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# Insight into the oligomeric structure of PORA from *A. thaliana*

Michal Gabruk [a,*], Zuzanna Nowakowska [b], Bozena Skupien-Rabian [c,d], Sylwia Kędracka-Krok [c,d], Beata Mysliwa-Kurdziel [a,1], Jerzy Kruk [a,*,1]

[a] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
[b] Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
[c] Department of Physical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
[d] Department of Structural Biology, Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7a, 30-387 Kraków, Poland

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ABSTRACT

Light-dependent protochlorophyllide oxidoreductase (POR, E.C. 1.3.1.33) is a plant enzyme that directly needs light to conduct a biochemical reaction. In the present paper we confirmed that POR forms large oligomers in solution before binding of substrates. We carried out the research using different techniques: cross-linking, native gel electrophoresis and FRET measurements. Mass spectrometry analysis of the cross-link products provided the first structural data about the organisation of the oligomer of POR. The results indicated that the catalytic motifs of the adjacent subunits become close to each other upon binding of substrates. Moreover, we identified two mutations of POR that disturbed its oligomerisation properties: Δ85–88 and Δ240–270. Additionally, a complete loss of the catalytic activity was observed for the following mutations: Δ189–194, Δ240–270, Δ318–331 and Δ392–393.



Light-dependent protochlorophyllide oxidoreductase (POR, E.C. 1.3.1.33) is a plant enzyme catalysing the penultimate reaction in the chlorophyll biosynthetic pathway in Angiosperms [1–3], namely the conversion of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) using NADPH as an electron donor [4]. However, the presence of the substrates is not sufficient to trigger the reaction. It was recently demonstrated that the reconstituted ternary complex NADPH:POR:Pchlide is stable *in vitro* in darkness for hours [5]. The reaction is initiated by photons absorbed by the Pchlide molecule within the complex [5], that was earlier shown for etiolated seedlings [6]. Consequently, the double bond between C17 and C18 of the Pchlide molecule is reduced, forming Chlide and $NADP^+$. The photoactive complexes Pchlide:POR:NADPH are accumulated by plants in the early developmental stage in the etioplasts within a highly structured lipid lattice called a prolamellar body (PLB) [3,7]. The light-dependent activity of POR is its unique feature and plays an important regulatory role in plant development and physiology [3,8].

POR is a member of the short dehydrogenase/reductase family (SDR) [9]. One of the characteristic features of the enzymes of this family is a catalytic motif *YxxxK*, where the tyrosine residue plays the role of proton donor [10]. Many SDR enzymes operate as dimers or oligomers and it was supposed that POR might also form such complexes. However, decades of research were necessary to indirectly confirm this hypothesis. The circular dichroism spectra of isolated PLBs showed exciton interactions between the Pchlide molecules bound by the enzyme [11]. This indicated that after binding of substrates, POR occurs in the aggregated form. Cross-linking experiments with two different linkers performed on etioplast membranes isolated from wheat revealed that POR *in vivo* indeed forms large oligomers, too large to be separated by SDS-PAGE [12]. The complexes of POR aggregates were successfully separated using gel filtration of solubilized PLB [13]. However, none of these studies did answer the question about the molecular mechanisms of the oligomers formation, especially what is the reason that triggers oligomerisation of POR: binding of substrates, interaction with lipids or just solely the intrinsic property of the enzyme. In the model proposed by Schoefs and Franck [6], the substrates are factors that trigger dimerization of the enzyme. Measurements of the activation volume of POR within PLBs revealed that the enzyme may disaggregate from oligomeric form or from the lipid membranes after the reaction [14]. *In vitro* studies using reconstituted complexes of recombinant POR isoforms of barley, *i.e.* PORA and PORB, revealed formation of hetero-oligomers upon binding of zinc-derivatives of Pchlide but only in the presence of liposomes [15,16]. However, the interpretation of these results in the light of their physiological significance remains controversial due to the fact that the accumulation of Pchlide *b* was not confirmed *in vivo* [17,18]. Homology modelling of POR and non-denaturing blots showed that POR from barley can form a hexamer

*Abbreviations:* FP, fluorescent proteins (CFP, YFP); Fx, fluorescence intensities at x nm; IL, In the loop, *i.e.* between residue 247 and 248 of POR sequence; CFP/YFP, cyan/yellow fluorescent protein; POR, light-dependent protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; FRET, Förster Resonance Energy Transfer; bs3, bissulfosuccinimidyl suberate; ACN, acetonitrile; ABC, ammonium bicarbonate; TFA, trifluoroacetic acid.

