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Light-Dependent Protochlorophyllide Oxidoreductase: Phylogeny, Regulation, and Catalytic Properties

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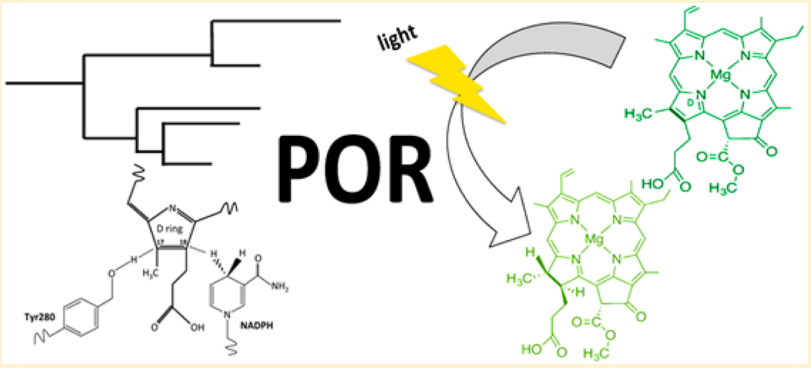

ABSTRACT: This Current Topic focuses on light-dependent protochlorophyllide oxidoreductase (POR, EC 1.3.1.33). POR catalyzes the penultimate reaction of chlorophyll biosynthesis, i.e., the light-triggered reduction of protochlorophyllide to chlorophyllide. In this reaction, the chlorin ring of the chlorophyll molecule is formed, which is crucial for photosynthesis. POR is one of very few enzymes that are driven by light; however, it is unique in the need for its substrate to absorb photons to induce the conformational changes in the enzyme, which are required for its catalytic activation. Moreover, the enzyme is also involved in the negative feedback of the chlorophyll biosynthesis pathway and controls chlorophyll content via its light-dependent activity. Even though it has been almost 70 years since the first isolation of active POR complexes, our knowledge of them has markedly advanced in recent years. In this review, we summarize the current state of knowledge of POR, including the phylogenetic roots of POR, the mechanisms of the regulation of *POR* genes expression, the regulation of POR activity, the import of POR into plastids, the role of POR in PLB formation, and the molecular mechanism of protochlorophyllide reduction by POR. To the best of our knowledge, no previous review has compiled such a broad set of recent findings about POR.

It has been almost 70 years since the first successful isolation of the enzymatically active complexes of protochlorophyllide oxidoreductase (hereafter, POR; in some papers, it is indicated as LPOR) from etiolated leaves.[1] Since that time, POR has remained one of the very few enzymes that are "driven" by light.[2,3] POR is unique in the need for its substrate to absorb photons to induce the conformational changes in the enzyme that are required for its catalytic activation.[4,5] This special property of the enzyme makes it an interesting and useful subject of study for ultrafast enzyme kinetics research.[6] Because of its light-dependent activity, POR plays a regulatory role in plant cells, participating in a "molecular switch" between scoto- and photomorphogenesis, although its main function is chlorophyll biosynthesis.[7,8] Chlorophyll *a* is the most abundant natural pigment on Earth.[9] Its biosynthetic pathway involves more than a dozen different enzymes controlled by a complex regulatory network of transcription factors and allosteric interactions (reviewed in refs 10−17).

In the case of all plants, chlorophyll biosynthesis takes place in chloroplasts and starts with a glutamate molecule[10,15] (Figure 1A). Subsequent condensation and redox reactions form 5-aminolevulinic acid (ALA), which is the first common biosynthesis intermediate of all tetrapyrroles in living organisms.[14] Next, two ALA molecules are asymmetrically condensed forming the first pyrrole compound, porphobilinogen. In subsequent reactions, further condensation takes place, leading to a more complex and larger molecule consisting of four pyrrole rings, protoporphyrin IX, which lies at the branch point of chlorophyll and heme synthesis.[14] The incorporation of iron into the tetrapyrrole ring leads to heme formation, while the incorporation of magnesium is the next step on the chlorophyll biosynthesis pathway.[14,15] This reaction is followed by the methylation of the Mg-protoporphyrin IX and the formation of isocyclic ring E of divinyl protochlorophyllide *a*.[18,19] A specific reductase can reduce one vinyl group leading to monovinyl protochlorophyllide *a*.[19,20] Both divinyl and monovinyl protochlorophyllide can be substrates for POR, although some minor differences can be observed in the spectral properties of POR:Pchlide complexes.[21] After pigment binding and photon absorption, POR reduces one of the

