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In vitro analysis of the plastid terminal oxidase in photosynthetic electron transport

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ABSTRACT

The plastid terminal oxidase PTOX catalyzes the oxidation of plastoquinol (PQH_2) coupled with the reduction of oxygen to water. *In vivo* PTOX is attached to the thylakoid membrane. PTOX is important for plastid development and carotenoid biosynthesis, and its role in photosynthesis is controversially discussed. To analyze PTOX activity in photosynthetic electron transport recombinant purified PTOX fused to the maltose-binding protein was added to photosystem II-enriched membrane fragments. These membrane fragments contain the plastoquinone (PQ) pool as verified by thermoluminescence. Experimental evidence for PTOX oxidizing PQH_2 is demonstrated by following chlorophyll fluorescence induction. Addition of PTOX to photosystem II-enriched membrane fragments led to a slower rise, a lower level of the maximal fluorescence and an acceleration of the fluorescence decay. This effect was only observed at low light intensities indicating that PTOX cannot compete efficiently with the reduction of the PQ pool by photosystem II at higher light intensities. PTOX attached tightly to the membranes since it was only partly removable by membrane washings. Divalent cations enhanced the effect of PTOX on chlorophyll fluorescence compared to NaCl most likely because they increase connectivity between photosystem II centers and the size of the PQ pool. Using single turnover flashes, it was shown that the level of reactive oxygen species, generated by PTOX in a side reaction, increased when the spacing between subsequent double flashes was enlarged. This shows that PTOX generates reactive oxygen species under limited substrate availability.
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1. Introduction

The plastid terminal oxidase PTOX is encoded by higher plants, algae and some cyanobacteria. PTOX is a plastid-localized plastoquinol oxygen oxidoreductase that was discovered through the Arabidopsis *immutans* mutation which shows a variegated leaf phenotype [1,2]. PTOX was shown to be essential for carotenoid biosynthesis in plants [3,4]. However, PTOX is also involved in photosynthetic electron transport [5,6] and chlororespiration [7] and may act as a safety valve protecting plants against photo-oxidative stress. Especially under harsh environmental conditions like with alpine plants [8,9], plants exposed to extreme temperatures [10,11] or to high salinity [12], the PTOX protein level is increased indicating a function in stress acclimation. However, overexpression of PTOX in Arabidopsis did not attenuate the severity of photoinhibition [13] or, when overexpressed in tobacco, even increased the production of reactive oxygen species and exacerbated photoinhibition [14,15]. It has been shown recently that the PTOX activity is too low to compete efficiently with electron flux through linear electron transport under high light intensities [6] suggesting that the major role of PTOX is in the control of the stromal redox poise thereby modulating the partition between linear and cyclic electron flow.

Biochemical analysis of PTOX has been performed with membranes isolated from *Escherichia coli* expressing the Arabidopsis IMMUTANS gene [16] and with the isolated enzyme at liposomes [17]. Isolated recombinant PTOX from rice fused to the maltose-binding protein (MBP-OsPTOX) was highly active and catalyzed the complete reduction of oxygen to water over a wide range of decyl-plastoquinol substrate concentrations [17]. However, under substrate limitation PTOX generated reactive oxygen species (ROS) at pH 6.0 while ROS production was detected at pH 8.0 when the substrate concentration was high [17].

In the present study we investigated the role of PTOX in photosynthetic electron transport using an *in vitro* approach. Addition of purified MBP-OsPTOX (hereafter PTOX) to PSII-enriched membrane fragments resulted in an efficient electron transfer from water at the donor side of PSII to oxygen catalyzed by PTOX. To show PTOX activity, we followed chlorophyll fluorescence induction and reoxidation kinetics in the

Abbreviations: chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; decylPQ, decyl-plastoquinone; EPR, electron paramagnetic resonance; MBP, maltose binding protein; 4-POBN, 4-pyridyl-1-oxide-N-tert-butylnitrone; PTOX, plastid terminal oxidase; PSI, photosystem I; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors in PSII; ROS, reactive oxygen species; S_n, oxidation states of the $[CaMn_4]$ cluster (oxygen evolving complex) in PSII; TL, thermoluminescence

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presence of PTOX. Furthermore, we investigated whether PTOX attaches tightly to the membrane fragments. The substrate dependence of O_2 reduction and ROS generation catalyzed by PTOX was demonstrated by generating a defined number of quinol molecules at a given time. To achieve this, PSII-enriched membrane fragments were excited with a series of saturating double flashes spaced with dark intervals of 1 s or 5 s between the double flashes. Using these approaches we demonstrate that PTOX is capable in accepting electrons from PQH_2 provided by PSII in the light and that PTOX generates ROS only under limited substrate availability.

2. Material and methods

2.1. Material

PSII-enriched membrane fragments from market spinach were obtained from thylakoid membranes according to Berthold et al. [18] with modifications to keep the Q_B site intact as described in [19]. PSII-enriched membrane fragments were resuspended in 20 mM MES, pH 6.5, 5 mM NaCl and 0.3 M sucrose. If not mentioned especially, experiments were performed in a buffer containing 20 mM MES, pH 6.5, 5 mM $CaCl_2$ and 0.3 M sucrose.

MBP-OsPTOX representing PTOX from rice translationally fused with the maltose binding protein was expressed in *E. coli* and purified as described in [17]. The protein content was determined using the Bradford assay.

