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Decay kinetics of tyrosine radical ($Y_Z^{\bullet}$) in chloride anion-depleted photosystem 2 studied by time-resolved EPR

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Abstract

The decay of tyrosine cation radical was found to be biphasic at 253 K. The fast phase corresponds to the $Y_Z^{\bullet}$ component while the slow phase corresponds to the tyrosine D radical ($Y_D^{\bullet}$) component. At 253 K, the $t_{1/2}$ value was ~28.6 s for the fast phase and ~190.7 s for the slow phase. The fast phase is attributed to the recombination of charges between $Y_Z^{\bullet}$ and Q_A^-. The activation energy for the reaction of Y_Z with Q_A^- between 253 and 293 K was 48 kJ mol^{-1} in Cl^--depleted photosystem 2 (PS2) membranes. Both the decay rate and the amplitude of the PAR -induced signal of $Y_Z^{\bullet}$ were affected by addition of chloride anion. Change in the decay rate and the amplitude of the PAR-induced signal of $Y_Z^{\bullet}$ was observed when other anions like Br^-, I^-, F, HCO_3^-, NO_3^-, PO_4^{3-} were substituted in the Cl^--depleted PS2.

Additional key words: bicarbonate; bromide; EPR; fluoride; iodide; nitrate; phosphate; photosystem 2; spinach; tyrosine radical; sulphate.

Introduction

Photosynthetic oxidation of water with concomitant production of molecular oxygen takes place in photosystem 2 (PS2), a multi-polypeptide enzyme complex of higher plants, algae, and cyanobacteria. Chloride anion is an essential cofactor in the oxygen evolving complex of PS2. The rate of oxygen evolution is inhibited if PS2 membrane particles are depleted of chloride anion, and is restored by addition of chloride anion (Critchley 1985, Britt 1996).

While a great deal is known about the phenomenology of the chloride anion requirement, there are still speculations about the mechanism by which it exerts its influence. Chloride anion is not required for the primary photochemistry in the PS2 reaction but is involved in the water-oxidising mechanisms (see volume edited by Govindjee *et al.* 1983). It probably does not undergo direct oxidation itself, since other anions substitute for it, but it influences the structure and redox properties of the Mn cluster which catalyzes water oxidation.

Evidence that chloride anion is bound close to the oxygen evolving complex (OEC) is provided by the fact that chloride-depleted PS2 membranes exhibit altered S_2-state EPR signals and that chloride is required for the S_2-to-S_3 transition (Ono *et al.* 1986). In addition, chloride-depleted PS2 membranes display a broadened radical EPR signal centred at $g = 2.0$ when irradiated at temperatures above 250 K and quickly cooled to 77 K. This signal is attributed to the $S_2Y_Z^{\bullet}$ state, which accumulates under steady-state irradiation because of the requirement for chloride anion in the S_2-to-S_3 transition (Wincencjusz *et al.* 1996).

Electron transfer between the OEC and P680 is mediated by a tyrosine residue, $Y_Z^{\bullet}$, which is a tyrosine-161 of the D1-polypeptide (Debus *et al.* 1988b). A second redox active tyrosine $Y_D^{\bullet}$ has been identified as tyrosine-161 of the D2 polypeptide (Debus *et al.* 1988). $Y_Z^{\bullet}$ and $Y_D^{\bullet}$ EPR signals can be distinguished on the basis of their decay rates because $Y_D^{\bullet}$ is typically present as a dark stable neutral radical and takes several minutes to decay at room temperature. On the contrary, $Y_Z^{\bullet}$ decays very fast in oxygen evolving PS2, in a time range faster than 1 ms at room temperature (Hoganson and Babcock 1988). The kinetics of oxidation of tyrosine D by the S_2-state in Cl^--depleted PS2 membranes is 10 times slower than in Cl^--sufficient PS2 (Deák *et al.* 1994). In the case of active, oxygen evolving PS2, Y_Z is not only rapidly