* Corresponding authors.
*E-mail addresses:* michal.gabruk@uj.edu.pl (M. Gabruk), jerzy.kruk@uj.edu.pl (J. Kruk).

[1] BMK and JK equally contributed as senior authors.

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consisting of five PORA subunits and one PORB molecule, while isoforms from *Arabidopsis* form dimers only [19]. On the other hand, our recent study revealed that all three isoforms of POR from *Arabidopsis* (*i.e.* PORA, PORB and PORC – the isoform found so far only in *Arabidopsis*) can form a fully assembled complex with NADPH and Pchlide [20]. Moreover, these data indicated that POR might form oligomers.

Studies performed so far, do not indicate clearly whether POR is prone to form oligomers in solution without the presence of lipids. Moreover, the role of the substrates in the oligomerisation process is unknown, as well as the organisation of the oligomers. In the present study we tried to address these questions using cross-linking combined with a mass spectrometry analysis, site-directed mutagenesis and FRET measurements. Using these techniques we successfully showed that PORA from *Arabidopsis* forms large oligomers before the binding of the substrates and without the lipids. Moreover, we identified two mutations of the enzyme that altered its oligomerisation properties.

## 1. Materials and methods

### 1.1. Protein expression and purification

The expression of PORA and its muteins was performed as described in [20]. The expression conditions of the POR fused with FP were similar. The purification was performed as described previously [20] with the use of AmiconPro units (Millipore).

### 1.2. Site-directed mutagenesis

The construction of the expression vector of *Arabidopsis thaliana PORA* was described previously [20]. Deletions were introduced into the pET15b–AtPORA expression vector using PCR and Pfusion DNA polymerase under the conditions recommended by the manufacturer (Thermo Scientific). The reaction mixture was digested with *Dpn*I at 37 °C for 1 h and then it was used to transform *E. coli* DH5α competent cells. The transformants were selected on a lysogeny broth with agar supplemented with 100 mg/l of ampicillin. The plasmids were isolated using a plasmid purification kit (Bio Basic INC) and the mutations were verified by sequencing (Genomed). Detailed information about annealing temperatures and the type of PCR and the sequence of the primers are summarised in Supplementary Table 1.

### 1.3. Cross-linking protocol and mass spectrometry analysis

Purified PORA was transferred into a WEB buffer (50 mM phosphate pH 7.0, 300 mM NaCl and 7 mM 2-mercaptoethanol) using SpinTrap G-25 columns (GE Healthcare). The cross-linking reaction was performed in a mixture containing 11 μM PORA and bs3 cross-linker in a concentration range 0.12–0.73 mM for 30 min at room temperature in the absence or in the presence of the substrates (5 μM Pchlide, 0.2 mM NADPH). This linker selectively binds to primary amines, so in the case of proteins to lysine residues and the N-terminal amine group. The reaction was stopped by 50 mM Tris pH 7.5 for 15 min at room temperature. The products of the reaction were separated by 12% SDS-PAGE and visualized by Coomassie Brilliant Blue staining. The bands of the cross-linked product were cut out of the gel and destained using 25% acetonitrile (ACN)/25 mM ammonium bicarbonate (ABC) and 50% ACN/25 mM ABC alternately. Then, the proteins were reduced with 50 mM DTT/25 mM ABC for 45 min at 37 °C and subsequently alkylated with 10 mg/ml iodoacetamide /25 mM ABC for 1 h at room temperature in the dark. After that, the bands were washed twice with 50% ACN/25 mM ABC, dehydrated in 100% ACN and air-dried. The bands were re-swelled in 25 mM ABC containing sequencing grade modified trypsin (Promega). Digestion was performed overnight at 37 °C. The reaction was stopped with trifluoroacetic acid (TFA). Peptides were collected, vacuum dried and suspended in a loading buffer (2% ACN, 0.05% TFA).

