Trichloroacetate Affects the Redox Active Tyrosine 160 of the D2 Polypeptide of the Photosystem II Core

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Trichloroacetate (TCA) affects the redox property of $Y_D$ (tyrosine-160 on D2 polypeptide) after the removal of 33 kDa extrinsic polypeptide from the photosystem II (PSII) core. However, TCA has no obvious effect on the redox property of $Y_Z$, confirming that the environment around $Y_D$ and $Y_Z$ are quite different. The conclusion on the effects on $Y_D$ is based primarily on the observation that $Y_D^+$ is not detected as the EPR signal $I_{\text{slow}}$ when 33 kDa polypeptide is released by TCA-treatment. Dialysis of the TCA-treated sample that allows the rebinding of mostly 33 kDa polypeptide restores $Y_D^+$, showing that the loss of the EPR signal $I_{\text{slow}}$ takes place after the removal of 33 kDa polypeptide but not the release of manganese (Mn). Additionally, treatment of several halogenated acetates on Tris-washed PSII particles shows that the degree of their effects on suppressing EPR signal $I_{\text{slow}}$ is correlated with their hydrophobicity. It is postulated that $Y_D$ becomes more accessible to some small hydrophobic molecules depending upon their hydrophobicity when 33 kDa polypeptide is removed.

\textbf{Introduction}

Photosystem II (PSII) includes at least seven intrinsic polypeptides (D1, D2, the $\alpha$ and $\beta$ subunits of cytochrome b559, CP47, CP43, and the psbl gene product) and three extrinsic polypeptides with molecular masses of 33, 23, and 17 kDa as the key components of this photosystem (see reviews by Bricker and Ghanotakis, 1996; Rogner et al., 1996). The 23 and 17 kDa polypeptides appear to play a role in the regulation of the action of calcium and of chloride, two cofactors involved in oxygen evolution. The 33 kDa extrinsic polypeptide is much more tightly associated with the intrinsic PSII polypeptides than other polypeptides and is considered to be a manganese-stabilizing polypeptide (Debus, 1992; Seidler, 1996).

Two tyrosine residues, denoted as $Y_Z$ and $Y_D$, have been identified as redox active components at the oxidizing side of the PSII electron transport chain (Barry and Babcock, 1987). Although they occur at symmetrical positions in the D1 and D2 polypeptides that form the PSII core (see e.g. Xi-ong et al., 1996), they are kinetically and functionally different (Babcock et al., 1989; Hoganson et al., 1995). Oxidation of the tyrosine $Y_Z$ gives rise to characteristic EPR signal $I_{\text{very fast}}$ (decays in time scale of micro- to millisecond ) and the appearance of EPR signal $I_{\text{slow}}$ (decays in time scale of hours) is due to another oxidized tyrosine $Y_D$. These two EPR signals have similar spectral characteristics indicative of similar protein environment around the two tyrosines (Babcock et al. 1989; Svensson et al., 1991). However, there are subtle and important differences between them (Svensson et al., 1991). Although the $Y_D/Y_D^+$ couple is highly oxidizing($E_m=-720–760$ mV at

\textbf{Abbreviations}: Chl, chlorophyll; DCA, dichloroacetate; EPR, electron paramagnetic resonance; MBA, monobromoacetate; MCA, monochloroacetate; Mes, 2-(morpholino)-ethanesulfonic acid; MFA, monofluoroacetate; MIA, monoiodoacetate; OEC, oxygen-evolving complex; PSII, Photosystem II; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetate; Tris, tris(hydroxymethyl)-aminomethane; $Y_D$, redox-active tyrosine 160 of the D2 polypeptide; $Y_Z$, redox-active tyrosine 161 of the D1 polypeptide.

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The results provide evidence that TCA affects $Y_D$, not $Y_Z$, after the removal of the 33 kDa extrinsic polypeptide. Thus, not only a difference in the environments of $Y_D$ and $Y_Z$ is confirmed, but an interaction of 33 kDa polypeptide and $Y_D$ is suggested.

Materials and Methods

PSII particles were prepared from spinach (Spinacia oleracea) leaves, as previously described (Xu et al., 1995). For TCA-treatment, PSII particles were incubated (0.5 mg Chl/ml) with different concentrations of trichloroacetate (TCA, pH 6.0) at 0° C for 30 min in room light $(3.2 \times 10^{-3} \text{ J cm}^{-2} \text{s}^{-1})$. Collected at centrifugation at 40 000$g$ for 30 min, PSII particles were washed once and resuspended in SMN buffer (0.4 M sucrose, 50 mM Mes-NaOH, 15 mM NaCl) at pH 6.0 with added 5 mM MgCl$_2$, and then stored in liquid nitrogen until use. SDS-PAGE results (see Xu et al., 1995) showed that 80, 250, or 500 mM TCA should be chosen as appropriate concentrations to release 90% of 17, 23 or 33 kDa polypeptides from PSII particles sequentially.