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; OEC, oxygen-evolving centre; P680, the primary electron donor of PS2; PAR, photosynthetic active radiation; PS2, photosystem 2; Q_A, the primary electron acceptor quinone of PS2; Q_B, the secondary electron acceptor quinone of PS2; Y_Z, tyrosine-161 of D1 polypeptide; Y_D, tyrosine-161 of D2 polypeptide.
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oxidized by P_{680}^+, but the oxidized Z^+ is, in turn, rapidly reduced by water molecules through the turnover of the S-states. Only when the electron-transfer pathway from the OEC is blocked and PS2 loses its oxygen evolution ability, the reduction of $Y_Z^{\bullet}$ becomes much slower. After Tris-treatment, if Tyr Z is already oxidized by a pre-flash, the charge recombination between $P680^+$ and Y_Z takes 200 µs (Conjeaud and Mathis 1986) but upon acetate treatment it takes around 500 µs (Saygin *et al.* 1986). Decay kinetics of $Y_Z^{\bullet}$ is dependent on the temperature and pH (Shigemori *et al.* 1997, Kuhne and Brudwig 2002). Kinetic properties of $Y_Z^{\bullet}$ in Ca^{2+}-depleted and Cl^--depleted PS2 membranes were studied by Boussac *et al.* (1992) and Simentiris *et al.* (1998). However, earlier works have been done at room temperature where the decay of $Y_Z^{\bullet}$ was fast and difficult to observe.

In this paper, we report the changes in decay kinetics of $Y_Z^{\bullet}$ in Cl^--depleted PS2 membranes as a function of temperature by time-resolved EPR measurements. We tried to understand the role of Cl^- on the donor side of PS2 by monitoring the reduction kinetics of tyrosine Z ($Y_Z^{\bullet}$) in Cl^--depleted PS2 membranes. We measured the yield of $Y_Z^{\bullet}$ formation and its decay kinetics in order to characterize chloride anion-induced effects on the donor-side of PS2. Changes in the decay rates of $Y_Z^{\bullet}$ were observed after replenishing Cl^--depleted PS2 with chloride and other anions such as bromide, fluoride, *etc.* We also calculated the $t_{1/2}$ values of the decay of $Y_Z^{\bullet}$ after re-substituting with different anions. Our data suggest two common binding sites for chloride and other anions on PS2.

Materials and methods

Sample preparation: Oxygen evolving PS2 membranes were prepared from market spinach using the method described in Kuwabara and Murata (1982). The membranes were stored at 77 K. After thawing, the particles were washed twice in low-chloride medium composed of 0.4 M sucrose, 4 mM Mes-NaOH (pH 6.5), and 2 mM NaCl, and centrifuged at 35 000×*g* for 10 min. Chlorophyll (Chl) concentrations of approximately 8–9 g m^{-3} were determined according to Porra (1990). All steps of preparations were performed under dim green light.

Chloride depletion was performed by a substitution by sulphate as described in Ono *et al.* (1986). The particles were suspended in 0.4 M sucrose, 50 mM Na_2SO_4, and 40 mM Hepes-NaOH (pH 7.5) at a concentration of 0.4 g(Chl) m^{-3}, incubated for 10 min in the dark, and then centrifuged at 35 000×*g* for 10 min. The resulting pellet was re-suspended in the same medium and was used as Cl^--depleted PS2 membrane particles. We checked the extent of chloride-depletion in our PS2 preparations by measuring the multi-line signal at 6 K (Ono *et al.* 1986). No multi-line signal corresponding to the S_2 state was observed in our samples irradiated at 200 K. Addition of chloride induced the formation of the multi-line signal (data not shown). Anion substitutions were performed following the method described in Ono *et al.* (1987). Na-salts of various anions was added to the Cl^--depleted PS2 membranes suspended in 0.4 M sucrose and 40 mM Hepes-NaOH (pH 7.5), and incubated for 10 min. The suspension of anion-substituted PS2 membranes was centrifuged at 35 000×*g* for 10 min and the resulting pellet was re-suspended in the same medium.

Oxygen evolution: The oxygen-evolving activity of PS2 was measured using a Clark-type electrode at 20 °C under continuous saturating irradiation through a *Toshiba R50* filter and an 8 cm thick water filter. In the presence of 600 µM *p*-phenylbenzoquinone as electron acceptor, the rate of oxygen evolution in normal PS2 membranes was about 110 mmol(O_2) kg^{-1}(Chl) s^{-1}. Cl^--depleted PS2 membranes showed activity of 11–14 mol(O_2) kg^{-1}(Chl) s^{-1}. Oxygen evolving activity of PS2 membranes was restored to approx. 90 % of the control upon addition of chloride anion.

Time-resolved EPR measurements were performed using *Varian E-109* system X-band spectrometer and a home made nitrogen gas-flow cryostat with a temperature controller. Samples were directly irradiated using *Hoya-Schott MegaLight-100* source to excite $Y_Z^{\bullet}$ EPR signals.

