>Z</sub> radical are required, as complements for those from Y<sub>D</sub> radical.

In this report we describe a PELDOR method applied to non-oriented and

oriented membranes of spinach PS II to determine the distance between  $Y_D$  and  $Y_Z$ , and  $Y_D$  and the Mn multiline center, and a mutant deficient of  $Y_D$  ( $Y_D160$ -D2) that were genetically constructed in *Chlamidomonas reinhardtii*. The relative positions of the electron transfer components  $Y_Z$  and  $Q_A$  in PS II were determined, and the results obtained for by pulsed EPR methods were discussed together with those for other radical species in relation to the crystallographic structure of PS II complex studied by X-ray [Zouni et al. 2001].

## Materials and Methods

The  $Y_D$  residue (the 160th tyrosine of the D2 protein) was replaced with phenylalanine by site-directed mutagenesis by the use of the His-tagged *psbD* plasmid, pBD302H. The  $Y_D160$ -D2 mutation was introduced according to "Megaprimer" method using oligo-nucleotide, 5'-TTCCTAATTTTCCCATTAGGT-3'. The mutated plasmid was used to transform the D2-deficient strain,  $\Delta D2$ -2. The resultant mutant strain of *C. reinhardtii*,  $Y_D160$ -D2, was used throughout this study. The  $Y_D160$ -D2 mutant cells were grown in liquid TAP medium under low light ( $\sim 5 \mu E/m^2/sec$ ) at 25 °C with continuous aeration. Cells were harvested at a mid-logarithmic phase, resuspended in a buffer containing 25 mM MES, 100 mM NaCl, 12.5 % glycerol, pH 6.5, and isolated with a Ni-column as described previously [Sugiura et al. 1998].

Tris-treated PS II were prepared to remove Mn atoms. For Zn-substitution, the Tris-treated PS II particles were suspended in  $ZnCl_2$  containing buffer, 24 mM  $ZnCl_2$ , 0.2 M sucrose, 20 mM MOPS, 20 mM NaCl, to substitute the non-heme iron by  $Zn^{2+}$  [Jegershoeld et al. 1999] (Zn-substituted PS II). Tris-treated and Zn-substituted PS II particles were suspended in a solution of 0.2 M sucrose, 20 mM NaCl, 1 mM EDTA and MOPS/NaOH (pH6.8 at 20 °C) containing 50 % glycerol (w/v), with the final chlorophyll concentration at 3.2 mg/ml.

Oxygen-evolving PS II was illuminated at 200 K for 10 min to obtain the  $S_2$ -state multiline EPR signal. The  $S_0$ -state was obtained by reduction of spinach PS II with 120  $\mu M$   $NH_2OH$ . Tris-treated spinach PS II membranes was illuminated for 20 s at 253 K with a 500 W tungsten halogen lamp through a 10 cm thick water filter and immersed immediately in a cool bath at 200 K to trap  $Y_Z$ . The Zn-substituted PS II was illuminated for 20 sec at 253K and immediately put into the 200 K bath in order to trap  $Y_Z \cdot Q_A^-$  radical pair.

CW (continuous wave) EPR measurement was carried out on a Varian E-109 X-band EPR spectrometer equipped with a TE<sub>102</sub> rectangular cavity at 77 K to observe the signal due to trapped radicals  $Y_Z \cdot$  and  $Q_A^-$ . The PELDOR measurements were performed on a pulsed EPR spectrometer ESP-380 (Bruker)

using a three pulses sequence. In PELDOR m.w. (microwave) magnetic field amplitude,  $H_1$ , in three pulses was adjusted to provide the spin rotation angles of  $90^\circ$ ,  $180^\circ$  and  $180^\circ$ , respectively. The first and third pulses of frequency  $\omega_1$ , separated by a time interval  $\tau$ , form the primary ESE (Electron Spin Echo) of one of the spin species. The second pulse of frequency  $\omega_2$ , separated from the first one by the time interval  $\tau' (\leq \tau)$ , changes orientations of the other radical spin. If the magnetic dipole interaction between the pair-wise distributed radicals is noticeable, flip of one of the spins changes the local magnetic field at its partner in the pair. As a result, the magnetization after the third pulse cannot be completely refocused at the time  $2\tau$  and the amplitude of the ESE signal exhibits the dependence on the second pulse position (i.e.,  $\tau'$ ). The following expression describes this dependence [Astashkin et al. 1998]:

$$V(\tau, \tau') \propto [\cos(2\pi D \tau') - 1] \quad (\tau' \leq \tau), \quad (1)$$

where

$$D = D_0(1 - 3\cos^2 \theta), \quad \text{with } D_0 = g_1 g_2 \beta^2 / \hbar r^3 \quad (2)$$

