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# Study on the effects of chloride depletion on photosystem II using different chloride depletion methods

**Anjana Jajoo · Sonal Mathur · Pooja Mehta · Miho Yoshoika · Suleyman I. Allakhverdiev · Yasusi Yamamoto**

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**Abstract** Chloride is an indispensable factor for the functioning of oxygen evolving complex (OEC) and has protective and activating effects on photosystem II. In this study we have investigated mainly by EPR, the properties of chloride-sufficient, chloride-deficient and chloride-depleted thylakoid membranes and photosystem II enriched membranes from spinach. The results on the effects of different chloride depletion methods on the structural and functional aspects of photosystem II showed that chloride-depletion by treating PS II membranes with high pH is a relatively harsh way causing a significant and irreparable damage to the PS II donor side. Damage to the acceptor side of PS II was recovered almost fully in chloride-deficient as well as chloride-depleted PS II membranes.

**Keywords** Chloride · Oxygen evolving complex · EPR · Photosystem II

**Abbreviations**

| | |
|---|---|
| Chl | Chlorophyll |
| CP43, CP47 | Core antenna subunits of PSII |
| DCMU | 3-(3`,4`-Dichlorophenyl)-1,1-dimethylurea |
| EPR | Electron paramagnetic resonance |
| ML | multiline EPR signal |
| PS II | Photosystem II |
| P680 | Primary electron donor |
| $Q_A$, $Q_B$ | Plastoquinones |
| $Y_Z$, $Y_D$ | Redox active tyrosines of the D1 and D2 proteins |

A. Jajoo (✉) · S. Mathur · P. Mehta
School of Life Sciences, Devi Ahilya University,
Indore 452 001 M.P, India
e-mail: anjanajajoo@hotmail.com

M. Yoshoika · Y. Yamamoto
Natural Science and Technology, Okayama University,
Okayama 700-8530, Japan

S. I. Allakhverdiev
Institute of Basic Biological Problems,
Pushchino, Moscow Region 142290, Russia

## Introduction

Transformation of light energy into chemical energy in the course of oxygenic photosynthesis takes place with the contribution of sequentially functioning photosystem II (PS II) and photosystem I (PS I), the thylakoid membrane complexes containing reaction center (RC) and the core antenna. PS II is a light dependent water-plastoquinone oxidoreductase enzyme which uses light energy to oxidize water and is mainly located in the appressed grana stacks. X-ray crystallographic investigations of cyanobacterial photosystem II have provided high resolution structures at 2.9 Å (Guskov et al. 2009) that explain the general arrangement of the protein matrix and cofactors. The structural and functional aspects of PS II are interrelated.

The central core of PS II is composed of a heterodimer of the D1 and D2 proteins, the two chlorophyll binding proteins CP43 and CP47, Cyt b559, and other small membrane spanning proteins. The D1/D2 heterodimer retains the main cofactors involved in the electron transfer in PS II. These include the primary electron donor (P680), the primary electron acceptor pheophytin (Pheo), the primary and secondary quinone electron acceptors, $Q_A$ and $Q_B$ and non heme $Fe^{2+}$. Electron transfer in PS II starts with excitation of $P_{680}$ ($P_{680}^*$), resulting in oxidation of the $P_{680}$ ($P_{680}^+$) and sequential reduction of Pheo, $Q_A$ and ultimately $Q_B$. The D1/D2 heterodimer also binds the

