Trichloroacetate affects the EPR Signal II$_{slow}$ and Signal I in the thylakoid of *Chlamydomonas reinhardtii*

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Abstract One electron paramagnetic resonance (EPR) signal, named Signal II$_{slow}$, originates from the oxidized Tyrosine 160 ($Y_D^{\bullet}$) of D2 polypeptide of photosystem II reaction center. After adding high concentration trichloroacetate (TCA) to the *Chlamydomonas reinhardtii* thylakoid suspension, this signal was abolished in a minute. Treatment of TCA also removes a few of polypeptides, including three extrinsic polypeptides of oxygen-evolving complex, from the thylakoid membrane. Based upon the analysis of the microenvironment around Y_D with a three-dimensional model, it is indicated that relatively high hydrophobicity of this microenvironment may be the essential prerequisite for TCA to affect Y_D. It has been observed that TCA treatment also retards the decay of the Signal I, produced by the oxidized reaction center chlorophyll dimer ($P700^+$) of photosys- tem I.

Keywords: thylakoid, Y_D, EPR, trichloroacetate (TCA), three-dimensional model.

The photosystem II (PS II) reaction center complex, that catalyzes electron transport from H_2O to the plastoquinone pool, consists of several intrinsic polypeptides and a number of accessory components. Two intrinsic core polypeptides of PS II, D1 and D2, are homologous in amino acid sequence to L and M subunits of the purple bacteria reaction center. The PS II complex also connects with three extrinsic polypeptides with apparent masses of 33, 23 and 17 ku to form an entity and go through the process of oxygen evolution[1].

After light energy is absorbed and transformed to the reaction center chlorophyll P680, charge separation takes place and the radical pair $P680^+Pheo^-$ is produced. Oxidized primary electron donor $P680^+$ is then reduced by the redox active tyrosine residue, Y_Z. It has been proved by site directed mutagenesis that Y_Z is Tyrosine 161 of D1 polypeptide[2]. Another electron donor of $P680^+$, Tyrosine 160 (Y_D) of D2 polypeptide, is also discovered by the same methods. The photooxidized Y_Z and Y_D produce EPR spectra with a similar line shape[3], but being easily distinguished by different kinetics. EPR signals, which are produced by $Y_Z^{\bullet}$ and decay on the time scales of microsecond (when the donor side of PS II is intact) and millisecond (when the donor side of PS II is not intact), are called Signal II$_{very\ fast}$ and Signal II$_{fast}$ respectively. $Y_D^{\bullet}$ is much more stable than $Y_Z^{\bullet}$. Its EPR signal, Signal II$_{slow}$, decays in several hours in the dark. The identical EPR line shape of $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ implies that the distribution of the unpaired electron spin density and the orientation of their tyrosine phenol rings in polypeptide backbone are the same[4]. It has become one major research topic why Y_Z and Y_D, although symmetric on structure, are so different in redox properties in primary photochemical reaction.

We have found that in the PS II membrane of high plant spinach, TCA of high concentration selectively quenches the Signal II$_{slow}$, but leaves the Signal II$_{fast}$ unaffected[5]. In this study, the same effect was confirmed in the thylakoid of green algae *Chlamydomonas reinhardtii*. It could be concluded that the effect of TCA on Signal II$_{slow}$ exists not only in high plant, but also in algae, and it could be used as a useful tool for studying Y_D. The possible mechanism of the TCA effect was discussed based on a three-dimensional model of the microenvironment around Y_D. It was discovered that TCA can also stabilize Signal I which is produced by the oxidized chlorophyll dimer ($P700^+$) in the reaction center of photosystem I (PS I).

1 Materials and methods

(i) Isolation of *Chlamydomonas reinhardtii* thylakoid. The cells of the *C. reinhadtii* were grown