2.2. Activity assays

Measurements of O_2 evolution and consumption were performed in a Liquid-Phase Oxygen Electrode Chamber (Hansatech Instruments, Norfolk, England). Electron transport activity of the PSII-enriched membrane fragments was measured as O_2 evolution in the presence of 1 mM 2,6-dichloro-1,4-benzoquinone. PSI activity was measured as O_2 consumption in the presence of 10 μM DCMU, 5 mM ascorbate, 30 μM 2,6-dichlorophenol-indophenol, 500 μM methylviologen and 10 mM NH_4Cl as uncoupler.

Activity of recombinant PTOX (10 μg ml^{-1}) was determined as O_2 consumption with decylPQ (100 μM) as substrate in a coupled assay with DT diaphorase (10 μg ml^{-1}) [17]. The reaction was started by adding NADH (200 μM). At 20 °C, the activity was 3 ± 0.5 μmol O_2 mg $protein^{-1}$ min^{-1}.

2.3. Room temperature chlorophyll fluorescence

Room temperature chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (DUAL-PAM, Walz, Effeltrich, Germany). If not especially mentioned, the intensity of the actinic red light was 22 μmol quanta m^{-2} s^{-1}. The intensity of the measuring light was sufficiently low so that no increase in the fluorescence (F_0) was observed upon set-on of the measuring light. Prior to the measurement, the samples, containing 20 μg chl ml^{-1}, were dark-adapted for 3 min. 10 μM DCMU was added to block the electron transfer from Q_A to Q_B; 10 μM octyl gallate was added to inhibit PTOX.

2.4. Thermoluminescence

The presence of Q_B was demonstrated and the size of the plastoquinone (PQ) pool was determined by thermoluminescence. Thermoluminescence was measured with a home-built apparatus [20] on PSII-enriched membrane fragments (0.1 mg chl ml^{-1}) that were dark-adapted for 3 min. PSII was excited with single turnover flashes at 1 °C spaced with a 1 s dark interval. Samples were heated at a rate of 0.4 °C s^{-1} to 60 °C and the light emission was recorded. The data were analyzed according to [21].

2.5. Room-temperature EPR

Spin-trapping assays with 4-pyridyl-1-oxide-N-tert-butylnitrone (4-POBN) (Sigma-Aldrich) to detect the formation of hydroxyl radicals were carried out using PSII-enriched membrane fragments at a concentration of 40 μg chl ml^{-1} and recombinant PTOX at 5 μg ml^{-1}. In the presence of 50 mM 4-POBN, 4% ethanol and 50 μM Fe-EDTA, samples were illuminated with a series of 24 or 48 double flashes with a dark interval of 1 s or 5 s in between each double flash using a Xenon-single turnover flash lamp from Walz. The dark interval between the single turnover flashes of the double flash pair was 1 s. EPR spectra were recorded at room temperature in a standard quartz flat cell using an ESP-300 X-band spectrometer (Bruker, Rheinstetten, Germany). The following parameters were used: microwave frequency 9.73 GHz, modulation frequency 100 kHz, modulation amplitude: 1 G, microwave power: 6.3 milliwatt, receiver gain: 2×10^4, time constant: 40.96 ms, and number of scans: 6.

2.6. Statistics

Data represent means or representative curves from measurements repeated four to nine times on independent preparations of PTOX and PSII-enriched membrane fragments. SD values are shown in Figs. 3, 4, and 6 and Tables 1 and 2.

3. Results

To study the relationship between PTOX activity and photosynthetic electron transport through the observation of chlorophyll fluorescence, PSII-enriched membrane fragments were reconstituted with purified recombinant PTOX. Illumination of PSII-enriched membrane fragments leads to a rise in chlorophyll fluorescence which reflects the reduction of the electron acceptors Q_A, Q_B and PQ. In fluorescence induction curves the maximum level of fluorescence is reached when Q_A is in its reduced state. To show PTOX activity, PSII-enriched membrane fragments were used instead of thylakoid membranes to avoid a competition between the reduction of O_2 by photosystem I (PSI) and by PTOX. PSII-enriched membrane fragments that contained the PQ pool were obtained by using a lower detergent concentration than in the classical protocol for PSII-enriched membrane fragments according to Johnson et al. [19]. The PSI content of the used preparations was negligible as shown by activity measurements (Table 1). Thermoluminescence (TL) measurements were used to show the presence and size of the PQ pool by following the oscillation of the so-called B-band ($S_{2,3}Q_B^-$ recombination) in dependence on the number of single turnover flashes (Fig. 1). In dark-adapted PSII reaction centers excitation by a single turnover flash leads to the formation of $S_2Q_B^-$ (when S_1Q_B is present in the dark, with S_n being oxidation states of the Mn cluster of the oxygen evolving complex and Q_B being the secondary quinone acceptor in PSII (for explanations on TL see Suppl. Fig. 1). Recombination of this charge pair leads to the B-band with a temperature maximum between 30 °C and 40 °C. The area under the B-band was calculated and showed the typical period-four oscillation pattern as a function of the flash number [22]. The period four oscillation pattern reflects both, the oxidation states of the

Table 1
PSII and PSI activities of PSII-enriched membrane fragments in comparison to thylakoid membranes.
PSII activity was measured as oxygen evolution in the presence of 2,6-dichloro-1,4-benzoquinone. PSI activity was measured as oxygen consumption in the presence of methylviologen, ascorbate, 2,6-dichlorophenol-indophenol and DCMU. 20 μg chl ml^{-1} was used for activity measurements.