$D_0$  is the dipole interaction constant between the two spins, in which  $r$  is the distance between the radical pair.  $\theta$  is the angle between the external magnetic field and the vector  $\mathbf{r}$  joining the two spins. Eq. (1) is averaged over angles  $\theta$  to fit the observed time profile. The constant  $D_0$  was estimated and the distance  $r$  was determined. When the frequencies  $\omega_1$  and  $\omega_2$  coincide, the method is called as ‘2+1’ pulse sequence, that also shows similar time dependence as Eq. (1)

## Results

Fig. 1 show the PELDOR time profile observed for the Mn-multiline in the  $S_2$ -state in non-oriented spinach PS II membranes. Fig. 1A shows a field sweep ESE for the field range including Cyt  $b_{599}$ , the multiline and  $Y_D$  radical signals. The derivative curve shows a similar line shape as observed by CW EPR method. ESE of the multiline was observed at the position shown by an arrow at the frequency of  $\omega_1$  and the center of  $Y_D$  radical signal excited by the frequency  $\omega_2$ . The observed time profile is shown in Fig. 1B. By simulations using Eqs. (1) and (2) and integration over the angle  $\theta$ , the best fitted value of distance was obtained to be 27 Å.

Fig. 2 shows the time profiles observed by ‘2+1’ pulse sequence in Tris-treated PS II oriented membranes, where  $Y_Z$  radical was trapped by illumination at 253 K. The spectrum A was observed for the magnetic field direction parallel to the membrane normal, while the spectrum B shows that observed with the magnetic field

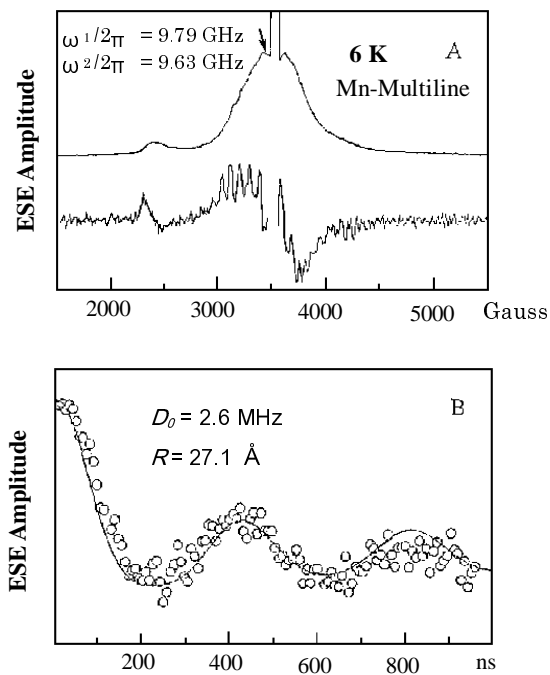


Fig.1. **A**. Field sweep ESE of the  $S_2$ -state Mn multiline and  $Y_D$  radical signals. Its derivative curve is similar to CW EPR. In **B** ESE is observed at the position, satisfying condition  $\omega_1 = g\beta B_0$  shown by the arrow in **A**, and  $Y_D$  radical signal is excited at the frequency  $\omega_2$ .