at 25℃(for culture medium see ref. [6]), with continuous bubbling of sterile air under bright fluorescence lamp (10×20 W). The cells in the late log phase of growth were harvested with centrifugation at 700 × *g* for 5 min and rinsed with a medium solution, containing 0.35 mol/L sucrose, 2 mmo/L $MgCl_2$, 20 mmol/L Hepes-KOH, pH 7.5. Then the cells were suspended in the same solution to meet a final Chl concentration of 1.5 mg/mL. Cells, incubated in ice, were broken with a CSF-1A ultrasonicator for 3 min (6× 0.5min) intermittently. After centrifugation at 700×*g* for 2 min, the supernatant was centrifuged at 45 000 × *g* for 10 min, and the pellets were resuspended with a medium, containing 10 mmol/L EDTA, 20 mmol/L Hepes-KOH, pH 7.5. Suspension was added to 2.2 mol/L sucrose to get a final sucrose concentration of 1.75 mol/L. Then it was placed in centrifuge tubes and overlaid with 20 mmol/L Hepes-KOH, pH 7.5, containing 0.5 mol/L sucrose and then centrifuged at 100 000× *g* for 1 h. Membranes at the top of the 1.75 mol/L sucrose layer were collected. They were resuspended in 10 vol. of 15 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L EDTA, 1 g/L BSA, 20 mmol/L MES，pH 6.0, and centrifuged at 12 500 × *g* for 10 min. The pellets were resuspended with SMN solution (0.35 mol/L sucrose, 15 mmol/L NaCL, 5 mmol/L $MgCl_2$, 20 mmol/L MES-NaOH, pH 6.0) and stored in liquid nitrogen until use.

(ⅱ) EPR spectra measurement. Pre-illumination with light intensity of 800 μmol photons • m^{-2} s^{-1} was given to the samples (1.5 mg Chl mL) at room temperature for 2 min. The same light was used for continuous illumination in measurement. TCA solution used for treatment was pre-adjusted to pH 6.0 with NaOH. EPR spectra were recorded at 77 K with a Varian E-112 spectrometer at X-band. Other spectral conditions were given in the legends of figures. The magnetic field was determined by an H-NMR field-meter and the microwave frequency by a superhigh frequency-meter.

(ⅲ) SDS-PAGE. Thylakoid of *C. reinhardtii* was adjusted with SMN solution to keep a Chl concentration of 3 mg/mL. After adding the same vol. of 2 mol/L TCA (pH 6.0), the mixed suspension was centrifuged at 20 000×*g* for 3 min immediately. Supernatant and pellet were collected separately and analyzed with SDS-PAGE. A slab gel with 6 mol/L urea in both stacking gel (5%) and resolving gel (13.75%) was used. After electrophoresis, gel was stained with Coomassie brilliant blue R-250.

(ⅳ) Analysis of the microenvironment around Y_D with a three-dimensional model. The three-dimensional model was drawn from the primary knowledge-based model of *C. reinhardtii* PSⅡ reaction center established by Xiong et al.[7]. In their work, a homology modeling procedure for the D1/D2 protein and accessory components of the PSⅡ reaction center of *C. reinhardtii* was carried out, based on the crystal structure of the L and M subunits of the purple bacteria reaction center. The QUANTA/CHARMm (version 4.1) molecular modeling program was used for the modeling.

2 Results

(ⅰ) TCA treatment abolished Signal $Ⅱ_{slow}$ in *C. reinhardtii* thylakoid. A typical EPR Signal $Ⅱ_{slow}$ was measured in the dark at 77 K, after the thylkoid of *C. reinhardtii* was pre-illuminated at room temperature for 2 min (fig. 1-1). It has a *g* value of 2.0046 and a linewidth of 2.0 mT. This signal was very stable and its intensity showed no change after the sample was kept in the dark for 10 min at room temperature (fig. 1-2). When the measurement was taken under continuous illumination, a single peak signal could be distinguished

Fig. 1. EPR signal of C. *reinhardtii* thylakoid, detected in the dark or with continuous illumination after pre-illumination. 1, EPR spectrum was immediately measured in the dark; 2, measured in the dark after 10 min dark adaptation; 3, measured with continuous illumination. The arrow indicates visible Signal $Ⅱ_{slow}$ overlapping on Signal Ⅰ. Sample Chl concentration: 1.5 mg/mL. Other conditions for EPR measurement: *T* = 77K; micro- wave frequency, 9.13 GHz; microwave power, 2.00 mW; modulation amplitude, 0.4 mT; modulation frequency, 100 kHz.

from the EPR spectrum of Signal II_{slow} (fig. 1-3). This single peak signal was recognized as the so-called Signal Ⅰ, produced by the oxidized state of PS Ⅰ reaction center chlorophyll dimer $P700^{+}$[3, 8]. Normally, it is detected only with continuous illumination, since $P700^{+}$ decays rapidly in several seconds in the dark. It was also found that Signal II_{slow} kept the peak intensity unchanged with continuous illumination, implying that the Signal $\text{II}_{\text{very fast}}$ decayed very fast and was hard to be detected when the donor side of the PS Ⅱ was perfect in thylakoid suspension[3].