	Activity [μmol O_2 mg chl^{-1} h^{-1}]	
	PSI	PSII
Thylakoid membranes	410 ± 15	300 ± 50
PSII-enriched membrane fragments	6 ± 2	500 ± 100

Table 2
Attachment of PTOX to PSII-enriched membrane fragments.
PTOX (10 μg ml^{-1}) was added to 20 μg chl ml^{-1} and chlorophyll fluorescence induction curves were measured. Then samples were washed twice in 20 mM MES pH 6.5, 0.3 M sucrose containing either 10 mM NaCl, 5 mM $CaCl_2$ or 5 mM $CaCl_2$ and 0.2% Tween-20. In case of pH 7.5, Tris instead of MES was used. The PTOX activity of the first supernatant was determined as oxygen consumption measured in the presence of decylPQH_2. Fluorescence induction was measured on the resuspended sample after the last washing. The amount of PTOX attached to the membrane is calculated from the PTOX activities. The PTOX activity was 3 ± 0.5 μmol O_2 mg^{-1} min^{-1} before adding it to the PSII-enriched membrane fragments. The differences in the fluorescence rise were determined by the time $t_{50\%}$ measured with and without PTOX prior and after washing of the samples.

	PTOX activity in control [%]	PTOX activity in supernatant [% of control]	PTOX membrane attached [μg ml^{-1}]	Δ $t_{50\%}$ +/− PTOX prior to washing[s]	Δ $t_{50\%}$ +/− PTOX after washing [s]
pH 6.5 NaCl	100 ± 3	70 ± 4	3.0 ± 0.27	0.42 ± 0.19	0.51 ± 0.13
pH 6.5 $CaCl_2$	90 ± 9	80 ± 5	2.0 ± 0.1	1.56 ± 0.12	1.67 ± 0.13
pH 7.5 $CaCl_2$	95 ± 16	69 ± 9	3.1 ± 0.28	1.45 ± 0.18	1.60 ± 0.2
pH 6.5 Tween	101 ± 2	100 ± 6	0	0	0

water-splitting complex and the reduction state of Q_B (Suppl. Fig. 1). As shown in Fig. 1, the oscillation of the B-band is damped after the 8th flash, and it can be concluded that the PSII-enriched membrane fragments used here contain 4 PQ molecules. Alternatively, the size of the PQ pool can be calculated by comparing the areas above chlorophyll fluorescence induction curves measured in the absence and presence of the herbicide DCMU. DCMU binds to the Q_B binding site in PSII, blocks the electron transfer from Q_A to Q_B, and induces a fast rise of fluorescence (Fig. 2). The areas above the induction curves with and without DCMU differ by a factor of 6–8, showing that 3–4 PQ are present, which is in accordance with the TL data.

To investigate whether PTOX is able to oxidize PQH_2 generated in the light by PSII, chlorophyll fluorescence induction curves were measured in the presence and absence of PTOX. At the light intensity used, the fluorescence induction was significantly decelerated by the addition of PTOX and the maximum fluorescence level was lower (Fig. 2), showing oxidation of the plastoquinol pool. The presence of PTOX also resulted in a stronger lag-phase in the fluorescence induction curve upon the onset of actinic light. To demonstrate that PTOX indeed oxidizes PQH_2, fluorescence induction curves were measured in the presence of DCMU. Addition of DCMU leads to a faster fluorescence rise in the PSII-enriched membrane fragments and addition of PTOX did not change the kinetics (Fig. 2B), thus proving the absence of unspecific side effects. It is to be noted that DCMU led to 25% quenching of the maximum fluorescence level, independent of PTOX.

The difference in the time needed to reach 50% of the fluorescence ($\Delta t_{50\%}$) between PSII samples and those supplemented with PTOX depends on the salt that was present in the buffer. When the buffer contained 10 mM NaCl, the difference in the fluorescence rise $\Delta t_{50\%}$ was 0.42 ± 0.19 s (Table 2, Suppl. Fig. 2). When 5 mM $CaCl_2$ was used instead of 10 mM NaCl, a much larger difference ($\Delta t_{50\%}$ = 1.56 ± 0.12 s) was seen. Therefore, all further measurements were done in the presence of 5 mM $CaCl_2$.

PTOX lowered the extent of variable fluorescence only at low light intensities up to 50 μmol quanta m^{-2} s^{-1} (Fig. 3). No effect of PTOX was observed at higher intensities of actinic light. This indicates that PTOX activity cannot compete with the kinetics of light-driven input of electrons at light intensities higher than 50 μmol quanta m^{-2} s^{-1}. Next, the dependence of the PTOX concentration on the time needed

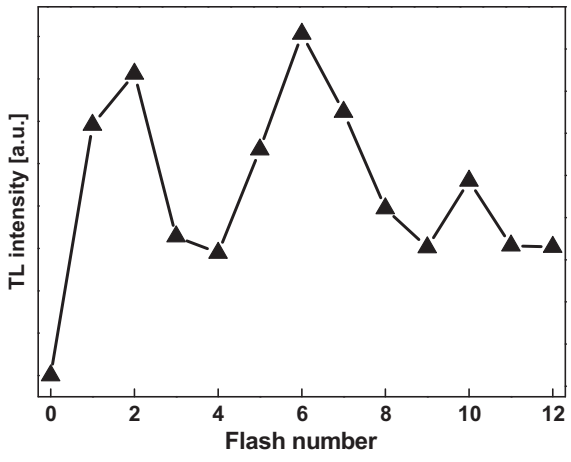

Fig. 1. Dependence of the intensity of the thermoluminescence B-band on the number of single turnover flashes in PSII-enriched membrane fragments. The integrated area of the B-band is shown. Before excitation the samples were dark-adapted for 3 min. Single turnover flashes were spaced with 1 s interval.

