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Effect of redox mediators on the flash-induced membrane potential generation in Mn-depleted photosystem II core particles

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Abstract An electrometrical technique was used to investigate flash-induced electron transfer reactions between Mn-depleted spinach photosystem II core particles incorporated into liposomes and redox mediators. Besides the fast increase in the transmembrane electric potential difference associated with electron transfer between the redox active tyrosine (Y_Z) and the primary quinone acceptor Q_A, an additional electrogenic phase was observed in the presence of *N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine and 2,6-dichlorophenol-indophenol. The latter phase is attributed to vectorial electron transfer from the redox dye(s) to the protein-embedded Y_Z. The data obtained suggest an electrically isolated location of the Y_Z from the external water phase.

Keywords Mn-depleted photosystem II · Proteoliposomes · Redox mediators · Photoelectric response · Electrometrical technique

Abbreviations

PS II	Photosystem II
RC	Reaction center
D1 and D2	Polypeptides consisting the anchoring site of the main cofactors for PS II
P_{680}	Primary electron donor in PS II
Q_A (Q_B)	Primary (secondary) plastoquinone acceptor
Y_Z	Tyrosine-161 on D1 of PS II
OEC	Oxygen-evolving complex
$\Delta\psi$	Transmembrane electric potential difference
MES	2-(*N*-morpholino)ethanesulfonic acid
TMPD	*N*,*N*,*N*′*N*′-tetrametyl-*p*-phenylenediamine
DCIP	2,6-Dichlorophenol-indophenol
MB	Methylene blue
τ	Characteristic time constant
E_m	Midpoint redox potential

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Introduction

Photosystem (PS) II, a large pigment–protein complex embedded in the thylakoid membranes of green plants, eukaryotic algae, and cyanobacteria uses light energy to drive two chemical reactions: the oxidation of water to molecular oxygen and the reduction of plastoquinone to plastohydroquinone. PS II consists of more than 20 polypeptides and a number of cofactors associated with the electron-transfer chain. The innermost part of the photoenzyme consists of the reaction center (RC), which is composed of the D1 and D2 proteins, where the primary charge separation occurs. The initial excitation of the primary donor, P_{680}, is followed by a sequential electron transfer from P_{680} to a pheophytine, a primary (Q_A) and then to a secondary (Q_B) plastoquinone acceptors. Re-reduction of P^+_{680} occurs through a redox-active tyrosine-161 (Y_Z) of the D1 subunit. The electrons that re-reduce Y_Z originate from the oxygen-evolving complex (OEC), which cycles through so-called S-states, thereby extracting four electrons from two molecules of water. Inorganic cofactors (Mn^{2+}, Ca^{2+}, and Cl^-) along with the

Y_Z and other critical residues in the D1 subunit comprise the OEC of PS II.

Mn cluster on the donor side of PS II prevents the oxidation of exogenous electron donors by redox-active Y_Z and facilitates the high rate of electron transfer from Y_Z to P^+_{680}. Extraction of Mn, which normally also removes Ca^{2+}, modifies the protein environment around Y_Z. As a consequence, Y_Z becomes accessible to the exogenous reductants, including Mn^{2+} (Babcock and Sauer 1975; Schmidt 1976; Yerkes and Babcock 1980; Delrieu 1995; Chroni and Ghanotakis 2001), and results in almost 1,000-fold decrease in the electron transfer rate between Y_Z and P^+_{680} at neutral pH (Conjeaud and Mathis 1980; Hays et al. 1990). Under such conditions, oxidized Y_Z is reduced with a half-time $\sim$20–800 ms, depending on the exogenous donor (Blubaugh and Cheniae 1990). These observations indicate a rapid access to the Y_Z site from the bulk phase, which becomes possible in the absence of the Mn cluster.

In PS II, the electron transfer from Y_Z to Q_A takes place across the dielectric core of the thylakoid membrane. This reaction is electrogenic (Pokorny et al. 1994; Hook and Brzezinski 1994; Haumann et al. 1997; Mamedov et al. 1999). The generation of a transmembrane electric potential difference ($\Delta\psi$) due to the reduction of Y_Z^{ox} by Mn cluster upon the first flash was demonstrated in thylakoids by measurement of the electrochromic absorption changes and liposome-reconstituted PS II core particles with active OEC using electrometrical technique (Haumann et al. 1997; Mamedov et al. 1999).