After pre-illumination, thylakoid samples were mixed with TCA solution (pH 6.0) of different concentrations and their EPR spectra were measured. No change was found for EPR spectra after treating thylakoids with 300 mmol/L TCA (comparing fig. 2-1 with fig. 1-1). But when 700 mmol/L TCA was used to treat thylakoids, an additive spectrum of Signal Ⅰ and a decreased Signal II_{slow} were obtained (fig. 2-2). When the TCA concentration was raised to 1.0 mol/L, only Signal Ⅰ, with a *g* value of 2.0026 and a linewidth of 0.75 mT, was detected (fig. 2-3). Signal II_{slow} disappeared completely. This indicated that the $Y_D^{\bullet}$, produced in the pre-illumination, was reduced with high concentration TCA treatment.

The influence of TCA on Signal II_{slow} was also time-dependent. An EPR spectrum, similar to that measured immediately after the addition of 700 mmol/L, was obtained when thylakoid was treated with 300 mmol/L for 15 min in the dark (fig. 2-4).

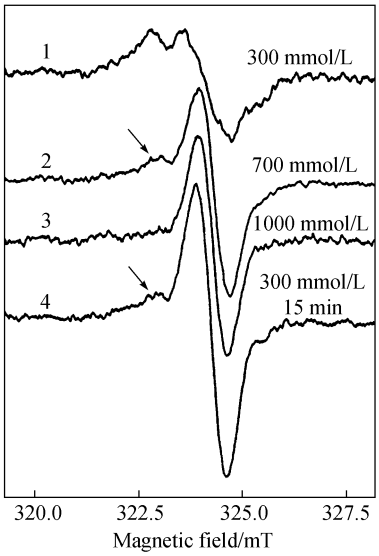

Fig. 2. The influence of TCA treatment on Signal II_{slow} of *C. reinhardtii* thylakoid. All samples were pre-illumi- nated first. 1—3, Adding 300, 700 mmol/L and 1.0 mol/L TCA (pH 6.0) separately to the sample suspension; 4, adding 300 mmol/L TCA to the sample, and keeping it in the dark for 15 min. EPR spectrum was measured in the dark. Arrows indicate visible Signal II_{slow} overlapping on Signal Ⅰ. For EPR measurement conditions see fig. 1.

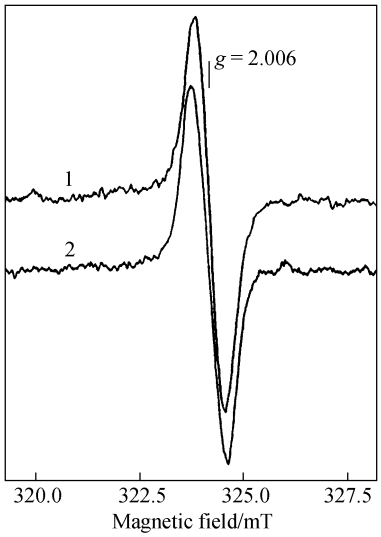

Fig. 3. The influence of TCA on Signal Ⅰ. 1, After adding 1.0 mol/L TCA (pH 6.0) and illuminating samples for 2 min, EPR spectrum was immediately measured in the dark; 2, measured in the dark after 5 min dark adaptation. For EPR measurement conditions see fig. 1.

(ⅱ) TCA treatment stabilized the EPR Signal Ⅰ in *C. reinhardtii* thylakoid. Signal Ⅰ, produced when thylakoid is illuminated, normally decays in time scale of second. It was discovered that when TCA of high concentration was added to the pre-illuminated *C. reinhardtii* thylakoid suspension, Signal Ⅰ was easily detected in the dark (see fig. 2). This implied that TCA of high concentration stabilizes the oxidized state of $P700^{+}$. To confirm this effect, 1.0 mol/L TCA (pH 6.0) was added to the thylakoid suspension just before pre-illumination, then the EPR spectra of illuminated thylakoid were measured in the dark. Signal Ⅰ with identical intensity of that measured with continuous illumination was produced (comparing fig. 3-1 with fig. 1-3). Signal Ⅰ also kept unchanged when the samples were kept

in the dark for 5 min before measurement (fig. 3-2). This shows that after treating thylakoid with TCA of high concentration, almost all $P700^+$, produced after illumination, is stable in the dark for a long time.