oxygen evolving center (OEC) of PS II. This complex is located at the lumenal side of the thylakoid membrane and includes ions of Mn, Ca and Cl. Two tyrosine residues, $Y_Z$ and $Y_D$, are symmetrically located on the D1 and the D2 proteins respectively. In higher plant PSII, three extrinsic polypeptides PsbO, P and Q, having relative molecular masses of 33, 23 and 17 kDa play important roles in the stability of OEC. The PsbP and Q contribute in the retention of functional chloride and calcium (Homann 1988). A cluster of four manganese (Mn) ions plays a central role in water oxidation capacity of PS II. The oxidation potential for oxidation of water produced by light-induced charge separation in the PS II reaction centers accumulates in the Mn cluster, through five successive redox states of OEC, named $S_0$, $S_1$,….$S_4$ (Kok et al. 1970). The two tyrosine residues play quite different roles. $Y_D^{\bullet}$ is a dark-stable neutral radical, which does not play a definite role in the normal electron transfer reaction in PS II. On the other hand, $Y_Z$ functions as an electron transfer intermediate between the reaction center $P_{680,}$ and the OEC. In the oxygen evolving PS II, $Y_Z^{\bullet}$ decays in a time range faster than 1 ms at room temperature and it is reduced by an electron from the OEC (Hoganson and Babcock, 1988) and its EPR signal has been called Sig. $II_{vf}$ (Babcock and Sauer, 1975). Detection of the EPR signal from $Y_Z^{\bullet}$ is difficult in native PS II membranes having high oxygen evolving activity. However $Y_Z^{\bullet}$ signal was visible in PS II membranes when normal electron transfer from the Mn cluster to $Y_Z^{\bullet}$ was inhibited by hydroxylamine (Sonneveld et al. 1979), or Tris-treatment (Klimov et al. 1982; Dekker et al. 1984; Allakhverdiev et al. 1986). In these systems, $Y_Z^{\bullet}$ radical could be trapped and subsequently its EPR signal was observed.

Calcium and chloride are crucial for optimal function of PSII and the loss of any one of these factors causes inactivation of PSII activity (Ke 2001). Chloride is required for high oxygen evolution in PS II and for the normal electron paramagnetic resonance (EPR) properties of the $S_2$ state (Wincencjusz et al. 1996, Ono et al. 1986). Studies on chloride depleted PSII membranes suggested that the presence of bound $Cl^-$ protected the donor side of PSII from the heat-induced damage (Wydrzynski et al. 1982; Coleman et al. 1988, Tiwari et al. 2007).

Three types of thylakoid membrane preparations have been commonly used to study the effects of chloride on PS II: chloride-sufficient, deficient and depleted membranes. Chloride-sufficient membranes contain intrinsic as well as externally added chloride. Chloride-deficient membranes have only intrinsic chloride and no externally added chloride, while in chloride-depleted membranes chloride was removed from the intrinsic sites. According to the earlier literatures, chloride depletion can be carried out in several ways including treatment with high concentrations of sodium sulphate (Ono et al. 1986) or with high pH (Homann 1988). We have prepared chloride-depleted thylakoid membranes by using these methods and have tried to differentiate the effects of chloride on the donor and acceptor sides of PS II in the different types of membrane preparations. For this purpose, we measured the rates of oxygen evolution, yield of $Y_Z^{\bullet}$ formation and the decay kinetics of $Y_Z^{\bullet}$, $S_2$ state multiline (ML) EPR signals, SDS-PAGE and Western blotting to monitor the reaction center-binding protein D1 and PsbO, P and Q.

## Materials and methods

### Preparation of PS II membranes

Oxygen evolving PS II membranes were prepared from market spinach using the method described by Kuwabara and Murata (1982). The membranes were suspended in a buffer solution containing 0.2 M sucrose, 20 mM MOPS, 20 mM NaCl, and 50 % (v/v) glycerol (pH 6.9) at chlorophyll concentration of approximately 4–6 mg $ml^{-1}$, and stored at 77 K until use. Chlorophylls were determined according to Porra et al. (1989).

### Preparation of chloride-sufficient, deficient and depleted PS II membranes

Prior to the experiment, the PS II membranes were slowly thawed at 0–4 °C and 1 ml each of the membrane samples was washed twice in 20 ml of the medium described below. Two types of suspending media were used. The first type medium contained $Cl^-$ in the form of 20 mM NaCl, 0.2 M sucrose and 50 mM MOPS (pH-6.9) and these preparations were termed as the "$Cl^-$-sufficient". The second type medium contained only 0.2 M sucrose and 50 mM Mops (pH-6.9) but no additional NaCl and was meant to remove non-specifically associated or loosely bound $Cl^-$. These preparations were termed "$Cl^-$-deficient ($Cl^-$ free)". All the steps were carried out in dim green light at low temperature. Chloride depletion was performed with $Cl^-$-free PS II membranes. Chloride depletion by sulphate treatment was performed following the method described by Ono et al. (1986). Briefly, PS II enriched membranes were suspended in 0.4 M sucrose, 50 mM $Na_2SO_4$, 40 mM Hepes-NaOH (pH 7.5) at a chlorophyll concentration of 0.4 mg Chl $ml^{-1}$, incubated for 10 min in the dark and then centrifuged at 35,000 x g for 10 min. The resulting pellet was resuspended in the $Cl^-$-free medium and was used as chloride-depleted (by sulphate) membranes. Chloride depletion by high pH treatment was performed following the method described by Vliet and Rutherford (1996). Briefly, the pH was increased by addition of 15 mM CAPS to pH 10. After

incubating it for 30 sec, the pH was lowered to pH 6.9 by addition of Mops (pH 6.9) and the sample was further incubated for 10 min.