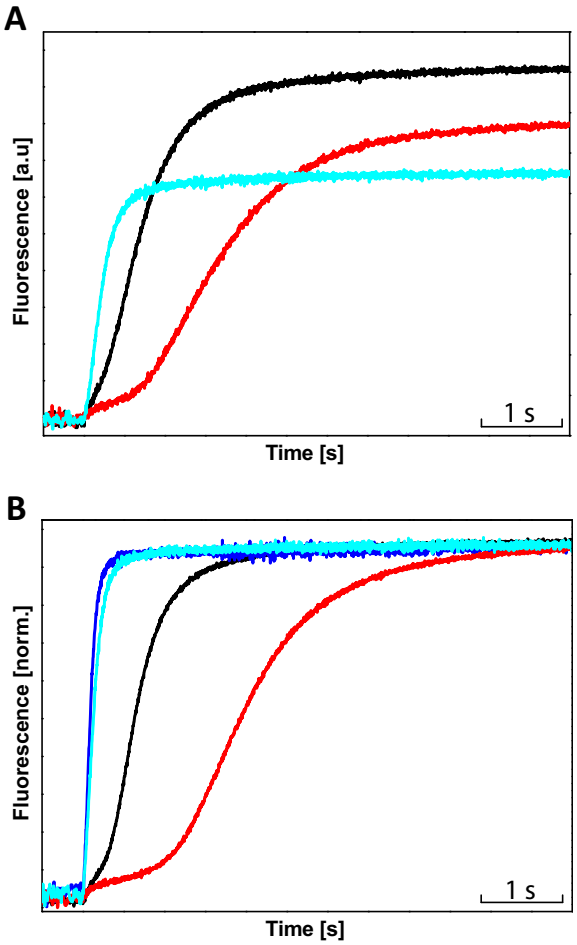

Fig. 2. Measured (A) and normalized (B) chlorophyll fluorescence induction curves of PSII-enriched membrane fragments in the presence (red) and absence (black) of PTOX. First, samples were measured in the absence of DCMU. Then DCMU was added to both samples after 5 min dark adaptation and the measurement was repeated (light blue, sample with PTOX; dark blue, sample in the absence of PTOX). The light intensity was 22 μmol quanta m^{-2} s^{-1}. Only the variable part of the fluorescence is shown. Measurements were performed with 20 μg chl ml^{-1}, 10 μg PTOX ml^{-1} in a buffer containing 5 mM $CaCl_2$, 0.3 M sucrose, 20 mM MES, and pH 6.5.

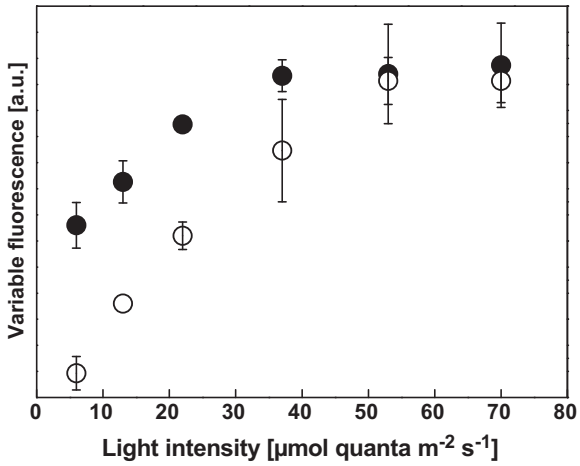

Fig. 3. Dependence of the variable fluorescence F_v of PSII-enriched membrane fragments on the light intensity in the absence (filled circles) and presence (open circles) of PTOX. For sample composition see Fig. 2.

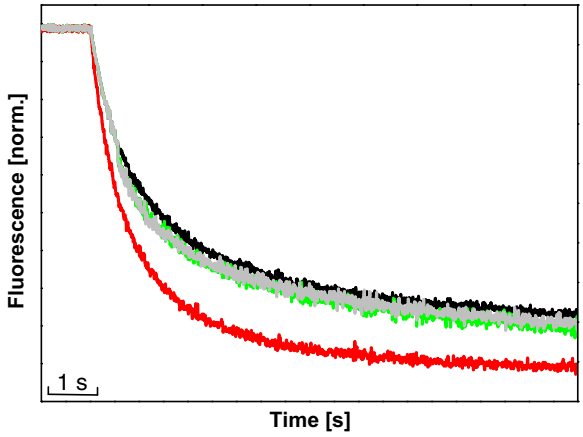

Fig. 5. Effect of PTOX on the decay of variable fluorescence. Normalized chlorophyll fluorescence decay curves of PSII-enriched membrane fragments after illumination in the absence (black) and presence (red) of PTOX and in the presence of 10 µM octylgallate with PTOX (green) and without PTOX (light gray). For sample composition and measuring conditions see Fig. 2.

to reach 50% of the fluorescence ($t_{50\%}$) was investigated (Fig. 4). Saturation was reached at about 2 µg ml^{-1} of PTOX when 20 µg chl ml^{-1} was used. This corresponds to 1 PTOX tetramer per 14 PSII, assuming that 250 chlorophyll molecules bind to 1 PSII and relying in the homotetrameric (80 kDa per monomer) association of the fusion protein described previously [17].

