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Functional and molecular characterization of plastid terminal oxidase from rice (*Oryza sativa*)

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ABSTRACT

The plastid terminal oxidase (PTOX) is a plastohydroquinone:oxygen oxidoreductase that shares structural similarities with alternative oxidases (AOX). Multiple roles have been attributed to PTOX, such as involvement in carotene desaturation, a safety valve function, participation in the processes of chlororespiration and setting the redox poise for cyclic electron transport. We have investigated a homogenously pure MBP fusion of PTOX. The protein forms a homo-tetrameric complex containing 2 Fe per monomer and is very specific for the plastoquinone head-group. The reaction kinetics were investigated in a soluble monophasic system using chemically reduced decyl-plastoquinone (DPQ) as the model substrate and, in addition, in a biphasic (liposomal) system in which DPQ was reduced with DT-diaphorase. While PTOX did not detectably produce reactive oxygen species in the monophasic system, their formation was observed by room temperature EPR in the biphasic system in a $[DPQH_2]$ and pH-dependent manner. This is probably the result of the higher concentration of DPQ achieved within the partial volume of the lipid bilayer and a higher Km observed with PTOX-membrane associates which is ≈47 mM compared to the monophasic system where a Km of ≈74 μM was determined. With liposomes and at the basic stromal pH of photosynthetically active chloroplasts, PTOX was antioxidant at low $[DPQH_2]$ gaining prooxidant properties with increasing quinol concentrations. It is concluded that in vivo, PTOX can act as a safety valve when the steady state $[PQH_2]$ is low while a certain amount of ROS is formed at high light intensities.

1. Introduction

The plastid terminal oxidase gene, initially identified through transposon tagging [1] and shown to map to the *immutans (im)* locus of *Arabidopsis thaliana* [2], codes for a plastid quinol:oxygen oxidoreductase, termed plastid terminal oxidase (PTOX). This catalytic function is indicated by (limited) sequence similarity to mitochondrial alternative oxidase (AOX). However, functional residues such as the four glutamates and two histidines, for instance, thought to coordinate two Fe ions, are highly conserved [3]. Their function in providing the structural basis for the active diiron carboxylate center has recently been confirmed by the structural elucidation of AOX from *Trypanosoma brucei* [4]. In addition, hydroquinone oxidation by PTOX was also shown in vivo [5] and in vitro [6].

The lack of PTOX caused by the *im* mutation leads to a variegated leaf phenotype i.e. with sectors showing either a bleached or wild-type appearance. White sectors accumulate phytoene; they are defective in phytoene desaturation catalyzed by phytoene desaturase (PDS) and these areas are therefore amenable to photobleaching [7]. This corroborates older data showing that PDS—directly or indirectly—requires quinones for activity ([8,9]. The white/green sectors are thought to arise during a crucial early phase in chloroplast development during which an optimal carotenoid complement is critically important. Only those cells with plastids successfully escaping this phase by eventually developing chloroplasts develop green sectors documenting that PTOX is then largely dispensable in carotene desaturation, the redox regulation of the plastoquinone pool being dominated by photosynthetic electron transport. PDS, requiring the midpoint potential of the PQ/PQH_2 redox pair for optimal function [10] is thus largely PTOX-independent in mature chloroplasts; conversely it is fully PTOX-dependent in non green plastids. Accordingly, tomato fruit defective in PTOX (*ghost)* have white fruit under high light conditions while mature leaves are hardly affected [11].