Results and discussion

In the presence of functional oxygen evolving complex (OEC) and at room temperature, the reduction of $Y_Z^{\bullet}$ is normally too fast ($t_{1/2}$<1 ms) to allow observation of the species attributed to $Y_Z^{\bullet}$. In Cl^--depleted PS2 membranes, OEC is severely inhibited and the decay kinetics of $Y_Z^{\bullet}$ are slowed, enabling its detection on irradiation. In time-resolved EPR measurements, the PAR-induced EPR signal included not only $Y_Z^{\bullet}$ components but also $Y_D^{\bullet}$. As expected, the PAR-induced EPR spectrum of $Y_Z^{\bullet}$ revealed a biphasic decay, the pattern of which changed with temperature. On the basis of their optical and EPR studies, Dekker *et al.* (1984) ascribed the fast decay phase to a charge recombination between $Y_Z^{\bullet}$ and Q_A^-, and the slow phase to an electron transfer through the added acceptor in the presence of ferricyanide and DCMU.

In Cl^--depleted PS2 membranes, OEC is severely inhibited and the decay kinetics of $Y_Z^{\bullet}$ is slowed, enabling its detection on irradiation. The decay of $Y_Z^{\bullet}$ in Cl^--depleted PS2 membranes showed a biphasic pattern at room temperature, as reported earlier (Boussac *et al.*

1992, Simentiris *et al.* 1998). We investigated the temperature dependence of $Y_Z^{\bullet}$ decay in Cl^--depleted PS2 membranes. Fig. 1 shows EPR traces of the decay of $Y_Z^{\bullet}$ as a function of temperature from 200 to 293 K. In normal oxygen evolving PS2 membrane particles (untreated), the $Y_Z^{\bullet}$ decay kinetics was too fast to be measured even at low temperatures (Fig. 1). Below 245 K, the $Y_Z^{\bullet}$ decay became almost monophasic, probably because both the fast and the slow phases decayed too slowly to be separated into two separate phases. At room temperature, it was difficult to observe the stable $Y_Z^{\bullet}$, but at 253 K the $Y_Z^{\bullet}$ decay slowed down and became amenable to study. The best temperature to carry our further experiments was 253 K. In Cl^--depleted PS2 membranes, the half time ($t_{1/2}$) of the fast phase was ~28.6 s while that of the slow phase was ~190 s.

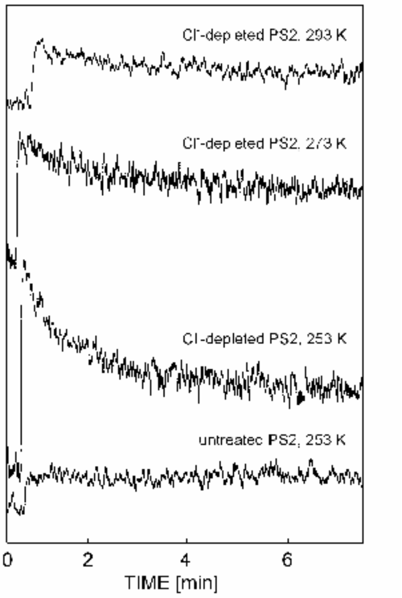

Fig. 1. EPR traces of the decay kinetics of $Y_Z^{\bullet}$ in chloride anion-depleted photosystem 2 membranes at different temperatures. Experimental conditions: microwave frequency 9.31 GHz, microwave power 2 mW, modulation amplitude 5 G.

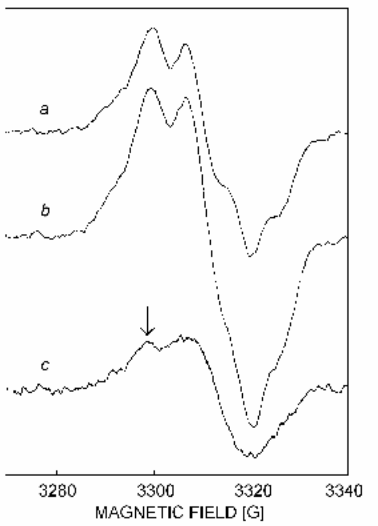

Fig. 2. $Y_D^{\bullet}$ spectra recorded at 253 K. *a* – spectrum recorded in the dark, *b* – after irradiation, *c* – irradiated minus dark spectrum. *Arrow* indicates the position of the magnetic field fixed for the kinetic experiments. Experimental conditions as in Fig. 1.