If Y_Z is electrically isolated from the external water phase, it is safe to suggest that an electrogenic character of Y_Z^{ox} reduction is not specific for Mn^{2+} as the electron donor. In the present work, we investigated the effect of redox-mediators, such as *N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine (TMPD), 2,6-dichlorophenol-indophenol (DCIP) and methylene blue (MB) on the kinetics of the photoelectric responses in Mn-depleted PS II core particles by electrometrical technique.

Materials and methods

Photosystem II core particles were prepared from spinach using the method described in (Ghanotakis et al. 1987). Preparation of the Mn-depleted PS II samples was carried out by a brief incubation in alkaline buffer as described previously resulting in depletion of Mn^{2+} (>95%), Ca^{2+} and the three extrinsic proteins (Ahlbrink et al. 2001). Incorporation of PS II core particles into proteoliposomes was carried out as described earlier (Haumann et al. 1997; Mamedov et al. 1999).

Transmembrane electric potential difference generation was measured as described previously (Semenov et al. 2006). Light flashes were provided by a frequency-doubled Quantel Nd-YAG laser (wavelength, 532 nm; pulse half-width, 15 ns; flash energy 50 mJ). The instrument rise time was 200 ns. The photoelectric responses were not saturated under these conditions. The variations of laser flash energies did not exceed 10%. All experiments were done at 23°C. The kinetic traces were resolved into individual exponents using GraphView (Kalaidzidis et al. 1997).

Results and discussion

Figure 1 shows laser-induced photoelectric response of the proteoliposomes containing Mn-depleted PS II core particles absorbed on the surface of the phospholipid-impregnated collodion film. The flash excitation leads to the generation of $\Delta\psi$ corresponding to the negative charging of the interior of the proteoliposomes. The negative sign of the photoelectric response indicates that the donor side is located at the external surface of the membrane and therefore the proteoliposomes containing PS II core particles might serve as a convenient system for studying the electron donation to oxidized Y_Z by exogenously added reductants.

It is well known that sodium dithionite is a strong reductant which reduces Q_A^- and poorly penetrates through

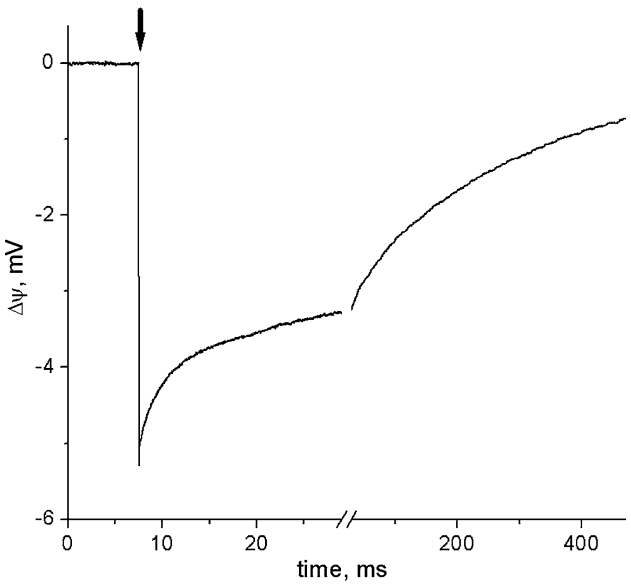

Fig. 1 Voltage changes of proteoliposomes containing Mn-depleted PS II core particles absorbed onto the phospholipid-impregnated collodion film following the single laser flash. The assay mixture contains 20 mM HEPES-NaOH buffer (pH 7.2), 10 mM NaCl, 0.3 M sucrose, 10 mM sodium ascorbate. *Arrows* here and further indicate laser flashes

the proteoliposomal membrane. The lack of the effect of sodium dithionite on the amplitude of the photoelectric response indicates that in proteoliposomes containing PS II the acceptor side is almost entirely oriented inside the vesicles (data not shown).

