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

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Z. Naturforsch. **52c,** 782–788 (1997); received June 30/August 20, 1997

TCA-Treatment, Halogenated Acetates, 33 kDa Polypeptide, Y_D/Y_Z, EPR Signal II_{slow}

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 environments around Y_Z and Y_D 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 II_{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 II_{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 II_{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.

Introduction

Photosystem II (PSII) includes at least seven intrinsic polypeptides (D1, D2, the α and β subunits of cytochrome b559, CP47, CP43, and the psbI 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. Xiong *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 $II_{very\ fast}$ (decays in time scale of micro- to millisecond) and the appearance of EPR signal II_{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(Em≈+720–760 mV at

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 dodecylsulfate 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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0939–5075/97/1100–0782 $ 06.00

pH≈6) (Boussac and Etienne, 1984; Vass and Styring, 1991), EPR signal II_{slow} is extremely stable in the dark, indicating that Y_D^+ is highly "insulated" from redox-active groups in the PSII complex as well as from the external medium (Babcock *et al.*, 1989; Svensson *et al.*, 1991). Detailed studies on chemical properties of both the tyrosines are required to understand their different behaviors in electron transport (Svensson *et al.*, 1991; Mino and Kawamori, 1994; Tang *et al.*, 1996).

A new method has been developed to release sequentially PSII extrinsic polypeptides: when PSII particles are incubated with increasing concentrations of trichloroacetate (TCA, pH 6.0), first 17, then 23 and finally 33 kDa polypeptide is released (Xu *et al.* 1995). The treatment is light and pH independent. In this paper, we have used this novel method to get further insight into the relationship between the 33 kDa polypeptide and Y_D. 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 TCA (pH 6.0) at 0° C for 30 min in room light (3.2×10^{-3} J.cm^{-2}.s^{-1}). Collected after 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-(0.8M, 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 (3.2×10^{-3} J.cm^{-2}.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 6M urea. A slab 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_{slow}) 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 9.5×10^{-3} J.cm^{-2}.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 (π) for acetic acid and halogenated acetic acids were estimated according Hansch and Leo (1979): $\pi=\log P_x - \log P_H$, where Px 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 π 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; Frankel 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 II_{slow}, an indicator of the oxidation of Y_D, was measured in PSII particles depleted of