(iii) TCA treatment removed a few of polypeptides from the thylakoid membrane. Our previous work showed that treatment with TCA of high concentration could sequentially release the three extrinsic polypeptides from the PS Ⅱ membrane[9]. It was also found, in this study, that the influence of TCA on Signal $Ⅱ_{slow}$ is time-dependent. This might indicate that the effect of TCA to deplete extrinsic polypeptides is the prerequisite of its influence on EPR signal. Therefore, we had to analyze protein patterns reflecting polypeptides released from thylakoid membrane after TCA treatment.

The *C. reinhardtii* tylakoid membrane (3 mg Chl/mL) was mixed with 1.0 mol/L TCA and then centrifuged at 20 000×*g* for 3 min. Fig. 4 shows the SDS-PAGE of pellets and suspension collected separately after centrifugation. A few of polypeptides, including three extrinsic polypeptides with apparent masses of 17, 23 and 30 ku (corresponding to the 33 ku manganese-stabilizing protein of high plant[10]) could be recognized to be partly released. The released proteins also consisted of 21-ku polypeptide belonging to the light-harvesting complex of PS Ⅰ (LHCI)[11], and a polypeptide with apparent mass of 49.5 ku. Besides these, some other polypeptides, whose masses shown as 60, 58, 51, 46, 36.5, 35.5, 18 and 16 ku, were also removed from the thylakoid membrane with trivial amount.

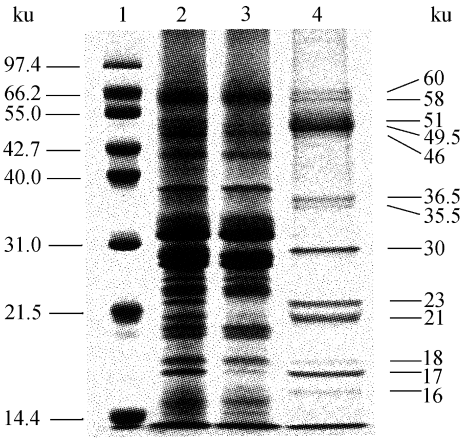

Fig. 4. SDS-PAGE shows the influence of TCA on polypeptide patterns of *C. reinhardtii* thylakoid membrane. 1, Protein markers (masses are indicated on the left); 2, control thylakoid; 3, thylakoid after 1.0 mol/L TCA treatment; 4, the extract after 1.0 mol/L TCA (pH 6.0) treatment of the thylakoid. Masses of extracted polypeptides are indicated on the right.

3 Discussion

Although Y_D and Y_Z are at analogous positions in D1 and D2, two homologous polypeptides of PS Ⅱ reaction center, they play very different roles in the energy transduction in PS Ⅱ. Contrary to that Y_Z is taking an active part in the photochemical reaction, very little is known about Y_D. The study of properties of Y_D is necessary for the comprehension not only of its potential function, but also of symmetric characteristic of PS Ⅱ polypeptides.

It was indicated that after treating the PS Ⅱ membrane of high plant with TCA of high concentration, three extrinsic polypeptides were removed from the membrane, and Signal $Ⅱ_{slow}$ was decreased[5]. By using *C. reinhardtii* thylakoid as a sample in this study, it was found that TCA released three extrinsic polypeptides of 17, 23, 30 ku from the thylakoid membrane and abolished Signal $Ⅱ_{slow}$ as well. A common effect of TCA to influence Y_D could be concluded.

Further study on the molecular properties of Y_D depends on the accurate analysis of its molecular structure in PS Ⅱ. But the establishment of three-dimensional structure of PD Ⅱ with high resolution is still a long-term project, since it is difficult to get crystal of PS Ⅱ complex of high quality. Recently, a relatively integrate three-dimensional model of *C. reinhardtii* PS Ⅱ reaction center was built by Xiong et al. with computer simulation[7]. This model is helpful to the study of the molecular mechanism of the effect of TCA. Amino acids existing around Y_D within 0.8 nm were drawn in fig. 5 based on the three-dimensional model of *C. reinhardtii* PS Ⅱ reaction center. Amino acids existing around Y_Z within 0.8 nm can be found in the previous study[12]. There are two major differences between microenvironment around Y_D and Y_Z. ⅰ) Y_D has a much higher atom density in its surrounding than Y_Z (177 atoms within 0.8 nm for Y_D and 137 atoms within 0.8 nm for Y_Z). Recent research indicated that some redox components of photosystem (like Q_B in purple bacteria) would produce geometric displacement during the redox reaction[13]. Little space restriction could promise active electron transport of Y_Z. ⅱ) Most