The samples lack natural electron donor(s) to oxidized Y_Z, Mn^{2+}, as well as the secondary quinone Q_B, so that on the acceptor side of the RC Q_A serves as a final electron acceptor (Ghanotakis et al. 1987). Therefore, a single-turnover flash results in charge separation across the RC complex and subsequent generation of the $Y_Z^{ox}Q_A^-$ state (Pokorny et al. 1994; Haumann et al. 1997; Mamedov et al. 1999). The decay kinetics of the photoelectric response could be approximated with four exponential phases with lifetimes (τ) of τ_1 ~14 μs (19%), τ_2 ~2.4 ms (18%), τ_3 ~20.4 ms (17%) and τ_4 ~275 ms (46%). The components with τ_3 and τ_4 correspond to the back reaction between Q_A^- and Y_Z^{ox}, the component with τ_2 corresponds to recombination between Q_A^- and P_{680}^+, while the faster component (τ_1) is likely due to back electron transfer from the pheophytine (Johnson et al. 1995; Garbes et al. 1998). In some cases, the small decay components with $\tau > 0.5$ s were observed and ascribed to passive discharge across the liposomal membrane. These components did not affect the lifetimes of the faster components. The presence of sodium ascorbate did not alter the kinetics of the photoelectric response (not shown).

Figure 2a shows laser flash-induced photoelectric responses in the absence and in the presence of TMPD/sodium ascorbate couple. Addition of 1 mM TMPD resulted in a significant increase in the contribution of the fast unresolvable phase and the appearance of an additional rise component. The insert to Fig. 2a shows that TMPD within the concentration range 0–10 μM has virtually no effect on the amplitude of the kinetically-unresolvable voltage change due to charge separation between Y_Z and Q_A. Further increase in the TMPD concentration results in approximately twofold rise in the fast phase amplitude and finally exhibits a saturation profile at >2 mM.

The relative contribution of the additional rise component amounted to ~30% of the fast phase attributed to $Y_Z^{ox}Q_A^-$ at 1–2 mM TMPD, while increasing concentration of TMPD resulted in a decrease in the contribution of the millisecond phase. This effect is presumably associated with the acceleration of passive discharge across the liposomal membrane caused by semireduced radical form of TMPD (Trebst and Reimer 1973). We attribute the appearance of an additional $\Delta\psi$ rise phase – to vectorial electron transfer from the redox dye to the protein-embedded Y_Z.

The exponential analysis of the kinetics of the decay and rising of $\Delta\psi$ is presented in Tables 1 and 2, respectively. From the Table 1 one can see that addition of TMPD