### Measurement of oxygen evolution

The oxygen-evolving activity of PS II was measured using a Clark-type oxygen electrode at 20°C under continuous illumination with a saturating light of 150 $Wm^{-2}$ (approximately 950 µmol photons $m^{-2}s^{-1}$), filtered through a Toshiba R50 and 8 cm thick water filters. The reaction mixture contained 0.33 M sucrose, 50 mM HEPES-NaOH (pH 7.5) and thylakoid suspension equivalent to 10 µg of Chl/ml. In the presence of 600 µM PPBQ as an electron acceptor, the rate of oxygen evolution in the control samples at pH 6.5 was about 400 µmol of $O_2$ (mg Chl)$^{-1}h^{-1}$.

### Measurement of chlorophyll a fluorescence induction kinetics

Chl *a* fluorescence transients were measured with a Plant Efficiency Analyzer (PEA Hansatech Ltd., King's Lynn, Northfolk, UK). The actinic light was provided by an array of three high-intensity light-emitting diodes, and the light was focused via lenses onto the sample surface to provide even illumination. The diodes provided red light of a peak wavelength of 650 nm. The fluorescence signal is received by a sensor head during recording and is digitized in a control unit using a fast digital converter. The reaction mixture contained 0.33 M sucrose, 50 mM HEPES-NaOH (pH 7.5) and the suspension of the PSII membranes at chlorophyll concentration of 10 µg Chl $ml^{-1}$. The experiment was done three times in triplicate.

### Measurement of EPR spectra

CW-EPR measurements were performed using a Bruker-300E X-band spectrometer, and an ST4102 standard cavity. An Oxford-900 continuous gas-flow cryostat and ITC-4 temperature controller were used to regulate the sample temperature at 6.0 K. To achieve the $S_2$ state, samples were illuminated with 500 mW tungsten-halogen lamp through an 8 cm thick water filter at 200 K in an ethanol/solid $CO_2$ bath (Olesen and Andreasson, 2003). The illuminated samples were quickly stored at 77 K and then $S_2$ state EPR signals were measured at 6 K. The intensity of the multiline EPR signal was measured as the sum of four hyperfine peak heights in the illuminated-minus-dark difference spectra.

Time-resolved EPR measurements were performed using a Varian E-109 system X-band spectrometer and a home-made nitrogen gas-flow cryostat with a temperature controller. We used a rectangular cavity with a window for illumination, and samples were directly illuminated in the cavity using a HOYA-SCHOTT Mega Light-100 to excite $Y_Z^{\bullet}$ signals. Analysis of the time-resolved EPR kinetic traces was done with an Origin v 6.1 software (Originlab Corporation, USA). Chlorophyll concentration used in all EPR measurements was 3 mg/ml.

### SDS page and western blotting

SDS/Urea-PAGE and Western blot analysis was carried out as described by Yamamoto et al. (2004). The concentration of the acrylamide in the resolving gel was 12.5% (w/v). Each lane contained sample equivalent to 2.5 µg chlorophyll. The antibodies against PsbO (33 kDa), PsbP (23 kDa) and PsbQ (17 kDa) and D1 protein were bought from Agrisera, Sweden. The bands that cross-reacted with the antibodies were detected by fluorography with enhanced chemiluminiscence (ECL) reagents (Amersham, Japan).