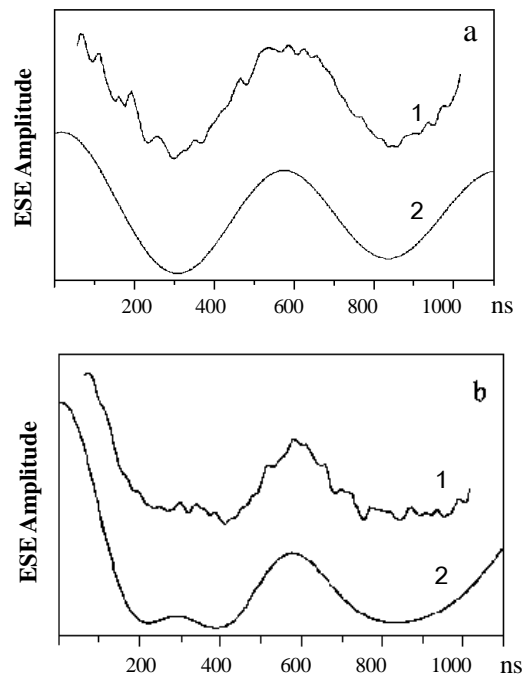


Fig.2. **a** The time  $\tau'$  dependence of '2+1' pulse sequence observed for  $Y_Z$  and  $Y_D$  radical pair in Tris-treated spinach PS II for magnetic field parallel to the membrane normal. **b** That observed for the magnetic field along the membrane plane. 1 shows experimental profiles, 2 shows the best fitted simulations with the values  $R = 29.5 \text{ Å}$ ,  $\theta = 80^\circ$  or  $110^\circ$ .

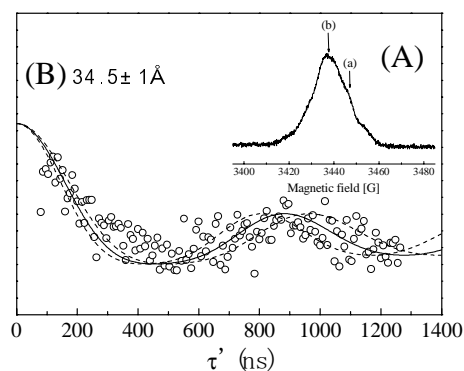


Fig. 3. (A) A field-sweep ESE spectrum observed in Zn-substituted *C. reinhardtii* PS II.  $Y_Z \cdot Q_A^-$  radical pair were trapped by illumination at 253 K for 20 s. (B) The dependence of the primary ESE amplitude on  $\tau'$ , measured for  $Y_Z \cdot Q_A^-$ . A full and dotted lines show the best fitted and 1 Å different values for simulation.

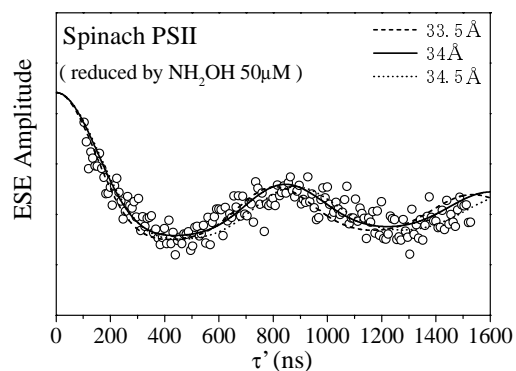


Fig.4. PELDOR time profile observed for the multiline in the  $S_0$ -state PS II and  $Y_D$  radical with the two frequency separation  $\omega_1 - \omega_2 = 0.1 \text{ GHz}$ . The 34 Å curve shows the best fitted simulation.

along in the membrane plane. Almost doubled frequency observed in this direction show that the  $Y_D$ - $Y_Z$  distance vector lies almost along the membrane plane. By simulations using the previously obtained distance 29.5 Å, the best fitted orientation was 80° or 110° from the membrane normal (**n**).

Fig. 3A shows the field-sweep ESE spectrum in the Zn-substituted PS II particles of  $Y_D$ -less mutant, which shows overlapped  $Y_Z^\bullet$  and  $Q_A^-$  signals. The  $\tau$  dependence of the primary ESE amplitude of  $Y_Z^\bullet$  radical is shown in Fig. 2B. The best-fitted simulation shown by a full line was obtained by using the value of 34.5 Å for the dipole interaction constant  $D_0$  in Eq. (2).

Fig. 4 shows a PELDOR time profile observed for the Mn-multiline in the  $S_0$ -state and  $Y_D$  radical. The best fitted value of distance was 34 Å, which is appreciably larger than that observed for the multiline center in the  $S_2$ -state.