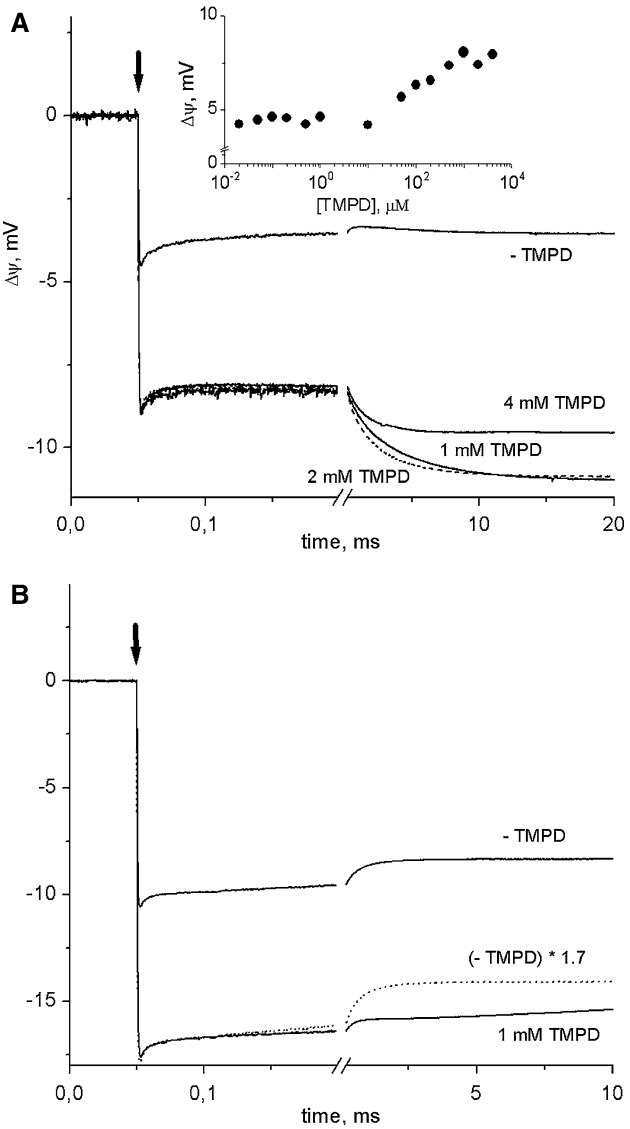

Fig. 2 Effects of reduced (**a**) and oxidized (**b**) TMPD on the kinetics of the photoelectric responses. Insert: dependence of the fast unresolvable phase amplitudes on the concentration of reduced TMPD. In experiments presented in (**b**), proteoliposomes were prepared with ferri- and ferrocyanide inside (10:1 mol/mol). The other experimental conditions as for Fig. 1

results in disappearance of the two fastest (τ_1 and τ_2) phases in the kinetics of the decay, which were ascribed to the back electron transfer from pheophytine and Q_A^- to P_{680}^+, respectively. In the presence of TMPD, the main contribution (73.2 %) was made by the slowest phase (τ_5), which appeared at the expense of kinetic phases τ_3 and τ_4 attributed to the back reaction between Q_A^- and Y_Z^{ox} and the faster phases. The appearance of the slowest phase is due to the prevention of the back reactions and can be ascribed to passive discharge across the liposomal membrane.

Table 2 shows the dependence of the kinetics of $\Delta\psi$ rise on concentration of TMPD. One can see that both kinetic phases accelerate upon increase of TMPD concentration.

Table 1 The exponential analysis of the flash-induced $\Delta\psi$ decay kinetics in the absence and in the presence of 2 mM TMPD

Sample	τ_1, ms	A_1, %	τ_2, ms	A_2, %	τ_3, ms	A_3, %	τ_4, ms	A_4, %	τ_5, ms	A_5, %
Control	0.014	19	2.4	18	20.4	17	275	46	–	–
+TMPD	–	–	–	–	26.7	6.3	64.5	20.5	607	73.2

τ_i characteristic times and A_i relative amplitudes of the decay kinetic phases

Table 2 The exponential analysis of the flash-induced $\Delta\psi$ millisecond rise kinetics in the presence of increasing concentrations of TMPD

TMPD concentration, mM	τ_1, ms	A_1, %	τ_2, ms	A_2, %
1	0.95	26	4.4	74
2	0.84	36	3.15	64
4	0.61	26	1.57	74

Note that the total amplitude of $\Delta\psi$ rise in Table 2 was normalized.

The effect of oxidized TMPD on the kinetics of the photoelectric responses of the proteoliposomes containing Mn-depleted PS II core particles is shown in Fig. 2b. Addition of 1 mM TMPD resulted in the increase of the unresolvable photoelectric response amplitude and essential slowing down of the decay.

The essential increase of the fast unresolvable photoelectric response in cases of both reduced and oxidized forms of TMPD might be explained by the existence of anionic semiquinone Q_A^- in a fraction of PS II before the flash, which is reoxidized directly by the oxidized TMPD (Bukhov et al. 2003). The midpoint redox potential (E_m) of TMPD/TMPD$^+$ redox couple is +260 mV at pH 7.0. In the presence of an excess of ascorbate ($E_m = +80$ mV) the concentration of the oxidized form of TMPD is three orders of magnitude lower than the concentration of reduced form. At 1 mM concentration of the reduced form of TMPD, the concentration of the oxidized form is $\sim$1 μM. This concentration $\sim$10 times exceeds the concentration of the RCs of PS II in the sample and therefore seems to be sufficient for equilibrium reoxidation of Q_A^-.

The effect of another redox mediator, MB, on the kinetics of the photoelectric response is shown in Fig. 3a. The additions of increasing concentration of MB gradually increased the amplitude of the fast phase of photoresponse. The effect of MB is similar to that of oxidized TMPD, which is not surprising, since MB is a well-known electron acceptor (Misran et al. 1994), but due to the rather low E_m value ($\sim$+10 mV at neutral pH) fails to serve as an electron donor to Y_Z^{ox} under our experimental conditions.