Abbreviations: DPQ, decyl-plastoquione; PTOX, plastid terminal oxidase; nOG, n-octyl β-D-glucopyranoside; DCPIP, 2,6-dichlorophenol-indophenol-Na; IPTG, isopropyl β-D-1-thiogalactopyranoside; BSA, bovine serum albumin; DoDm, n-dodecyl β-D-maltoside; DeDm, n-decyl β-D-maltoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; CMC, critical micelle concentration; GPC, gel permeation chromatography; SOD, superoxide dismutase

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What is the role of PTOX in chloroplasts, given that it is dispensable for carotenogenesis? The "safety valve" function, which is a protective function against over-reduced states under high light and other stress conditions has frequently been put forward. For instance, the alpine plant *Ranunculus glacialis* under light stress at increasing altitudes [12], the halophyte *Thellungiella halophila* under salt stress [13] and *Brassica fruticulosa* under temperature and light stress [14], all respond by increasing PTOX levels. The current interpretation is that PTOX acts as an alternative electron sink consuming excess photosynthetically generated electrons avoiding over-reduction of the quinone pool. Reduction of oxygen to water has been assumed thus preventing the formation of toxic ROS. However, this is not undisputed. PTOX has been reported to not protect from photoinhibition in overexpressing Arabidopsis plants [15]. Moreover, PTOX-overexpressing plants are not protected in high light; rather the opposite is true as witnessed by strongly increased superoxide and hydroxyl radical levels [16]. Similarly, overexpression of PTOX from *Chlamydomonas reinhardtti* achieved by chloroplast transformation of tobacco led to plants which were more sensitive to light than the wild type [17]. Moreover, PTOX activity measured non-invasively was shown under diverse conditions to be about two orders of magnitude lower than that of its competitor for hydroquinones, the linear electron transport, which is not compatible with a safety valve function [18].

Additional functions attributed to PTOX relate to its participation in the chlororespiratory pathway. Here, the reduction of the PQ pool by ferredoxin:quinone reductase (FQR), or non-photochemically by NADPH through the plastid-encoded NDH complex or an NADPH:plastoquinone oxidoreductase requires a terminal oxidase that is thought to be PTOX [19,5]. Moreover, based on functional measurements, PTOX is also thought to regulate the cyclic electron circuits around PSI by fine-tuning the redox state of electron carriers [20,18].

Thus, several vitally important PTOX functions have been indicated by the use of reverse genetics and by spectroscopic measurements: carotene desaturation, a safety valve function, involvement in chlororespiration and in cyclic electron transport around PSI. However, the necessary interpretations all suffer from the fact that there is hardly any knowledge on the intrinsic properties of PTOX. Investigations "close to the enzyme" have been presented by Josse et al. [6] using *Escherichia coli* expressed protein, however these experiments suffer from the fact that complex *E. coli* membrane preparations were used to which the protein is bound i.e. that PTOX was investigated in the presence of the respiratory redox chain resulting in a complex mix of a multitude of redox mediators.

We therefore set out to fill this research gap by investigating PTOX from *Oryza sativa* only using a minimum of components such as purified recombinant protein, hydroquinones in free form or embedded into liposomal membranes. The results obtained shed light on the oligomeric assembly of PTOX, its kinetic properties and identify conditions under which ROS can be produced.

2. Material and methods

2.1. Chemicals used

Phusion™ High-Fidelity DNA Polymerase was a product of Finnzymes. Amylose resin and restriction enzymes were from New England BioLabs (UK). n-Octyl β-D-glucopyranoside was purchased from AppliChem (Germany). Phenyl-p-benzoquinone, dimethoxy-5-methyl-1,4-benzoquinone, 2,5-dimethyl-benzoquinone, 2,6-dimethyl-benzoquinone, 3,5-di-tert-butyl-1,2-benzoquinone, 2,5-dichloro-benzoquinone, and 2,6-dichloro-benzoquinone were from Sigma, Fluka and Kodak. 2,3-dimethyl-benzoquinone was from SynChem OHG (Germany). Gel Filtration LMW and HMW Calibration Kits were purchased from GE Healthcare. The following quinones and other fine chemicals were purchased from Sigma-Aldrich: duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone), decyl-plastoquinone (2,3-methyl-5-decyl-1,4-benzoquinone), octyl-gallate (3,4,5-trihydroxybenzoic acid-n-octylester), DCPIP (2,6-dichlorphenol-indophenol-Na), p-benzoquinone, decyl-ubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), and vitamin K1 (2-methyl-3-phytyl-1,4-naphthoquinone).