The PAR-induced EPR signal of tyrosine radical includes the $Y_D^{\bullet}$ as well as the $Y_Z^{\bullet}$ component. The EPR spectrum of $Y_Z^{\bullet}$ is normally measured as the light-dark difference spectrum after a relatively short dark time, and hence the spectrum due to $Y_D^{\bullet}$, which is relatively stable in dark, can be subtracted. Fig. 2 shows the tyrosine EPR spectra recorded in dark (*a*) and after irradiation (*b*). The difference spectrum (*c*) shows the $Y_Z^{\bullet}$ spectrum recorded in Cl^--depleted PS2 membranes at 253 K. The arrow indicates the position of magnetic field fixed for the kinetic experiments.

Fig. 3 shows EPR signal arising from $Y_Z^{\bullet}$ and its subsequent reduction in the dark after replenishing the PS2 membrane particles with different concentrations of chloride anion. OEC inactivation by depleting PS2 membranes of its chloride anion was reversible, resulting in a faster decay of $Y_Z^{\bullet}$ on addition of the chloride anion. Addition of more than 100 mM Cl^- led to a very fast decay, which could not be observed even at 253 K, just as in case of normal PS2. The additions of Cl^- affected not only the decay rates, but also affected the PAR-induced intensity of the $Y_Z^{\bullet}$ signal, in a concentration dependent manner. There was a consistent decrease in the amplitude of the $Y_Z^{\bullet}$ signal with an increase in Cl^- concentration. The decay kinetics of $Y_Z^{\bullet}$ was related to the back reaction from Q_A^-. The decrease in the intensity of the $Y_Z^{\bullet}$ EPR signal after the addition of Cl^- may be ascribed to weaker inhibition at the acceptor side resulting in fast back reaction between $Y_Z^{\bullet}$ and Q_A^-.

The decay rates of $Y_Z^{\bullet}$ were calculated using the following equation:

$$I = A_1 \exp(-t/t_1) + A_2 \exp(-t/t_2) \qquad (1)$$

where t_1 is the half-time constant for fast phase corresponding to $Y_Z^{\bullet}$ components and t_2 is that for slow phase corresponding to $Y_D^{\bullet}$ components. Cl^--depletion affected not only the ratio of PAR -induced $Y_Z^{\bullet}$ but also the decay rate. The estimated $t_{1/2}$ values for the fast phase were about 28.6 s at 253 K. The $t_{1/2}$ values were remarkably greater than those of Tris-treated PS2 membranes which are about 0.5–0.6 s (Shigemori *et al.* 1997). The increase may be ascribed to the change in redox potential of Y_Z and/or Q_A.

The activation energy (E) was calculated using the formula

$$1/_{t1/2} = A \exp(-E/kT) \qquad (2)$$

There are two possibilities for the utilization of the electron stabilized in Q_A: a forward transfer to Q_B or

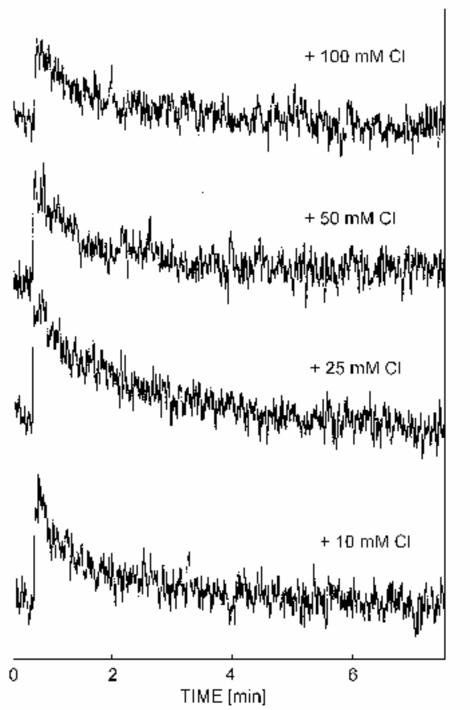

Fig. 3. EPR traces of decay kinetics of $Y_Z^{\bullet}$ at 253 K in chloride anion-depleted photosystem 2 membranes after replenishing with different concentrations of chloride. Experimental conditions as in Fig. 1.