The effect of another redox mediator, DCIP, on the kinetics of the photoelectric response is shown in Fig. 3b. In the presence of excess ascorbate, DCIP resulted in a slowing of the decay kinetics, which indicates an effective interaction between DCIP and oxidized Y_Z. At the dye concentrations higher than 4 mM, besides slowing of the decay, an additional slow rise of $\Delta\psi$ was observed. We propose that this slow $\Delta\psi$ rise is due to the electrogenic reduction of Y_Z^{ox}. DCIP serves as less effective donor than TMPD, most probably because the reduced form of the former is negatively charged at neutral pH, and the

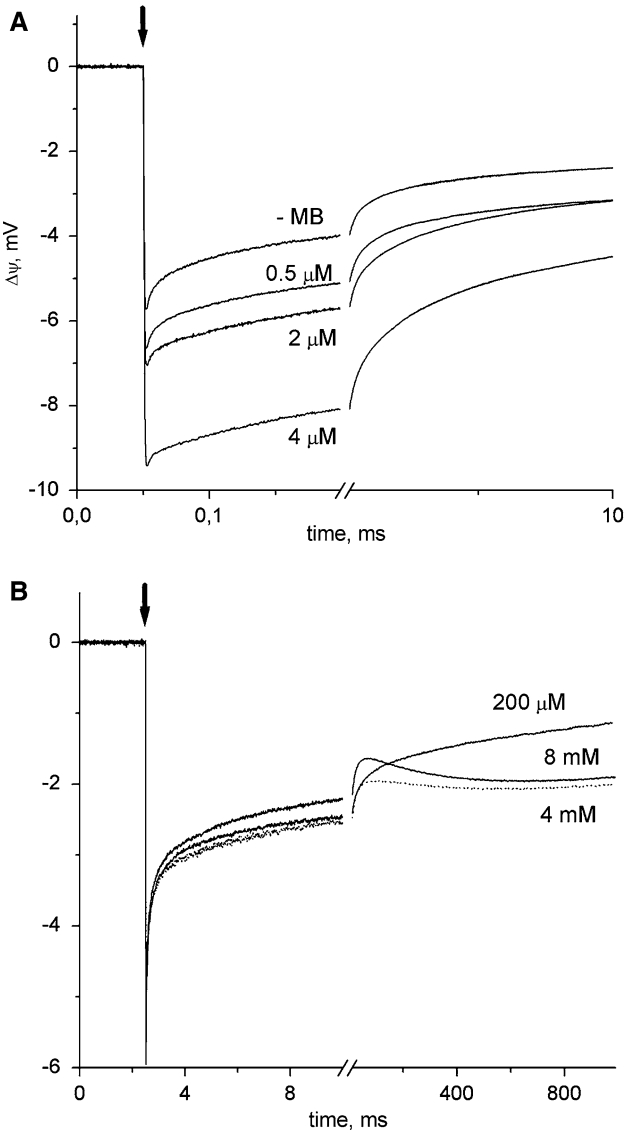

Fig. 3 Effects of MB (**a**) and DCIP (**b**) on the photoelectric responses. The other experimental conditions as for Fig. 1

existence of a negative charge in the vicinity of either Y_Z or the protein surface at the possible DCIP binding site may hamper its binding to the protein. The lack of the kinetically unresolvable phase increase in case of DCIP in contrast to TMPD may be due to the different charge state of $DCIP^{ox}$ compared to that of $TMPD^{ox}$.

Based on the results obtained in PS II core particles with active OEC and in thylakoids, the total electrogenicity on the donor side of the enzyme was tentatively attributed to: (1) transport of electrons from the Mn to Y_Z^{ox}; (2) proton transfer from amino acid X group in the vicinity of Mn cluster into the lumen, which constituted ~10.5% in PS II core particles and ~16% of the fast phase attributed to formation of $Y_Z^{ox}Q_A^-$ in thylakoids (Haumann et al. 1997). However, as the positions of Mn atom donating electron to Y_Z upon single flash and of the X group are insufficiently understood, the dielectrically weighted distance between the Mn-binding site and Y_Z could be underestimated. According to the X-ray structure of the PS II complex, the shortest distance between the binding site for the Mn atom remotest from P_{680} and protein–water surface in PS II core particles lacking the extrinsic proteins and Mn atoms is ~12Å (Loll et al. 2005). Examination of the hydrophobic profile of this protein's region revealed the presence of considerable quantity of nonpolar amino acids (Ferreira et al. 2004; Loll et al. 2005). Therefore, it cannot be excluded that the electron transfer between protein–water interface and Mn-binding site may contribute to the electrogenicity upon electron transfer from TMPD to Y_Z^{ox}.