2.2. Cloning and DNA constructs

To clone OsPTOX (Acc. AF085174.3) lacking a predicted 35-amino-acid transit peptide (ChloroP 1.1 software) the corresponding cDNA was synthesized by GenScript (Germany). Primers OsPTOX Fw1 5′-AGTCAT ATGGGTACCGTCGGCACCGTCGCC-3′ (*Kpn*I) and OsPTOX + Stop Rs1 5′-GCAAGCTTGGATCCTCACTCTTTACTCACAAGAG-3′ (*Bam*HI) were used for introducing restriction sites by PCR amplification using Phusion™ High-Fidelity DNA Polymerase. The purified PCR product was inserted into pBAD-TOPO vector (Invitrogen) by TA cloning and the resulting vector pBAD-OsPTOX + TGA verified by sequencing. The *Kpn*I/*Bam*HI fragment was inserted in-frame into a series of Gateway derived destination vectors as described [21,22]. The expression plasmids pHGW-OsPTOX, pHMGW-OsPTOX, pHGGW-OsPTOX, pHNGW-OsPTOX and pHXGW-OsPTOX encode the corresponding fusion proteins His6-OsPTOX; His6-MBP-OsPTOX (MBP: maltose-binding protein); His6-GST-OsPTOX (GST: glutathione S-transferase); His6-NusA-OsPTOX (NusA:N-utilizing substance A) and His6-TRX-OsPTOX (TRX: thioredoxin). The Gateway empty vector pHMGW which encodes only His6-MBP was used as control.

2.3. MBP-OsPTOX expression and purification

All plasmids were transformed into BL21(DE3) *E. coli* cells. 2 ml of overnight cultures of transformed cells was inoculated into 400 ml of 2*YT-medium, grown at 37 °C to an OD_{600} of 0.8 and induced with IPTG (0.2 mM). After induction overnight at 16 °C the cells were harvested and either used directly or frozen at −80 °C.

Purification was carried out on ice. Cells were resuspended in buffer A (25 mM sodium-phosphate buffer pH 7.6, $MgCl_2$ 2.5 mM, NaCl 300 mM, glycerol 15 vol.%) and disintegrated by three passages through a French Pressure Cell at 18,000 psi. After centrifugation at 28,000 *g* for 40 min the supernatant was solubilized for 30 min on ice by slowly adding 5 X CMC n-octyl β-D-glucopyranoside (nOG; 1 X CMC = 25 mM) and then applied to Amylose resin (BioLab). After washing thoroughly with buffer A containing 1 X CMC nOG; the elution was accomplished with buffer B (50 mM Tris–HCl pH 8.0, $MgCl_2$ 2.5 mM, glycerol, 10 vol.%) containing 1 X CMC nOG and 10 mM maltose.

Further purification of His6-MBP-PTOX was achieved by ion exchange chromatography using an ÄKTA-explorer FPLC (GE Healthcare) with MONO Q 5/50 GL column (GE Healthcare) equilibrated with buffer B containing 1 X CMC nOG and 180 mM NaCl. 500 μl of Amylose-purified PTOX was loaded with the same buffer and the column developed with a linear gradient using buffer B in the presence of 1 X CMC nOG and 500 mM NaCl. This was followed by a washing step with the same buffer containing 1 M NaCl.

The peak eluting at about 220 mM NaCl was collected and the purity was checked by SDS-PAGE using 10% polyacrylamide gels. Further purification and MW determination were achieved by gel permeation chromatography (GPC) using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer B containing 1 X CMC nOG and calibrated with the LMW and HMW Calibration Kit (GE Healthcare). Proteins resolved by SDS-PAGE were detected using Coomassie Brilliant Blue G250 (Sigma-Aldrich). Protein quantification was done using the Bradford reagent.

2.4. Enzyme assays and measurements

Protein-free liposomes containing the quinone acceptors were prepared with 10 mg/ml of soybean lecithin (Sigma-Aldrich) in buffer B, as described [23–25]. The quinone/lipid ratio was estimated with the

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