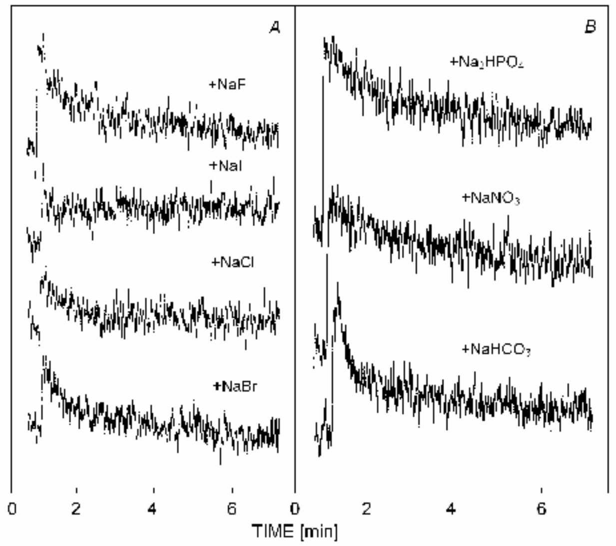

Fig. 4. EPR traces of $Y_Z^{\bullet}$ decay at 253 K in chloride anion-depleted photosystem 2 membranes after substituting with different anions (100 mM). Experimental conditions as in Fig. 1.

recombination with P_{680}^{+} in equilibrium with Y_Z. At the same time Y_Z receives electrons from the Mn cluster. Thus, there are two concurrent ways to reduce $Y_Z^{\bullet}$: either by the Mn cluster or by Q_A^-. $Y_Z^{\bullet}$ decay depends on the charge state of these components. Now let us consider three cases:

(*1*) If we assume that Cl^--depletion prevents electron transfer from Q_A^- to Q_B, the probability of charge recombination between Q_A^- and Y_Z increases, making the $Y_Z^{\bullet}$ EPR signal less visible because of small intensity and faster decay.

(*2*) If Cl^--depletion affects electron transfer only from the Mn cluster to Y_Z, the decay of $Y_Z^{\bullet}$ would be controlled by Q_A^-. Due to the electron vacancy in Q_B, recombination between Q_A and $Y_Z^{\bullet}$ would be slowed and intensity of $Y_Z^{\bullet}$ would be high.

(*3*) Cl^--depletion affects electron transfer between $Q_A^- \rightarrow Q_B$ and between Mn cluster$\rightarrow Y_Z$. In this case $Y_Z^{\bullet}$ decay will be controlled by Q_A^- as well as by changes in the recombination in the redox potential of Q_A.

There is a well known effect of Cl^- on the donor side of PS2 (Yocum 1992, Olesen and Andreasson 2003). Based on the observation that addition of chloride to Cl^--depleted PS2 membrane particles leads to a smaller intensity of the PAR-induced $Y_Z^{\bullet}$ EPR signal and to faster decay rates, we favour the third possibility, suggesting that Cl^- may bind to the donor side as well as to the acceptor side of PS2.

This possibility was further confirmed by experiments carried out in the presence of DCMU which inhibits electron transport from Q_A to Q_B. No significant change in the decay pattern in the presence of DCMU was observed in our samples (data not shown).

We also examined the decay of $Y_Z^{\bullet}$ in Cl^--depleted PS2 membranes after substituting various anions for Cl^-. The objective was to see whether other anions could indeed substitute for Cl^-. Br^-, I^-, F^-, HCO_3^-, NO_3^-, and HPO_4^{3-} were used as sodium-salts (100 mM). Fig. 4*A,B* shows the effects of these anions on the decay of $Y_Z^{\bullet}$: Br^-, I^-, and NO_3^- could substitute for Cl^- to a large extent, as the decay pattern and amplitude of the $Y_Z^{\bullet}$ signal and that with Cl^- were almost similar. The substitution restored the normal activity in the Cl^--depleted PS2 membranes leading to faster $Y_Z^{\bullet}$ decays. The activation energy E could not be calculated for the anion-substituted Cl^--depleted PS2 membranes as the decay was too fast to be resolved into two distinct components at temperatures higher than 253 K. E value for the reaction of $Y_Z^{\bullet}$ with Q_A^- between 253 and 293 K in untreated Cl^--depleted PS2 membranes was estimated to be 48 kJ mol^{-1}, which is significantly higher than the E values reported in Tris-treated PS2 membranes (38.8 kJ mol^{-1}, Shigemori *et al.* 1997) and in formate-treated PS2 membranes (28.7 kJ mol^{-1}, personal communication).