## Results and discussion

### Effects of chloride depletion on Oxygen Evolving Complex

The rates of oxygen evolution were measured in $Cl^-$-sufficient, deficient and depleted PS II membranes. The rates in µmoles of oxygen evolved per mg chl per hr in PS II membranes were 398 in $Cl^-$-sufficient, 300 in $Cl^-$-deficient, 56 in $Cl^-$-depleted by sulphate treatment and 45 in $Cl^-$-depleted by high pH treatment, respectively. Inhibition of oxygen evolution were probably due to release of Mn from the functional site of PS II as was evident from $S_2$ state multiline (ML) signal (Fig. 1). In chloride-sufficient sample, a large ML signal between 2500 and 4000 G has been observed while a little decline in the intensity of ML signal was observed in chloride-deficient samples. In chloride- depleted PS II membranes treated with sulphate, a small ML signal (about 25% of that of chloride sufficient) was observed while almost no ML signal was detected in high pH-treated samples. Thus, the chloride depletion inhibited the formation of the ML signal.

### Effect of chloride-depletion on Chl *a* fluorescence induction kinetics

Chlorophyll *a* fluorescence induction kinetics was measured in the four samples in order to probe the effects of chloride depletion on the donor and the acceptor sides of PS II (Fig. 2). Chloride-sufficient (control) PS II membranes show a characteristic induction curve, steps of which are designated O-J-I-P (shown in the inset). The OJIP curve represents the successive reduction of electron transport pool of PS II (shown on log scale to allow visualization of

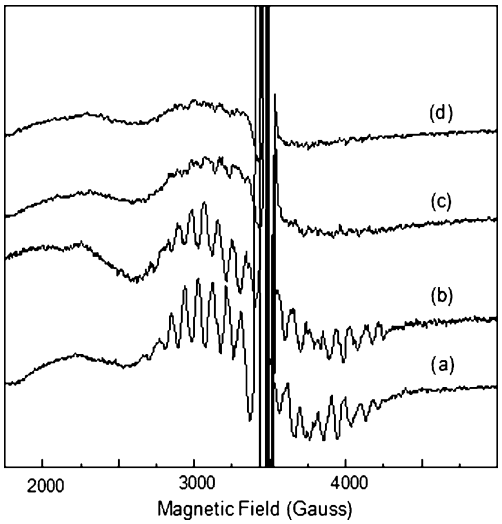


**Fig. 1** $S_2$ state-EPR spectra in the PS II membranes of chloride-sufficient **a**, chloride -deficient **b**, chloride depleted by sulphate **c**, and chloride-depleted by high pH **d**. The EPR spectra are presented as illuminated-minus-dark difference spectra. Experimental settings: temperature 6 K, microwave power 2 mW, microwave frequency 9.417, modulation amplitude 16 G. Chlorophyll concentration used was 4 mg $ml^{-1}$

the complete fluorescence transient) (Strasser et al. 2004). The O to J phase (ends at ~2 ms), the J to I phase (ends at ~30 ms) and I to P phase (ends at ~500 ms) were clearly observed (Fig. 1). The values of the minimal fluorescence (Fo), variable fluorescence (Fv) and maximal fluorescence (Fm) were calculated from the curves and various parameters like Fv/Fm, Fv/Fo, 1-Vj etc. (Table 1) were derived with the help of software Biolyzer HP 3. The ratio Fv/Fm which is a measure of the quantum efficiency yield of

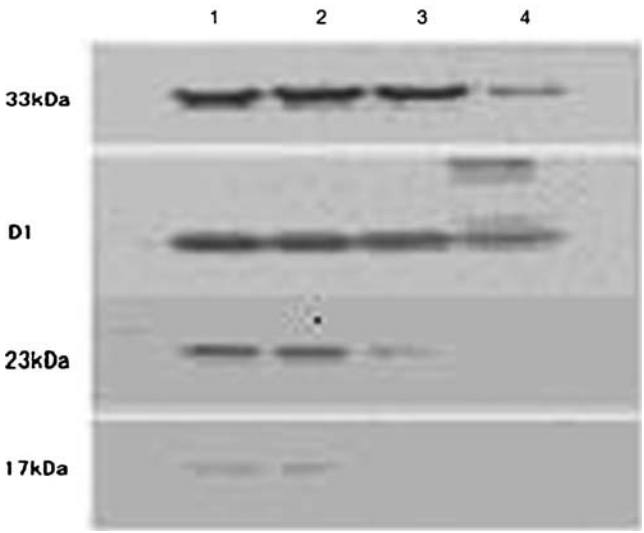


**Fig. 2** The OJIP Chl *a* fluorescence transient curve (log time scale) in the PS II membranes of chloride-sufficient **a**, chloride-deficient **b**, chloride-depleted by sulphate **c** and chloride depleted by high pH **d**. Inset shows a characteristic OJIP curve shown with normal PS II membranes