Peptides were analysed with the use of a Q-Exactive mass spectrometer (Thermo Scientific) coupled with nano-HPLC (UltiMate 3000 RSLCnano System, Thermo Scientific). Peptides were loaded onto a C18 trap column (AcclaimPepMap100 C18, Thermo Scientific; ID 75 μm, length 20 mm, particle size 3 μm, pore size 100 Å) in 2% ACN/0.05% TFA at a flow rate of 5 μl/min and then separated on a C18 analytical column (AcclaimPepMapRLSC C18, Thermo Scientific; ID 75 μm, length 150 mm, particle size 2 μm, pore size 100 Å) using a 90 min gradient of ACN from 2% to 40% in the presence of 0.05% formic acid at a flow rate of 300 nl/min. Peptides were ionized in a Digital PicoView 550 ion source (New Objective). The Top 8 method was used for mass spectrometry measurement with full MS and MS/MS resolution of 70,000 and 35,000 respectively. One sample of protein without substrates was analysed in a slightly different way for optimization purposes: the peptides were separated using a 30 min gradient and measured with the use of the Top 12 method with full MS and MS/MS resolution of 70,000 and 35,000 respectively. The type of method did not influence the results.

The data was analysed with StavroX software 3.5.1 [21]. The limits for mass deviations between calculated and measured mass were set to 3 ppm for precursor ions and 10 ppm for fragment ions. The signal-to-noise ratio was set to 2.0. The oxidation of methionine residues was considered as a variable modification, while carbamidomethylation of cysteine residues was a fixed modification. The mass of the bs3 linker was set on 172.07356 Da. A missed cleavage site was allowed for lysine residue bound with the linker. All cross-links identified by StavroX with the positive score were manually validated.

### 1.4. Fusion of fluorescent proteins with POR

Plasmids containing fluorescent protein tags were constructed using the InFusion method according to the protocol (Clontech). *CFP* and *YFP* genes were inserted into the sequence coding the extra loop of PORA, *i.e.* between codons corresponding to the 247 and the 248 residues (IL). The inserts and the vector were amplified by PCR using appropriate primers.

In order to obtain CFP and YFP not fused with POR but containing the same N-terminal His-tag, both *CFP* and *YFP* genes were inserted at the 5′ end of the *POR* sequence using the same method. The inserts were amplified by PCR, while the vector was linearized with the *Nde*I enzyme. Then, the STOP codon was introduced at the 3′ end of the *FP* sequences with the PCR (described in the site-directed mutagenesis paragraph).

All vectors were verified for correct insertion by PCR and sequencing (Genomed). For the sequence of the primers see Supplementary Table 1.

### 1.5. FRET measurements

Equimolar amounts of IL-CFP-POR and IL-YFP-POR (0.77 ± 0.04 μM) were mixed in a WEB buffer by pipetting into a quartz cuvette placed in the thermostatic sample holder of Perkin Elmer LS-50b set to 23 °C. Continuous measurement of 50 fluorescence spectra was performed immediately after sample mixing. Spectra were collected between 470 and 560 nm for excitation at 430 nm and 250 nm/min scan rate with both slits set at 10 nm. Measurements were conducted without stirring because it caused protein denaturation. The fluorescence intensities at 480 nm and at 527 nm were read (F480 and F527, the emission maxima of CFP and YFP, respectively). Finally, the parameter F527/F480 was calculated which is the ratio of F527 to F480. For blank measurements, equimolar amounts of CFP and YFP were mixed (0.86 ± 0.04 μM) in a cuvette or the FPs were measured separately. In that case, CFP was first measured alone, then YFP alone, and finally the corresponding spectra of CFP and YFP were added together. This allowed us to estimate the relative contribution of the overlapping fluorescence bands originating from different FP. Finally, the F527/F480 ratio was calculated as a function of the time for the CFP/YFP mixture with no FRET.