Tris-(0.8 M, pH 8.2) washed PSII particles were prepared as described by Tamura and Cheniae (1987). For further treatment with halogenated acetates (TCA, DCA, MFA, MCA, MBA or MIA), Tris-washed PSII particles were incubated with different concentrations of acetates (pH 6.0) at 0° C for 30 min in room light $(3.2 \times 10^{-3} \text{ J cm}^{-2} \text{s}^{-1})$, then collected as mentioned above.

For dialysis, the T-PSII particles (PSII particles kept for 30 min in 500 mM TCA, pH 6.0) were diluted with SMN buffer (1:4), and dialyzed twice against 25 mM Mes-NaOH (pH 6.0) at 4° C for 6 h in darkness. Pellets collected by centrifugation (40 000$g$, 25 min) were suspended in SMN buffer.

Polypeptide composition was analyzed by SDS-PAGE in the buffer system (see Laemmli, 1970) containing 6 M urea. A gel containing 5% (stacking gel) and 13.75% (resolving gel) acrylamide was used. The densitogram of the gel stained in Coomassie brilliant blue R-250 was obtained with a Digital Imaging System (IS-1000).

The abundance of Mn was measured with a Shimadzu atomic absorption spectrometer (AA-6501F). Samples (20 µl) were dried at 105° C for 30 s, ashed at 1000° C for 20 s, and atomized at 2500° C for 3 s before measurement.

The dark-stable EPR spectra (signal $II_{low}$) were measured with samples frozen in the dark after an illumination of 5 min and then a dark-adaptation of 30 min at room temperature. The light-induced EPR signal $II$ spectra were measured with samples frozen during continuous illumination after a 5 min-illumination at room temperature. The light intensity was $9.5 \times 10^{-3} \text{ J cm}^{-2} \text{s}^{-1}$. All spectra were recorded at liquid nitrogen temperature (77 K) with a Varian E-112 spectrometer at X-band. Other spectral conditions are given in the legend of Fig. 1. The magnetic field was determined by an H-NMR field-meter and the microwave frequency by a superhigh frequency-meter.

Dipole moments of (halogenated) acetic acids were calculated by using the MMX molecular mechanics (forcefield) calculation method with the PCMODEL molecular modeling program (Anonymous, 1990). Calculation was made in an apolar solvent with a dielectric constant of 1.5 to minimize the energy of the molecular model and to get an optimal geometry of the model. The hydrophobic constants ($\pi$) for acetic acid and halogenated acetic acids were estimated according Hansch and Leo (1979): $\pi = \log P_X - \log P_H$, where $P_X$ is the partition coefficient (see Rappoport, 1976) of a halogenated derivative of acetic acid and $P_H$ that of the parent molecule, acetic acid in this study. The $\pi$ for acetic acid (x=H) is defined as zero.
Results and Discussion

Although close contacts between the 33 kDa polypeptide and CP47 are generally accepted, it was also suggested that the 33 kDa polypeptide may interact or shield essentially all of the intrinsic polypeptides of the PSII core (Debus, 1992; Franke and Bricker, 1995). A specific interaction between the 33 kDa polypeptide and the D2 polypeptide was proposed based on the observation that changing Tyr-160 (Y_D) to Phe in the D2 polypeptide of Synechocystis sp. PCC 6803 destabilized the binding of the 33 kDa polypeptide to isolated PSII particles (Noren et al., 1991). Therefore, the relationship between the 33 kDa polypeptide and Y_D was studied in this paper.