**Table 1** Values derived from fluorescence induction curves obtained with different chloride treated PS II membranes. The values of the minimal fluorescence (Fo), variable fluorescence (Fv) and maximal fluorescence (Fm) and relative variable fluorescence (Vj) were calculated from the curves (Fig. 2.) and various parameters like Fv/Fm ratio (a measure of the quantum efficiency yield of primary photochemistry of PS II) , Fv/Fo (a measure of the the efficiency of electron donation to PS II reaction center) and 1-Vj (a measure of changes at the acceptor side of PS II) etc. were calculated

| Sample (PS II membranes) | Fv/Fm | Fv/Fo | (1-Vj) |
|---|---|---|---|
| $Cl^-$ Sufficient | 0.676±0.01 | 2.087±0.01 | 0.615±0.01 |
| $Cl^-$ deficient | 0.482±0.01 | 0.93±0.01 | 0.528±0.01 |
| $Cl^-$ depletion by sulphate | 0.232±0.01 | 0.302±0.01 | 0.211±0.01 |
| $Cl^-$ depletion by high pH | 0.224±0.01 | 0.288±0.01 | 0.059±0.01 |

primary photochemistry of PS II, decreased in chloride-deficient, sulphate-treated and high pH -treated PS II membranes by 29%, 66% and 69% respectively. The decrease in Fm and fluorescence at J, I, P may be due to two reasons, first by inhibition of electron transport at the donor side of the PS II which results in the accumulation of $P_{680}^+$ (Govindjee 1995) and second due to a decrease in the

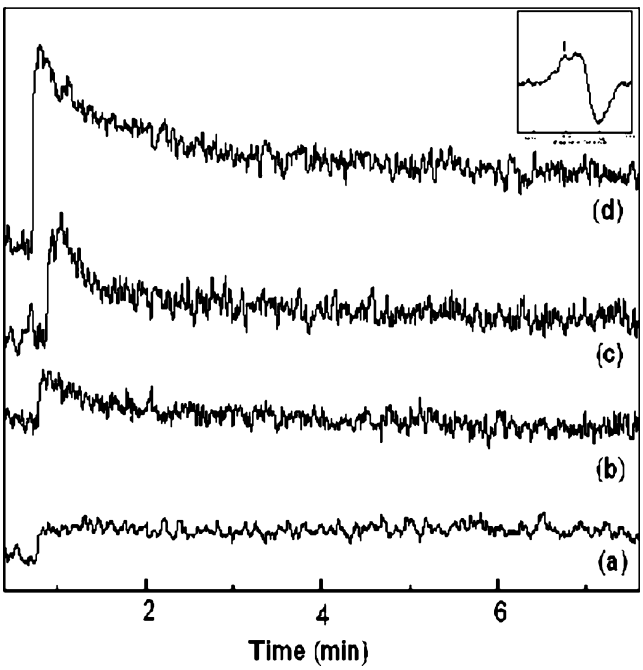


**Fig. 3** EPR traces of the decay kinetics of $Y_Z^{\bullet}$ at 253 K in the PS II membranes of chloride -sufficient **a** chloride-deficient **b** chloride-depleted by sulphate **c** and chloride- depleted by high pH **d**. Inset shows CW EPR difference spectrum (illuminated-minus-dark) of tyrosine recorded at 253 K. Arrow indicates the position of the magnetic field fixed for kinetic measurements. Experimental conditions: microwave frequency 9.31 GHz, microwave power 2 mW, modulation amplitude 5 G. The magnetic field was fixed at 3,414 Gauss for the kinetic experiments