In our samples, 100 mM NaF could restore the decay of the EPR signal of $Y_Z^{\bullet}$ in the Cl^--depleted PS2 membranes confirming the earlier results of Baumgarten *et al.* (1990) and Vliet and Rutherford (1996). I^- could partially substitute for Cl^-, we measured a decay constant of the fast phase around 3.53 s. In the presence of bicarbonate, the decay rate as well as the yield of $Y_Z^{\bullet}$ changed, but not to the same extent as with Br^- *etc.* (Fig. 4*B*).

Two distinct types of the effect were observed after anion substitution of Cl^- depleted PS2 membrane particles. In the presence of anions such as HCO_3^-, PO_4^{3-}, and NO_3^- (Fig. 4*B*), the amplitude of the PAR-induced intensity of $Y_Z^\bullet$ EPR signal was significantly greater than that observed with Cl^-, Br^-, or I^-. This suggested that some anions affected electron transfers between Mn cluster and Y_Z resulting in greater PAR-induced signal of $Y_Z^\bullet$. In the presence of bicarbonate, the $t_{1/2}$ value was 10.3 s. NO_3^- and PO_4^{3-} also caused an increase in the PAR-induced signal of $Y_Z^\bullet$, but the decay rate in them was faster. The order of effectiveness of various anions in substituting for Cl^- was $Br^- > I^- > F^- > NO_2^- \sim PO_4^{3-} > HCO_3^-$.

In our earlier work (Jajoo and Bharti 1993), besides the water oxidation site, Cl^- was suggested to have a site of action between Q_A and Q_B. Vliet and Rutherford (1996) observed distinct Cl^--depletion effects in PS2 observed under two different Cl^--depletion treatments indicating the presence of two distinct binding sites in PS2. Cl^- seems to have at least two distinct binding sites on PS2, which have different affinities for different anions.

Table 1 shows the changes in the $t_{1/2}$ values of $Y_Z^\bullet$ decay and changes in the $Y_Z^\bullet/Y_D^\bullet$ ratio with different treatments**.** The intensity ratio $Y_Z^\bullet/Y_D^\bullet$ is related to the donor side inhibition due to the inhibition of electron transport from the water oxidizing complex. The higher is this ratio, the greater is the inhibition at the donor side. In our experiments, $Y_Z^\bullet/Y_D^\bullet$ ratio decreased remarkably after re-substituting with Cl^- and other anions. This suggests that the inhibition at the donor side as imposed in Cl^--depleted PS2 membranes is reduced in the presence of Cl^- and other anions.

Styring *et al.* (2003) showed a pH dependence of the donor side reactions in Ca^{2+}-depleted PS2 membranes. Boussac *et al.* (1992) suggested that Cl^- depletion and Ca^{2+} depletion result in an essentially similar electron transfer block. Electrostatic effects were suggested to be responsible for it. A role of chloride in the charge separation reactions associated with the de-protonation events was also suggested. Our results support this hypothesis and we assign the effects observed in our experiments to electrostatic changes, since most of the anions could substitute for Cl^- and exhibited similar pattern of $Y_Z^\bullet$ decay and $Y_Z^\bullet/Y_D^\bullet$ ratio. It seems to be a charge dependent rather than specifically Cl^--dependent phenomenon. The observed effects of various anions on the $Y_Z^\bullet$ radical decay are probably due to a non-specific salt effect. The effectiveness of the anions was found to be different. It may depend on a number of factors including ionic radii, surface charge density, affinity for binding site, *etc*. However, further studies are required to elucidate the mechanism of action of different anions.

Table 1. $Y_Z^\bullet/Y_D^\bullet$ intensity ratio and the half-times of the biphasic decay of $Y_Z^\bullet$ in chloride anion-depleted photosystem 2 membranes at 253 K.

Treatment	Half-time of $Yz^\bullet$ decay ($t_{1/2}$) [s]		$Y_Z^\bullet/Y_D$
100 mM anions	fast phase	slow phase	
Control	28.6	197	0.63
+ NaCl	0.80	233	0.42
+ NaBr	1.20	230	0.46
+ NaI	3.53	223	0.43
+ NaF	8.30	227	0.54
+ $NaHCO_3$	14.90	231	0.83
+ $NaNO_3$	1.87	220	0.57
+ Na_2HPO_4	1.90	229	0.59
+ Na_2SO_4	2.45	220	0.56

This work presents findings about the $Y_Z^\bullet$ decay kinetics in Cl^--depleted PS2 membranes at different temperatures and opens a broad field for further research. In our knowledge, a very slow (in s-range) $Y_Z^\bullet$ decay rate has been reported for the first time. It would be interesting to understand the mechanism of action of anions in the presence and absence of chloride anion.

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