EPR signal I_{slow}, an indicator of the oxidation of Y_D, was measured in PSII particles depleted of extrinsic polypeptides by TCA-treatment on EPR signal I_{slow}, displaying Y_D+: (1) control; (2) -17 kDa; (3) -17,-23 kDa; (4) -17,-23,-33 kDa. Panel B. Effect of dialysis of PSII particles on the EPR spectra of signal I_{slow}: (a) control; (b) T-PSII particles (PSII particles incubated for 30 min in 500 mM TCA, pH 6.0); (c) dialyzed T-PSII particles. Panel C. Dark-stable (solid curves) and light-induced (broken curves) EPR signal II spectra of control and TCA-treated PSII particles, curve 1 and 2, control; curve 3 and 4, 500 mM TCA-treated PSII particles. Panel D. Effect of TCA on the EPR signal I_{slow} of Tris-washed (pH 8.2, 40 min) PSII particles: (a) control; (b) 0.8 M Tris-washed; (c) treated with 100 mM TCA (pH 6.0, 30 min) after the Tris-washing.
various extrinsic polypeptides. Fig. 1A shows the EPR signal $I_{\text{slow}}$ measured in the presence or absence of 17; 17 and 23; as well as 17, 23 and 33 kDa extrinsic polypeptides after TCA-treatment, as outlined by Xu et al. (1995) and in Materials and Methods. When the 17 and 23 kDa polypeptides were removed from PSII, the EPR signal $I_{\text{slow}}$ remained normal or only slightly modified (curves 2 and 3). However, when the 33 kDa polypeptide was also released, the amplitude of the EPR signal $I_{\text{slow}}$ was greatly decreased (curve 4), indicating that the presence of 33 kDa polypeptide is related to the change of the redox characteristic of Y$_D$.

The 33 kDa polypeptide has been known to be a Mn-stabilizing polypeptide (Seidler, 1996). Mn content in PSII core was, thus, measured in the samples used for the EPR signal $I_{\text{slow}}$ data. Table I shows that 500 mM TCA-treatment of PSII particles removes 96% of the Mn and is accompanied by the disappearance of EPR signal $I_{\text{slow}}$. Dialysis of the TCA-incubated PSII particles (T-PSII) was then made to study the relationship among 33 kDa polypeptide, Mn and Y$_D^+$. The SDS-PAGE analysis (Fig. 2A) shows that in 500 mM TCA-treated PSII particles, all three extrinsic polypeptides were released (lane 2). In dialyzed T-PSII particles, most of 33 kDa (and 30% of 23, 17 kDa) polypeptides had been reconstituted (lane 3 in Fig. 2B), and the EPR signal $I_{\text{slow}}$ was recovered (Fig. 1B). Importantly, the Mn content of the dialyzed T-PSII particles was not

<table>
<thead>
<tr>
<th>Samples</th>
<th>Extrinsic polypeptides</th>
<th>Mn content</th>
<th>EPR signal $I_{\text{slow}}$ ($Y_D^+$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>17 kDa</td>
<td>23 kDa</td>
<td>33 kDa</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>80 mM TCA-treated</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>250 mM TCA-treated</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>500 mM TCA-treated</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.8 M Tris-washed</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dialyzed T-PSII particles</td>
<td>–*</td>
<td>–*</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Indicates the presence, – indicates the absence, –* indicates retention of 30% of relative component in the control. T-PSII indicates PSII particles were incubated 30 min in 500 mM TCA, pH 6.0, in room light.
restored at all (Table I). It is, thus, obvious that the variability in the amplitude of the EPR signal $I_{\text{slow}}$ is not related to the Mn, but to the presence of 33 kDa polypeptide. We suggest two alternatives to explain these results: (1) the 33 kDa polypeptide may be more closely associated with D2 polypeptide than assumed so far and, thus, affecting $Y_D$ or (2) the release of the 33 kDa polypeptide induces some "conformational change" that indirectly affect $Y_D^+$, but not $Y_Z^+$ (see later discussion).

Fig. 1C shows comparisons of the dark-stable or the light-induced EPR signal II spectra between the control and the TCA-treated PSII particles. Curves 1 and 3 are repetition of curves 1 and 4 in Fig. 1A and arise from $Y_D^+$ but not from $Y_Z^+$ because the half-time decay of $Y_Z^+$ is far faster than that of $Y_D^+$. The light-induced spectra (broken curves) were recorded for samples illuminated for 5 min at room temperature and then frozen during continuous illumination, therefore both $Y_D^+$ and $Y_Z^+$ are observed in the control intact PSII particles (curve 2). It shows that the amplitude of the EPR signal II in the light-induced sample is 20% larger than that in the dark-stable samples. In the TCA-treated PSII particles (curve 4) in which the Mn complex had been removed (cf. data in Table I), the electron transport between Mn complex and $Y_D$ was blocked. This causes an accumulation of $Y_Z^+$. The amplitude of the EPR signal II shows a 20% decease after the TCA-treatment in the light-induced PSII particles, not the amount as the 85% decrease shown for EPR signal $I_{\text{slow}}$ affected by the TCA-treatment, implying that TCA has no obvious effect on the redox property of $Y_Z$, and only a specific effect on $Y_D$.