PTOX did not only slow down the fluorescence rise, but also accelerated the fluorescence decay 2–3 fold (Fig. 5). Addition of octylgallate, a specific inhibitor of PTOX [16], abolished this effect. The fluorescence decay was fitted with a 2-component exponential decay function. The fast component of the decay, t_1, reflects the $S_2Q_A^-$ recombination reaction which is known to take place with a $t_{1/2}$ of approximately 1 s [23], and this decay component was independent of PTOX. However, the half-time of the slower decay component, t_2, reflecting $S_{2,3}Q_B^-$ recombination, was accelerated by a factor of 2–3 in the presence of PTOX. In this assay, using 22 µmol quanta m^{-2} s^{-1}, 2 µg ml^{-1} of PTOX was saturating for 20 µg chl ml^{-1}, corresponding to 1 PTOX per 14 PSII (Fig. 6). This is the same PTOX to PSII ratio as calculated from fluorescence induction curves. As noted above in the case of fluorescence induction, PTOX had the strongest effect on the fluorescence decay in the presence of $CaCl_2$, followed by $MgCl_2$ (Fig. 7). When substituted with NaCl the decay kinetics were slower. $ZnCl_2$ had no effect. Divalent cations induce stacking of the thylakoid membranes [24,25] and also of PSII-enriched membrane fragments, as seen by the enhanced lag-phase in the fluorescence induction curve of a PSII sample when the buffer contained Ca^{2+} instead of Na^+ (Suppl. Fig. 2). In addition, Ca^{2+} or Mg^{2+} may assist in the association of PTOX to the PSII-containing membrane fragments. A structural role seems likely since PTOX activity measured potentiometrically in the absence of PSII-enriched membrane fragments using decyl-plastoquinol ($decylPQH_2$) as substrate showed that the enzyme activity is independent of different ions tested (see first column of Table 2 for Ca^{2+} and Na^+). However, 5 mM $ZnCl_2$ inhibited PTOX activity completely. PTOX activity was already strongly inhibited when 100 µM $ZnCl_2$ was added (data not shown). Similar inhibitory effects of Zn^{2+} have been reported previously for the alternative oxidase of mitochondria, AOX [26] and for other non-heme diiron proteins [27].

In analogy to AOX [28], PTOX is thought to form a four helix bundle which attaches to the surface of the thylakoid membrane. To test whether the recombinant PTOX binds tightly to the membrane, the PSII-enriched membrane fragments reconstituted with PTOX were washed thoroughly with the buffer. PTOX activity of the supernatant was measured by oxygen consumption using $decylPQH_2$ as substrate while PTOX activity in the pellet was assayed by chlorophyll fluorescence. When 0.2% Tween-20 was added prior to the washing, all PTOX activity was found in the supernatant and the effect of PTOX on the

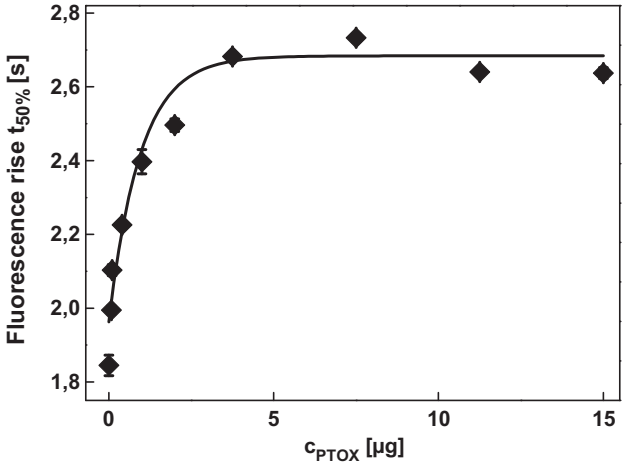

Fig. 4. Dependence of the fluorescence induction on the PTOX concentration. The time for 50% of fluorescence rise, $t_{50\%}$, was determined at the given PTOX concentrations. For measuring conditions see Fig. 2.

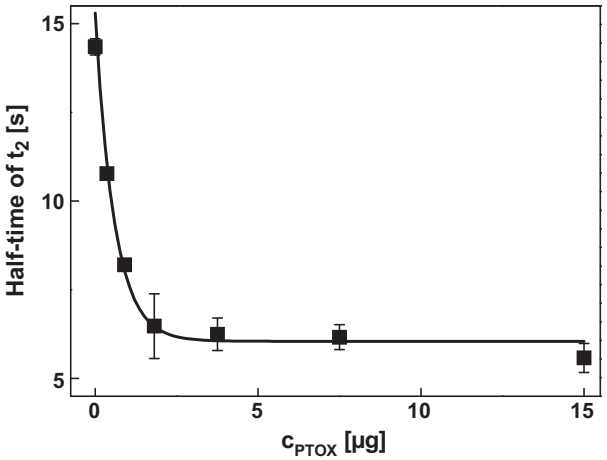

Fig. 6. Dependence of the half-time of the slower decay component t_2 of chlorophyll fluorescence decay on the PTOX concentration. Curves were fitted with two exponential decay curves (t_1 and t_2). For sample composition and measuring conditions see Fig. 2.

