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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.

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