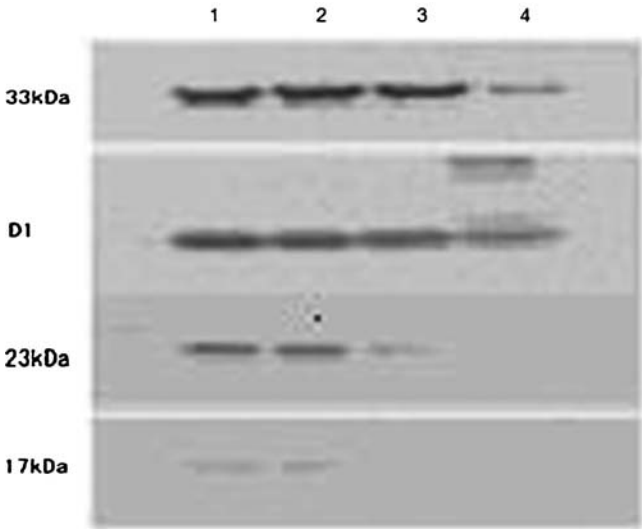


**Fig. 4** The effect of various chloride depletion treatments on the proteins in PS II as revealed by Western Blotting. The antibodies against PsbO, P and Q and the D1 protein were used. Different lanes designate: 1, chloride-sufficient PSII membranes; 2, chloride-deficient PSII membranes; 3, chloride-depleted PSII membranes obtained by sulphate treatment; 4, chloride-depleted PSII membranes obtained by high pH-treatment

pool size of $Q_A^-$. Fv/Fo ratio decreased in different chloride-depleted samples which reflects the structural alterations in PS II (Havaux and Lannoye 1985). The ratio Fv/Fo reflects the efficiency of electron donation to PS II reaction center and the rate of photosynthetic quantum conversion at PS II reaction center (Babani and Lichtenthaler 1996). To localize the effects of chloride depletion on the acceptor side of PS II the kinetics of relative variable fluorescence (Vj) was calculated. Vj is equivalent to (Fj-Fo/Fm-Fo). Decrease in the value of 1-Vj in chloride-depleted samples revealed a loss of $Q_A^-$ reoxidation capacity and inhibition of electron transport at the acceptor side of PS II (Congming and Vonshak 1999).

## Effect of chloride depletion on $Y_Z^\bullet$ decay kinetics

In order to investigate further the effects of chloride depletion on the donor side of PS II, Yz decay rates were measured at 253 K. In the presence of a functional OEC, the reduction of $Y_Z^\bullet$ at room temperature is normally too fast to allow observation of the species attributed to $Y_Z^\bullet$. In Cl- depleted PS II membranes, OEC was severely inhibited and the decay kinetics of $Y_Z^\bullet$ was slowed, which enabled the detection of $Y_Z^\bullet$ on illumination (Fig. 3). A continuous wave (CW) EPR spectrum of tyrosine radical was measured and the illuminated-minus-dark spectrum is shown in the Inset of Fig. 3. For tyrosine radical, the outer hyperfine line was used to distinguish it from other radicals. The arrow indicates the field position used for measuring the decay kinetics of $Y_Z^\bullet$. The light induced EPR spectrum of $Y_Z^\bullet$ revealed biphasic decay. As the decay kinetics of $Y_Z^\bullet$ is related to back reaction from $Q_A^-$, the decrease in the intensity of $Y_Z^\bullet$ in $Cl^-$ sufficient PS II membranes may be ascribed to a less inhibition at the acceptor side resulting in the fast back reaction between $Y_Z^\bullet$ and $Q_A^-$ (Dekker et al. 1984). The decay rates of $Y_Z^\bullet$ are calculated from the following equation:

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

where, $t_1$ is the time constant for the fast phase in the $Y_Z^\bullet$ decay and $t_2$ is that for the slow phase of the decay. Chloride-depletion affected not only the ratio of light-induced $Y_Z^\bullet$ but also the decay rate. In chloride depleted PS II membranes, the estimated $t_{1/2}$ values for the fast phase were about 27.4 s and for the slow phase was about 192 s, respectively, at 253 K. The $t_{1/2}$ values could not be calculated in control samples as the rate of decay was too fast to be measured. The increase may be ascribed to change in redox potential of $Y_Z$ and /or $Q_A$. Thus, there are two possibilities for the utilization of the electron stabilized in $Q_A$: forward transfer to $Q_B$ or recombination with $P_{680}^+$ in equilibrium with $Y_Z$. At the same time $Y_Z$ requires electron from Mn cluster. There are two concurrent ways to reduce $Y_Z^\bullet$: either by Mn cluster or by $Q_A^-$. $Y_Z^\bullet$ decay kinetics depends on the charge state of these components.