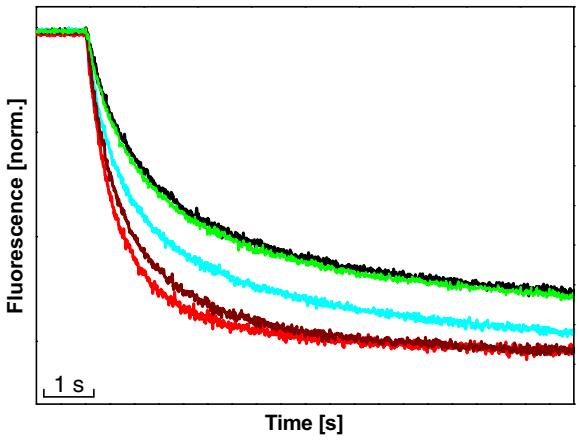

Fig. 7. Effect of monovalent and divalent cations on the decay of variable fluorescence. Measurements were performed with 20 μg chl ml^{-1}, 10 μg PTOX ml^{-1} in a buffer containing 5 mM divalent cations or 10 mM NaCl, 0.3 M sucrose, 20 mM MES, pH 6.5, 22 μmol quanta m^{-2} s^{-1} were used as actinic light. Black: PSII-enriched membrane fragments in 5 mM $CaCl_2$ and in the absence of PTOX; all other samples contained PTOX (10 μg ml^{-1}) and 5 mM $CaCl_2$ (red), 5 mM $MgCl_2$ (brown), 10 mM NaCl (turquoise), and 5 mM $ZnCl_2$ (green).

chlorophyll fluorescence was abolished (Table 2). In the absence of detergent the effect of PTOX on fluorescence induction and decay remained apparent after washing, meaning that PTOX tightly attached to the membrane. Nevertheless, the majority of PTOX activity was found in the supernatant (Table 2). Using 20 μg chl ml^{-1} and 10 μg ml^{-1} PTOX prior to washing, about 2 ± 0.1 μg ml^{-1} of recombinant PTOX was found in the pellet representing the PSII-enriched membrane fragments (Table 2). This corresponds again to 1 PTOX per 14 PSII and is in agreement with the results from the concentration dependency of PTOX effects on chlorophyll fluorescence (Figs. 4, 6). The amount of PTOX attached to the PSII-enriched membrane fragments was almost the same at pH 6.5 and pH 7.5 (Table 2). The measurements were performed at pH 6.5, thus at the optimum pH for the water-splitting activity and at pH 7.5 reflecting the stromal pH of photosynthetically active chloroplasts. PTOX activities in the supernatant (measured in the absence of PSII-enriched membrane fragments) were slightly lower in the presence of NaCl than in the presence of $CaCl_2$. This may indicate that in the presence of 10 mM NaCl a slightly higher amount of PTOX attaches to the membrane than in the presence of 5 mM $CaCl_2$ (Table 2). In both cases, the slowing down of the fluorescence rise was maintained or even slightly higher (Fig. 6) after the washing procedure compared to the induction curves measured prior to the washing.

We have shown previously that PTOX-containing *E. coli* membranes, tobacco plants expressing PTOX from Arabidopsis [14] and purified PTOX [17] all generate reactive oxygen species as a side product under certain conditions. With the PSII-enriched membrane fragments reconstituted with PTOX used here, it is possible to generate distinct substrate concentrations in a given time by illuminating the sample with single turnover flashes. One double flash, with a 1 s spacing between the flashes, generates one PQH_2. PTOX requires two PQH_2 to fully reduce oxygen to water. The 2nd flash pair generates the 2nd PQH_2. By varying the dark interval between the double flashes, the substrate is fed to the enzyme fast or with a delay between the generation of the next PQH_2 molecules. Measurements were done in the presence of the spin trap 4-POBN to detect the formation of reactive oxygen species. We used a total flash number of 24 or 48 double flashes which provides 24 or 48 molecules PQH_2 per PSII as substrate for PTOX. We did not increase the flash number further to avoid photo-oxidative damage of PSII. On the other hand, at least 24 double flashes were required to obtain a reasonable signal to noise ratio. 48 double flashes were chosen as the optimal condition. Fig. 8 shows the EPR spectra of the POBN-adduct obtained after illumination in samples containing PSII-enriched membrane fragments in the absence and the presence of PTOX. In this indirect spin-trapping assay, hydroxyl radicals are trapped which derive from $O_2^{\bullet-}/H_2O_2$ [29]. When the samples were illuminated with a 1 s dark interval between the double flashes, no significant difference in the signal size between PSII samples in the absence or presence of PTOX was observed. By enlarging the dark interval to 5 s, a significantly larger EPR signal was obtained in the presence of PTOX. This shows that PTOX produces more ROS as a side product when the number of plastoquinol molecules per dark interval is limiting so that the substrate is not provided fast enough. Increasing the dark interval even more (up to 20 s between the double flashes) did not further increase the difference in ROS formation between the two samples (data not shown). When the dark interval between the double flashes was 5 s, the ROS formation in the PSII sample in the absence of PTOX was also increased, but not to the same extent. This increase is caused by the quite slow conversion of H_2O_2 to hydroxyl radicals in the presence of 50 μM FeEDTA used in the spin trapping assay.