Received: June 24, 2015
Revised: July 30, 2015
Published: July 31, 2015

DOI: 10.1021/acs.biochem.5b00704
Biochemistry 2015, 54, 5255−5262

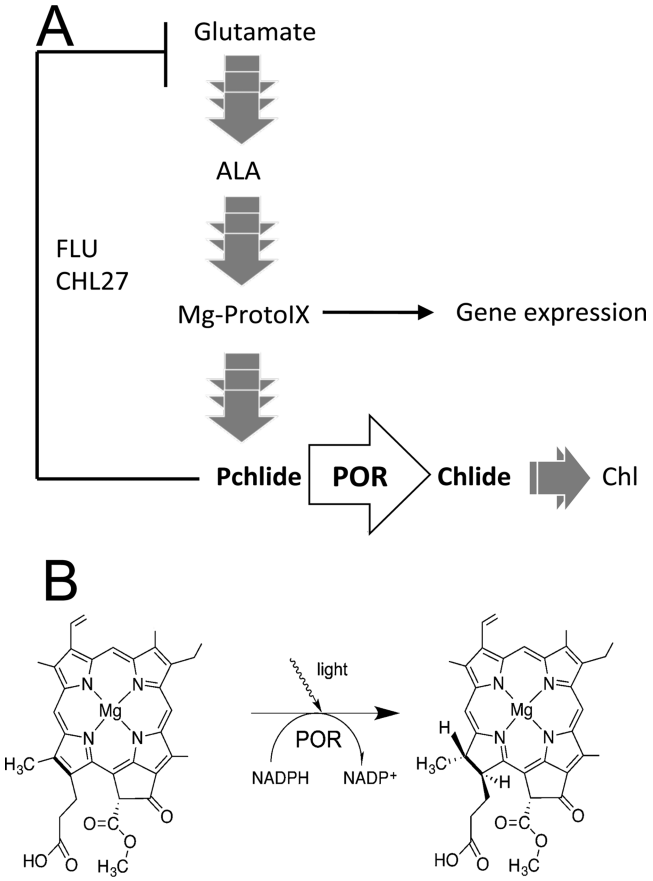

Figure 1. (A) Overall scheme of chlorophyll biosynthesis. The protochlorophyllide (Pchlide) reduction step is highlighted. The key intermediates and important regulatory mechanisms are marked. (B) POR-catalyzed reduction of Pchlide to chlorophyllide (Chlide).

double bonds of the fully unsaturated tetrapyrrole, porphyrin, using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (Figure 1B). In this reaction, the chlorin ring of the chlorophyll molecule is formed. Chlorins absorb red light better than porphyrins, which is crucial for the conversion of the energy from sunlight into chemical energy in photosynthesis. In the final step of the pathway, the product of the reaction, chlorophyllide (Chlide), is conjugated with phytyl pyrophosphate or geranylgeranyl pyrophosphate by chlorophyll synthase.[20,22]

PHYLOGENETIC ROOTS OF PCHLIDE REDUCTION

In nature, Pchlide reduction can be catalyzed by non-homologous but isofunctional enzymes. One of them is POR, and the other is the light-independent, dark-operative Pchlide oxidoreductase (hereafter, DPOR).[23]