**Table 1. The derived distances and angles of electron transfer cofactors in PS II by pulsed EPR**

| Paramagnetic Pairs               | Distances (Å)         | Angles (°) from <b>n</b>        | Methods              |
|----------------------------------|-----------------------|---------------------------------|----------------------|
| P680— $Q_A$                      | $27.4 \pm 0.3^{*1}$   | $21 \pm 5^{*2}$                 | ESEEM                |
| P680— $Y_D, Y_Z$                 | 20                    | 42                              | Graphic—derived      |
| $Y_D$ — $Q_A$                    | $38.5 \pm 0.8^{*3}$   |                                 | '2+1' pulse          |
| $Y_D$ — $Y_Z$                    | $29.5 \pm 0.5^{*4}$   | $80 \text{ or } 100 \pm 2^{*4}$ | '2+1' pulse          |
| $Y_D$ —Chl <sub>Z</sub>          | $29.4 \pm 0.5^{*3,5}$ | $54 \pm 5^{*5}$                 | '2+1' pulse          |
| $Y_D$ —Mn <sub>4</sub> ( $S_2$ ) | $27.1 \pm 0.2^{*4}$   | $70 \text{ or } 110 \pm 2^{*4}$ | ELDOR                |
| $Q_A$ —Cyt b559                  | $40 \pm 1?$           | $78 \pm 3$                      | ELDOR                |
| $Y_D$ —non-heme Fe               | $42 \pm 2^{*6}$       |                                 | Selective hole burn. |

<sup>\*1</sup> Hara et al., (1997) : <sup>\*2</sup> Yoshii et al., (1999): <sup>\*3</sup> Shigemori et al., (1998):

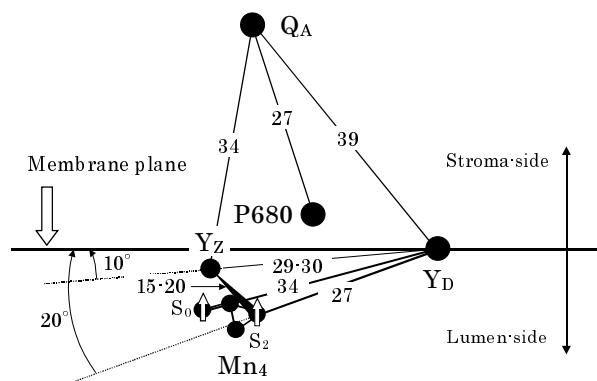
<sup>\*4</sup> Astashkin et al., (1998): <sup>\*5</sup> Tonaka et al., (2000): <sup>\*6</sup> Hara and Kawamori, (1997)

## Discussion

The distance between  $Y_Z^\bullet$  and  $Q_A^-$  radicals has been reported to be  $34 \pm 1$  Å by [Zech et al. 1999] using a spin polarized radical pair ESEEM in *Synechococcus elongates*. The distance obtained in this work is almost the same as that reported there, suggesting that these two radicals are similarly arranged in PS II complex in green alga and cyanobacteria. This value is, however, appreciably smaller than the distance between  $Y_D^\bullet$  and  $Q_A^-$  in spinach PS II (38.5 Å) [Shigemori et al. 1998].

The distances between pairs of electron transfer cofactors so far obtained by pulsed EPR are shown in Table I. Among these, relative positions of P680,  $Q_A$ ,  $Y_D$ ,

Fig. 5. A 3-d illustration of positions of the spin centers of the Mn cluster in  $S_0$ - and  $S_2$ -states and other main electron transfer cofactors. The unit of values is ( $\text{\AA}$ )



$Y_Z$  and the Mn-clusters are shown in Fig. 5. Comparing with the X-ray analysis data [Zouni et al. 2001] positions of most electron transfer cofactors seem to be coincident except for the distances between  $Y_Z$  and the Mn-cluster. This discrepancy may be ascribed to the difference in the measurement method. Pulsed EPR observes the relative positions of spin centers, while X-ray does electron densities. Furthermore, the method of relaxation measurement was not accurate for the large distribution of spins in the Mn-cluster. However, it is worthwhile to remark that the large movement of the spin center in the Mn-cluster occurred from the  $S_0$  to  $S_2$  state transition accompanied by oxidation of the Mn-cluster.

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