4. Discussion

Adding purified PTOX to PSII-enriched membrane fragments slowed down fluorescence induction curves (Fig. 2), reduced the maximum level of fluorescence (Fig. 3) and accelerated the decay of variable fluorescence in the dark (Fig. 5). This shows that PTOX can efficiently react with plastoquinol that had been reduced in the light by PSII. An effect of PTOX was also observed when similar experiments were performed with thylakoid membranes (data not shown) but the fluorescence rise was less affected than when PSII-enriched membrane fragments were used. An effect of PTOX on fluorescence induction was only seen at low light intensities (Fig. 3). Taking the PTOX activity measured as O_2 consumption in the presence of decylPQH_2 (3 ± 0.5 μmol O_2 mg protein^{-1} min^{-1}) and a molecular mass for the MBP-OsPTOX tetramer of 320 kDa, the catalytic turnover of PTOX is 16 O_2 per second, corresponding to 64 electrons per PTOX per second. When the light dependency of the fluorescence induction in the presence of DCMU was measured at 50 μmol quanta m^{-2} s^{-1}, the turnover of PSII was calculated to be 24 electrons per PSII per s. The comparison of the two turnover rates shows that the turnover of PTOX is 2.7 times higher than that of the reduction of the PQ pool by PSII. However, at 50 μmol quanta m^{-2} s^{-1} the effect of PTOX on fluorescence induction is already saturated (Fig. 3). This discrepancy may be explained by limited accessibility of PTOX to the PQ-pool in the stacked PSII-enriched membrane fragments. In addition, an effect of the MBP fusion on the attachment of PTOX to the membrane cannot be excluded.

In the presence of $CaCl_2$ PTOX shows the strongest effect on fluorescence induction and decay kinetics (Fig. 7 and Table 2). As shown in Table 2, the amount of PTOX attached to the membrane was slightly higher in the presence of NaCl than in the presence of $CaCl_2$. This may be due to the fact that membranes are less stacked than in the presence of Ca^{2+}. However, the differences found in the amount of PTOX attached to the membrane are small. This indicates that the effect of mono- and divalent ions on PTOX activity monitored as chlorophyll fluorescence depends rather on the degree of stacking of the membrane fragments than on differences in the attachment of PTOX to the membrane. Divalent cations like Mg^{2+} and Ca^{2+} induce stacking of thylakoid membranes and increase the connectivity between several PSII reaction centers [24,25,30]. Connectivity implies that several PSII centers share one large PQ pool [31] so that PTOX proteins attached to the membrane have higher accessibility to the substrate. In the case of low connectivity (when NaCl is present), the PQ molecules are shared between fewer reaction centers and the size of the PQ pools is smaller. Under this condition, PTOX is less efficient, although the number of PTOX attached per PSII is similar or even slightly higher when comparing PTOX attachment in the presence of NaCl and $CaCl_2$ (Table 2).

According to Figs. 4, 6 and Table 2, the ratio of PTOX per PSII can be estimated to be about 1:14; however, care needs to be taken when