Table I shows that 33 kDa polypeptide is removed not only in PSII particles by 500 mM TCA-treatment, but also by 0.8 mM Tris-washing, as is well known (Kuwabara and Murata, 1983). However, $Y_D^+$ is absent only in TCA-treated PSII particles; in the case of Tris-washing, the EPR signal $I_{\text{slow}}$ displays a normal shape and hyperfine structure of a band-width of 19 gauss (curve b in Fig. 1D), indicating that TCA-treatment differs from Tris-washing in some crucial aspects.

The EPR signal $I_{\text{slow}}$ was not detected in Tris-washed PSII particles after the treatment with lower concentration (100 mM) of TCA (curve c in Fig. 1D). It seems that after the removal of the 33 kDa polypeptide in TCA-treated PSII particles, TCA influences specially the redox property of $Y_D$. To further probe this effect, we systematically investigated effects of other halogenated acetates on redox property of $Y_D$ in the 0.8 mM Tris-washed PSII particles in which the 33 kDa polypeptide was already removed. Concentration, at which the hyperfine structure of EPR signal $I_{\text{slow}}$ start to dissipate, and most of the amplitude of EPR signal $I_{\text{slow}}$ is suppressed, was chosen as an critical concentration. It is observed that halogenated acetates have different critical concentrations to suppress the EPR signal $I_{\text{slow}}$ in Tris-washed PSII particles. The concentrations are in the order TCA < DCA < MBA < MCA < MFA (Table II).

By comparison with some of their physicochemical properties, we found that the degree of the suppression of the EPR

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Critical concentrations [mM]</th>
<th>Dissociation constants ($K_d$)</th>
<th>Hydrophobicity constants ($\pi$)</th>
<th>Dipole moment (Debye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>100</td>
<td>0.63</td>
<td>1.87</td>
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<tr>
<td>DCA</td>
<td>400</td>
<td>1.29</td>
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<tr>
<td>MIA</td>
<td>600</td>
<td>3.18</td>
<td>1.17</td>
<td>–</td>
</tr>
<tr>
<td>MBA</td>
<td>800</td>
<td>2.90</td>
<td>0.97</td>
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</tr>
<tr>
<td>MCA</td>
<td>1400</td>
<td>2.86</td>
<td>0.65</td>
<td>3.25</td>
</tr>
<tr>
<td>MFA</td>
<td>&gt;2000</td>
<td>2.58</td>
<td>0.06</td>
<td>3.14</td>
</tr>
</tbody>
</table>

DCA, dichloroacetate; MBA, monobromoacetate; MCA, monochloroacetate; MFA, monofluoroacetate; MIA, monoiodoacetate; TCA, trichloroacetate.
signal $I_{\text{slow}}$ (lowest to highest) correlates well with the hydrophobicity ($\pi$) of chemicals, but not with their dissociation constants ($K_d$) or dipole moments (Table II), both of which represent electrostatic or electrodynamic forces stabilizing the ordered, native structure of biomolecules. This indicates that a key property that affects the redox characteristic of Y$_D$ is the molecular hydrophobicity of the halogenated acetates used. It is therefore postulated that TCA alters the redox state of Y$_D$, relying upon its molecular hydrophobicity. This may act through accessibility involving either of the two alternatives mentioned earlier, i.e., direct or indirect.

Svensson et al. (1991) analyzed the protein environment in the cavities around Y$_D$ and Y$_Z$ based on the analysis of hydrophilicity index of amino acid, and indicated that the environment around Y$_D$ is more hydrophobic than that around Y$_Z$. This indication is taken by the authors to support our presumption concerning the hierarchy observed in the effect of various halogenated acetates with different hydrophobicities (Table II).

Although little is known about the function of Y$_D$, studies have shown that both the conformations of the Y$_D$ component and of its immediate microenvironment in the PSII reaction center play crucial roles in maintaining its unique biological properties (Tommos et al., 1993; Tang et al., 1996). Thus, the changes in the local protein environment surrounding Y$_D$ can influence its redox property. To summarize our results, we propose that the 33 kDa extrinsic polypeptide might help to keep special microenvironment around Y$_D$. When it is removed, the microenvironment around Y$_D$ but not around Y$_Z$ might become susceptible to attacks by small hydrophobic molecules or other factors.

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