Our results favor the possibility that $Cl^-$ is acting also at the acceptor side resulting in faster electron transfer rates between $Q_A \rightarrow Q_B$, as evident from smaller intensity of the light induced $Y_Z^\bullet$ signal and faster decay rates. This was further confirmed by carrying out experiments in the

**Table 2** Percentage recovery of the activity of PS II in various chloride-treated thylakoid membranes upon reconstitution with chloride. For reconstitution, the samples were incubated for 30 min in 100 mM chloride and then different measurements were made. The experiments were done three times in duplicates

| | Chloride deficient | Chloride depleted (sulphate treated) | Chloride depleted (high pH treated) |
|---|---|---|---|
| Oxygen evolution | 92±1 % | 83±2 % | 65±2 % |
| $S_2$ state ML EPR signal | 90±2 % | 77±2 % | 58±3 % |
| Fv/Fm | 90±3 % | 75±4 % | 63±3 % |
| Yz decay | 90±2 % | 75±3 % | 55±4 % |
| 1-Vj | 90±3 % | 90±2 % | 90±3 % |

presence of DCMU. DCMU inhibits the electron transport between $Q_A$ and $Q_B$. No significant change in the decay pattern in the presence of DCMU (data not shown) was observed in our $Cl^-$-depleted samples, probably because $Q_A \rightarrow Q_B$ was already inhibited due to $Cl^-$ depletion.

Effects of chloride depletion on protein profile

To observe changes in the polypeptide pattern due to chloride depletion, SDS-PAGE (data not shown) and Western blotting measurements were performed using antibodies against D1, Psb O, Psb P and Psb Q (Fig. 4). The PsbQ (17 kDa) was totally lost in the chloride depletion both by sulphate or high pH treatment of the PSII membranes. However, the PsbP (23 kDa) was partially retained in sulphate treatment but was totally lost when high pH treatment was given. The PsbO (33 kDa) was partially lost in high pH treatment while no significant loss occurred in sulphate-treated samples. The D1 protein seemed to be lost in high pH treated samples with a clear formation of some aggregates as seen in the Western blot.

Reconstitution with chloride

In order to understand if chloride caused reversible or irreversible decline in the activity of PS II, experiments of reconstitution with 100 mM chloride were performed (Table 2). Oxygen evolution was restored largely in the chloride-deficient and sulphate-treated samples and partially in high pH-treated samples. OEC inactivation by depletion of chloride was found to be reversible, as evident from a faster decay of $Y_Z^{\bullet}$ on addition of chloride. The decay kinetics of $Y_Z^{\bullet}$ is related to back reaction from $Q_A^-$. The decrease in the intensity of $Y_Z^{\bullet}$ after addition of $Cl^-$ may be ascribed to a less inhibition at the acceptor side resulting in the fast back reaction between $Y_Z^{\bullet}$ and $Q_A^-$. Addition of chloride to the PS II membranes largely restores the ML signal. The ability to form ML signal was restored by 80% by the addition of 100 mM chloride to the sulphate-treated thylakoid membranes and by 60% by the addition of chloride to the high pH-treated samples. Thus the ML EPR signal seems to be correlated to the functional binding of chloride to PS II. The Fv/Fm ratio was also restored largely by the addition of chloride. The parameter relating to the acceptor side effects such as 1-Vj, one of the parameters of chlorophyll fluorescence, was restored almost completely (> 90%) in all the three samples.

## Conclusion

In conclusion, removal of only extrinsic chloride (as in chloride-deficient samples) affected the donor side of PS II. Activity of the OEC was affected in a totally reversible manner, and thus no permanent damage to OEC occurred in chloride-deficient samples. D1 and other polypeptides were also intact in this preparation. Damage caused by chloride depletion by sulphate treatment caused temporary damage to PS II complex mainly on the OEC. The acceptor side recovers fully on addition of chloride. This is supported by western blot analysis where no damage to D1 protein appears and thus the loss of activity could be recovered on addition of chloride in sulphate-treated PS II membranes. Damage caused by chloride depletion by high pH-treatment caused more damage to the PS II complex mainly on the OEC. The acceptor side recovered fully on addition of chloride. Damage to D1 protein occurred which was shown in the Western blot by the formation of D1 aggregates.

Thus chloride depletion leads to changes in both the donor and the acceptor side of PS II. However different methods of chloride depletion may affect PS II to different extents. Our observations suggest that chloride depletion by high-pH treatment causes harsher damage to PS II.

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