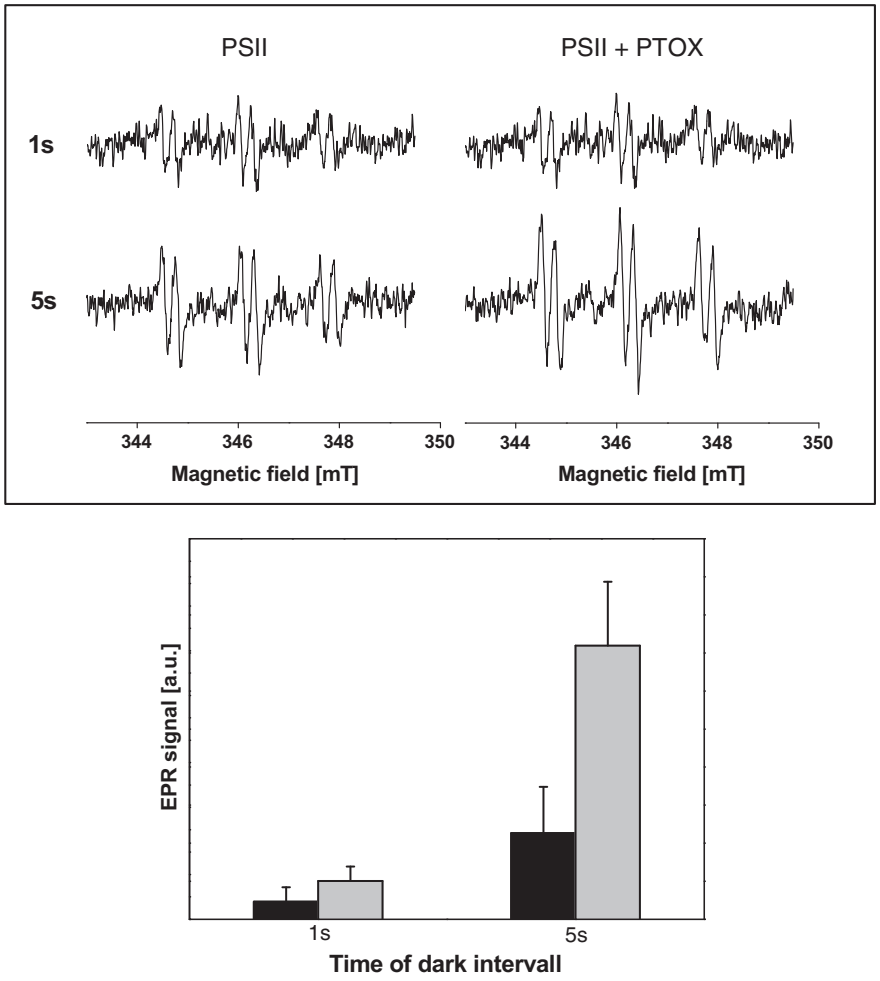

Fig. 8. Generation of reactive oxygen species by PTOX. Generation of hydroxyl radicals is shown by indirect spin trapping with 4-POBN/ethanol as trap. Samples in the presence and absence of PTOX were illuminated with a series of 48 saturating flash pairs of double turnover flashes. The spacing between the two flashes of one flash pair was set to 1 s; the spacing between the flash pairs was set to 1 s or 5 s. upper figure: typical EPR spectra; lower figure: difference in the signal sizes (mean ± SD, $n = 6$). Samples contained 40 μg chl ml^{-1} and 5 μg PTOX ml^{-1}.

drawing conclusions from the *in vitro* system used to the *in vivo* situation. According to the literature, the ratio between PTOX and PSII is much lower in leaves compared to the reconstituted system investigated here. Lennon et al. [32] reported one PTOX per 100 PSII reaction centers. However, the level of PTOX per PSII increases when plants acclimate to harsh environmental conditions. In sun leaves of the alpine plant *Ranunculus glacialis* the PTOX amount was found to be 3 times higher than in shade leaves [9]. In salt-treated *Thellungiella halophila*, the amount of PTOX was increased at least 4-fold [12].

The data obtained with the reconstitution system used here can contribute to the discussion on the potential of PTOX of acting as a safety valve under stress conditions. According to our data, even at the comparatively high PTOX/PSII ratio achieved, a function of PTOX as a direct safety valve to keep the photosynthetic electron transport chain more oxidized under stress conditions seems to be unlikely since the PTOX effect on fluorescence rise was already saturated at 50 μmol quanta m^{-2} s^{-1}. At high light intensities the reduction of the PQ pool by PSII was faster than its oxidation by PTOX. The relevance of the *in vitro* data shown here for the *in vivo* situation has, however, to be regarded with caution. One has to keep in mind that the accessibility of PTOX to plastoquinol *in vivo* may be facilitated in comparison to the situation here when stacked PSII-enriched membrane fragments are used. In addition, it cannot be excluded that the *in vitro* PTOX activity may be lower than *in vivo* due to the use of MBP-PTOX.

The physiological role of PTOX *in vivo* may rather lie in the fine tuning of the redox state of the PQ pool than in allowing a high throughput of electrons from PSII to oxygen via this enzyme. Such a fine tuning is highly important to keep the electron flow active and to adjust the optimal partition between linear and cyclic electron flow at a given light condition. In such sense PTOX may well help to protect a plant against photo-oxidative damage by setting the redox poise of the PQ pool under certain physiological conditions such as a sudden change in light intensity. Furthermore, PTOX plays an important physiological role in chlororespiration [33] and may be highly important under light-limiting conditions when the PQ pool is reduced by catabolic reactions.

The question has been raised previously whether PTOX generates ROS in a side reaction. Significant levels of ROS production have been found in tobacco plants overexpressing PTOX [14]. In these plants, increased photoinhibition compared to the wild-type was reported under high light conditions whereas a high level of PTOX protected against photoinhibition at low light intensities. Furthermore, it has been shown using PTOX with decylPQH_2 in liposomes as substrate that a small but significant amount of ROS was generated at pH 8.0 at high substrate concentrations and at pH 6.0 at low substrate concentrations [17]. Fig. 8 shows that, in the system using PSII-enriched membrane fragments with added PTOX, ROS were generated by PTOX when the availability of the substrate was limited by increasing the dark interval between double flashes. This experiment shows that PTOX generates ROS under certain circumstances but the physiological relevance of these data is questionable. PTOX is assumed to bind to the stromal side of the thylakoid membranes and will be exposed in

the light to slightly alkaline pH-values *in vivo* rather than to a slightly acidic pH-value as used here. The experiments shown in Fig. 8 had to be carried out at pH 6.5 because PSII loses its water-splitting activity when incubated at pH 8.0 [34].

In conclusion, the data presented here show that PTOX is active when bound to thylakoid membrane fragments that contain PSII and no PSI. PTOX attaches tightly to the membrane and can be a generator of oxygen radicals under substrate limitation. Further studies are needed to show whether PTOX is attached to the grana stacks *in vivo* or rather to the stroma lamellae as reported by Lennon et al. [32] and if so, by which protein–protein interaction such asymmetric distribution may be achieved. Furthermore, information is lacking on the regulation of PTOX activity. A yet unknown regulation mechanism might enhance PTOX activity under certain abiotic stress conditions and thereby permit the opening of a safety valve that prevents over-reduction of the photosynthetic electron transport chain at high light intensities. PTOX may also play an important role in retrograde signaling pathways since both, the redox state of the PQ pool and ROS act as signals. On one hand PTOX would help to set the redox poise of the PQ pool to a certain level by adjusting the PQ/PQH_2 ratio at a given condition. In addition, depending on the quinol concentration, PTOX may catalyze the generation of a small amount of ROS, thereby triggering a stress acclimation response of the plant.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.07.016.

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