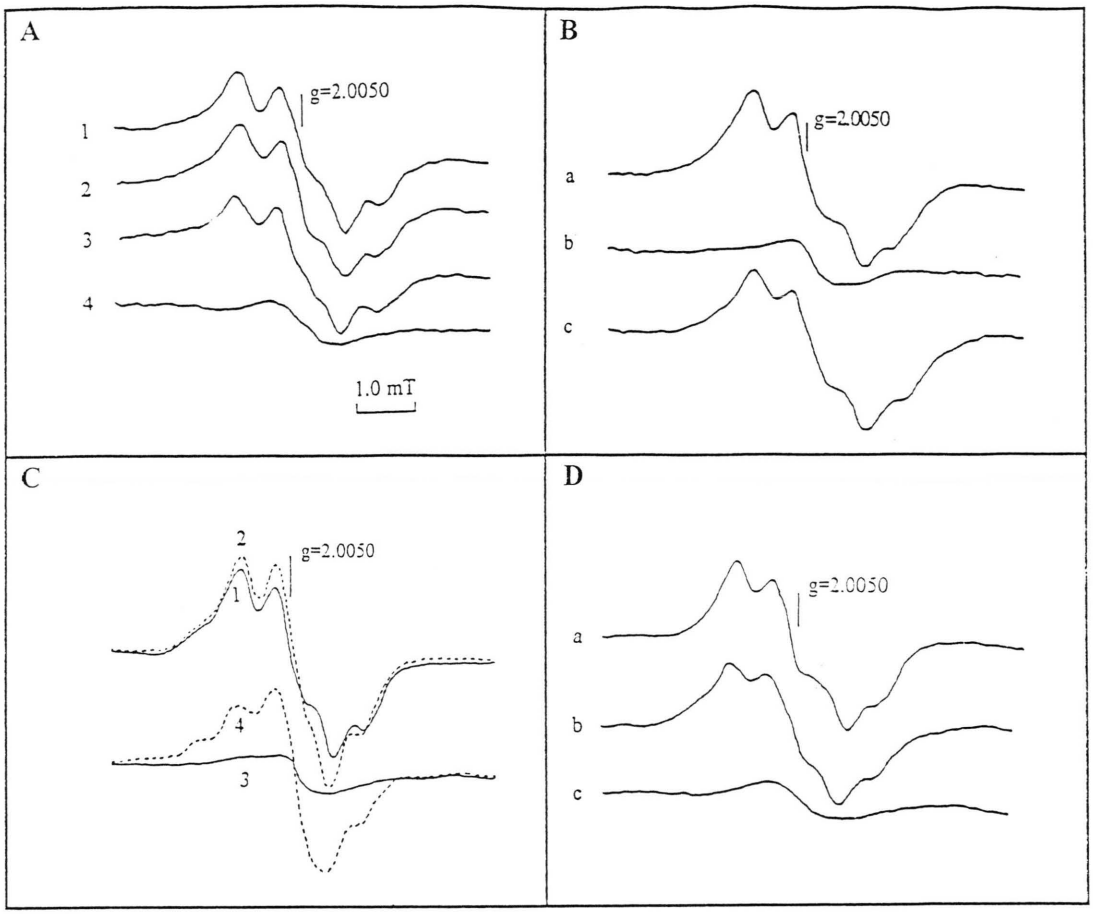

Fig. 1. EPR signals measured in PSII particles depleted of various extrinsic polypeptides. Chl concentrations are 3.6, 2.0, 3.0 and 3.0 mg/ml respectively in Panels A, B, C and D. Spectrometer conditions: modulation frequency, 100 kHz ; microwave frequency, 9.17 GHz; microwave power, 0.5 mW; response time, 0.25 s; center field set, 3.250×10^{-1} T; sweep width, 2.5×10^{-2} T. modulation amplitude was 3.2×10^{-4} T in Panels A, C and D or 1.6×10^{-4} T in Panel B; receiver gain was 8.0×10^{3} in Panels A, C and D or 4.0×10^{4} T in Panel B. Panel A. Effects of removal of extrinsic polypeptides by TCA-treatment on EPR signal II_{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 II_{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 particels. Panel D. Effect of TCA on the EPR signal II_{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 II_{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 II_{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 II_{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 kD 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 II_{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 II_{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 II_{slow} was recovered (Fig. 1B). Importantly, the Mn content of the dialyzed T-PSII particles was not

Table I. Comparison of the removal of extrinsic polypeptides, the preservation of Mn content and the appearance of the EPR signal II_{slow} (Y_D+) in various PSII particles.

Samples	Extrinsic polypeptides			Mn content		EPR signal II_{slow} (Y_D^+)
	17 kDa	23 kDa	33 kDa	Mn/300 Chl	%	
Control	+	+	+	3.96	100	+
80 mM TCA-treated	–	+	+	3.84	97	+
250 mM TCA-treated	–	–	+	3.48	88	+
500 mM TCA-treated	–	–	–	0.16	4	–
0.8 M Tris-washed	–	–	–	0.13	3	+
Dialyzed T-PSII particels	–*	–*	+	0.32	8	+

+ 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.

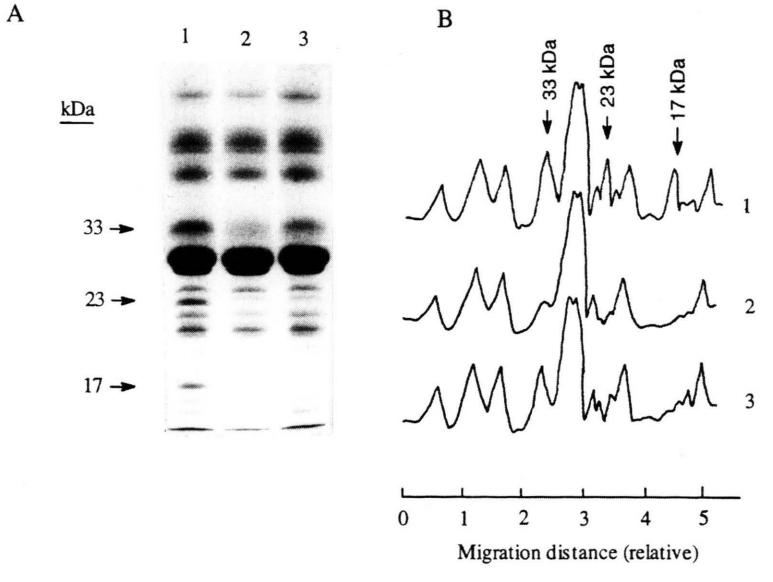

Fig. 2. SDS-PAGE electrophoretogram (A) and densitogram (B) of TCA-treated PSII particles: Lane 1, control; Lane 2, 500 mM TCA-treated; Lane 3, dialyzed T-PSII particles (T-PSII particles are PSII particles incubated for 30 min in 500 mM TCA, pH 6.0).

restored at all (Table I). It is, thus, obvious that the variability in the amplitude of the EPR signal II_{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_Z 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 II_{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 M 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 II_{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 II_{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 M Tris-washed PSII particels in which the 33 kDa polypeptide was already removed. Concentration, at which the hyperfine structure of EPR signal II_{slow} start to dissappear, and most of the amplitude of EPR signal II_{slow} is suppressed, was chosen as an critical concentration. It is observed that halogenated acetates have different critical concentrations to suppress the EPR signal II_{slow} in Tris-washed PSII particles. The concentrations are in the order TCA<DCA<MIA<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 II. Comparision of physicochemical properties and the critical concentrations of halogenated acetates, with each critical concentration of different acetates, the EPR signal II_{slow} is suppressed in the 0.8 M Tris-washed PSII particles.

Chemicals	Critical concentrations [mM]	Properties of chemicals		
		Dissociation constants (K_d)	Hydrophobicity onstants (π)	Dipole moment (Debye)
TCA	100	0.63	1.87	2.12
DCA	400	1.29	1.66	2.53
MIA	600	3.18	1.17	–
MBA	800	2.90	0.97	3.11
MCA	1400	2.86	0.65	3.25
MFA	>2000	2.58	0.06	3.14

DCA, dichloroacetate; MBA, monobromoacetate; MCA, monochloroacetate; MFA, monofluoroacetate; MIA, monoiodoacetate; TCA, trichloroacetate.

signal II_{slow} (lowest to highest) correlates well with the hydrophobicity (π) 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 heirarchy 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.

Acknowledgements

We thank Cheng-Ming Zhou for technical assistance and Tian-Duo Wang for a critical reading of the manuscript. This study was supported by the Director's Fund of Chinese Academy of Sciences, Shanghai Branch (1996–1997), and Shanghai Municipal Fundation of Science and Technology (1997).

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