Recently Haumann et al. (2005) observed the increase of the X-ray absorption with the onset of Mn reduction after deprotonation of a base close to the Mn complex. On this basis the authors proposed that S_4-state formation is identified with a deprotonation process preceding the subsequent electron transfer to Y_Z^{ox}. The authors attributed the deprotonating residue X to the Arg357 of the CP43 protein. Since this residue is located closer to the protein–water interface, than the Mn cluster, then the electron transfer from Mn to Y_Z and deprotonation of X group observed by electrochromic measurements may not cover the whole dielectric distance from the protein surface to Y_Z.

Figure 4 shows the proposed scheme of arrangement of PS II redox cofactors in the membrane of proteoliposome. According to the scheme, $TMPD^{ox}$ and MB are capable to oxidize partially reduced Q_A^- in the dark, and thus increase the number of open RCs. The fast phase of $\Delta\psi$ rise is attributed to charge separation between P_{680} and Q_A and subsequent reduction of P_{680}^+ by Y_Z, while the slower phase is ascribed to the electrogenic reduction of Y_Z^{ox} by reduced forms of TMPD and DCIP. The scheme also shows tentative location of XH group and Mn cluster (which is absent in our samples). Vertical grey arrows indicate the possible electrogenic steps accompanying the electron and

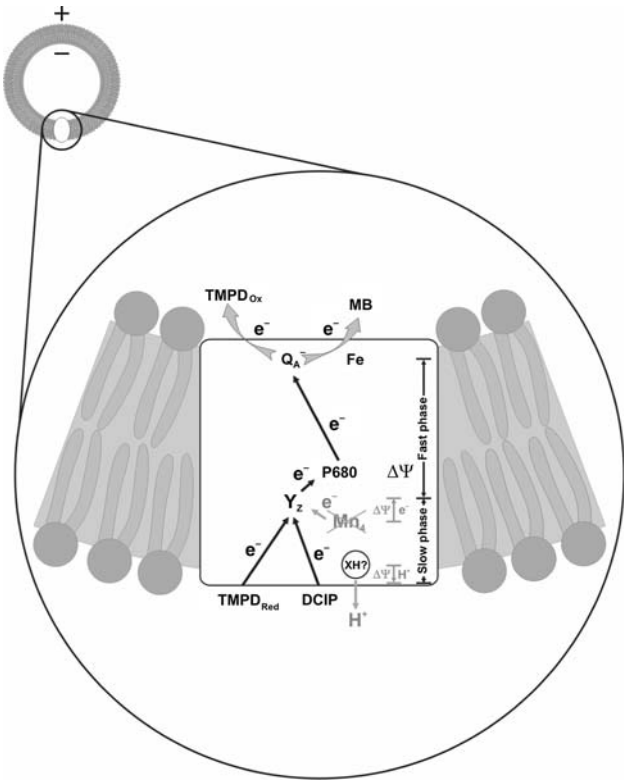

Fig. 4 The tentative scheme of arrangement of PS II redox cofactors in the membrane of proteoliposome. See details in the text

proton transfer in PS II core particles with active OEC. The arrangement of redox cofactors on the donor site of PS II is generally in line with the scheme proposed by (Haumann et al. 2005).

The results obtained in this work suggest that the electrogenic nature of the electron donation to oxidized Y_Z is not specific for Mn^{2+} as an electron donor, and the voltage changes observed in the presence of artificial donors are due to the vectorial electron transfer from the protein–water interface to Y_Z. This finding suggests an electrically isolated location of the Y_Z inside the protein molecule. Moreover, the contribution of electrogenic reduction of Y_Z^{ox} by redox mediators to the total electrogenesis in PS II (at least in Mn-depleted core particles) is higher than the contribution due to $Mn \rightarrow Y_Z$ electron transfer and accompanying proton ejection (see Haumann et al. 1997; Mamedov et al. 1999). This observation demonstrates that Mn-binding site is located in the protein domain with relatively low dielectric permittivity. The increase in the $\Delta\psi$ fast phase caused by an oxidized form of TMPD and MB indicates the existence of the partially reduced Q_A^- in the fraction of Mn-depleted PS II RCs.

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