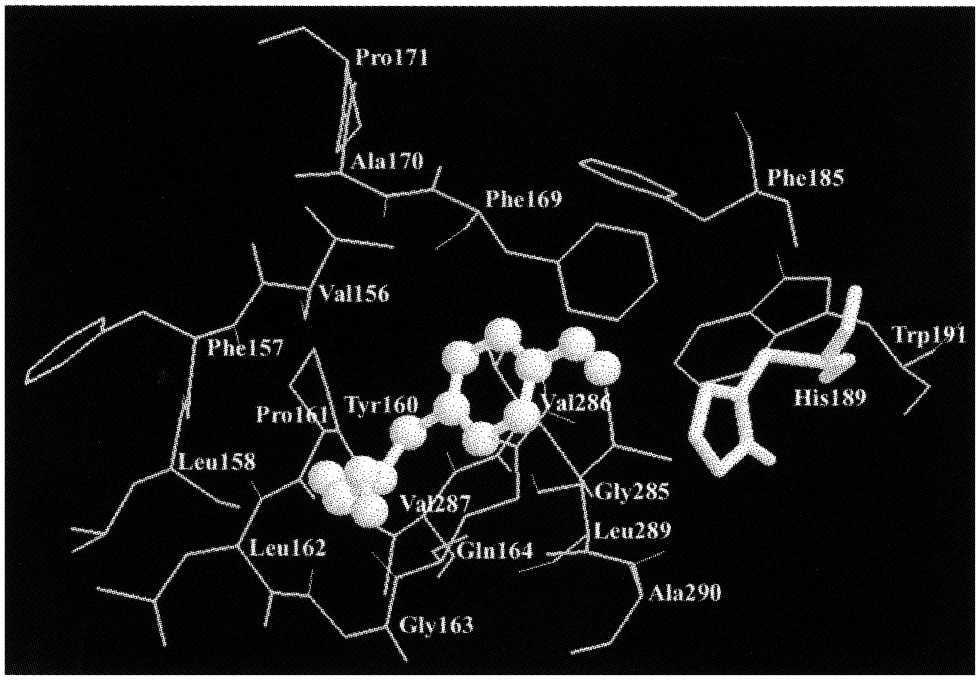

Fig. 5. Three-dimensional model of the 0.8-nm surrounding of Y_D (D2-Tyr160). The amino acid shown in ball and stick pattern is Tyr160, while the one shown in stick pattern is His189. A hydrogen bond was suggested to exist between those two residues[2].

amino acid residues around Y_D are hydrophobic residues, including Val156, Phe157, leu158, Leu162, Phe169, Ala170, Phe185, Trp191, Val286, Val287, Leu289 and Ala290. This means that Y_D is located in a relatively more hydrophobic isolated environment. This might be one reason that Y_D was not involved in the main electron flow of PS Ⅱ. As a result, Signal $Ⅱ_{slow}$ is relatively stable. Contrary to Y_D, Y_Z has a comparatively hydrophilic environment. This is consistent with the previous prediction in high plant[14].

Treatment of PS Ⅱ membrane of high plant with several kinds of halogenated acetates showed that the degree of their effects on suppressing EPR Signal $Ⅱ_{slow}$ is correlated well with their hydrophobicity[5]. The relatively high hydrophobicity of the environment around Y_D may be one of the important prerequisites for the hydrophobic molecular TCA to get in and influence the redox property of Y_D.

Besides the effect of TCA on Signal $Ⅱ_{slow}$, it was also found in this study that TCA could stabilize Signal Ⅰ, produced by the oxidized state of PS Ⅰ chlorophyll dimer $P700^+$. TCA might block the upstream electron transport of P700, and retard the reduction of $P700^+$ also. This finding indicates that TCA treatment could be used as another means to study the structure and function of PS Ⅰ.

Acknowledgements This work was supported by the State Key Basic Research and Development Plan (Grant No. G1998010100) and the National Natural Science Foundation of China (Grant No. 39730040).

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(Received April 21, 2000)