DPOR is phylogenetically the oldest enzyme that catalyzes Pchlide reduction. It is chloroplast-encoded, a multisubunit complex consisting of three different polypeptides: two L-protein dimers and an NB-protein heterotetramer. DPOR resembles nitrogenases in sequence and structure, although there are some differences in the structure of iron−sulfur clusters.[24,25] Like nitrogenase activity, DPOR activity is sensitive to oxygen.[26] DPOR first arose in anoxygenic photosynthetic bacteria.[27] DPOR can be also found in non-angiosperms, green algae, and red algae, although often in the form of pseudogenes[12,28] and accompanying one or several copies of the *POR* gene.[29] DPOR genes are extraordinarily highly conserved in sequence, even though they seem to be "optional" in organisms having both light-dependent and -independent oxidoreductase.[28] The maintenance of both POR and DPOR enzymes in some lineages may be connected with the catalytic capacity of light-dependent oxidoreductase under harsh conditions.[29] It was shown that Pchlide to Chlide photoconversion was 3−7 times more efficient for red light than for blue light,[30] although there was some controversy about the excitation wavelength used in the experiments and about calculations that neglected scattering.[31] Nevertheless, POR may be inefficient in deep or turbid water, where only blue light is available because of scattering.[29]

In contrast to DPOR, POR is a nucleus-encoded single-polypeptide enzyme, showing a high degree of similarity to the short-chain dehydrogenase-reductase (SDR) family. Although POR and DPOR are not phylogenetically related, they share a common sequence motif of unknown function.[32] POR is not sensitive to oxygen but requires light for enzymatic activation, which is considered its "Achilles' heel" by some authors.[29] Phylogenetic analysis shows that POR first arose in cyanobacteria probably under strong evolutionary pressure to find an oxygen-insensitive variant of Pchlide reduction.[33] The gene was inherited by land plants from cyanobacterial ancestors,[34] and now POR is the only Pchlide reductase in angiosperms. Some organisms derived the *POR* gene by horizontal gene transfer (HGT): from prasinophytes to stramenopiles[29] or from cyanobacteria to the anoxygenic phototrophic α-proteobacterium *Dinoroseobacter shibae* DFL12.[34] Unlike genes encoding DPOR, several genes of POR isoforms may exist within one genome. Those extra copies of *POR* may be the result of HGT or of genome duplication. Three algae lineages underwent genome duplications, resulting in *POR* duplication: dinoflagellates, chlorarachniophytes, and stramenopiles.[29] In the case of land plants, the number of *POR* copies varies between organisms but the origin of multiple *POR* copies is unknown. In the case of *Arabidopsis thaliana*, which underwent three whole genome duplications,[35] three isoforms of POR (PORA, PORB, and PORC) may be the outcome of these events. So far, *A. thaliana* is the only identified organism with three isoforms of POR.

REGULATION OF THE EXPRESSION OF *POR* IN *A. THALIANA*

The precise regulation of enzymes involved in the chlorophyll biosynthetic pathway is crucial because Pchlide, like other chlorophyll intermediates, is highly phototoxic.[36] The mechanisms of *POR* genes regulation are best known for *A. thaliana*. All genes involved in tetrapyrrole biosynthesis in this plant can be clustered into four categories (c1−c4), in which genes are similarly controlled in an endogenous rhythmic manner but also by a developmental program.[13] *PORC* belongs to the c2 cluster with 15 other enzymes, while *PORA* and *PORB* are the only members of the c4 cluster.[13]

The genes of the c4 cluster, *PORA* and *PORB*, are expressed at the early stage of plant development during etiolation. Their expression is positively regulated by ethylene, a gaseous plant hormone, through ethylene insensitive 3 (EIN3) and EIN3-like 1 (EIL1).[36] The effect is so strong that the application of ethylene to an *A. thaliana* mutant overproducing Pchlide rescues its defect.[37] After illumination, the level of *PORA* expression dramatically decreases in *A. thaliana* seedlings, because light, acting through phytochrome A (PhyA), is a

DOI: 10.1021/acs.biochem.5b00704
Biochemistry 2015, 54, 5255−5262

strong negative regulator of this gene.[38] It was shown that in an *A. thaliana* mutant lacking CBL-interacting protein kinase 14 (CIPK14), the level of PORA was barely detectable and the expression of both PORB and PORC was downregulated.[39] These results indicate that CIPK14 is involved in the PhyA-mediated inhibition of seedling greening. The expression of PORB is still the subject of extensive studies.

In contrast to *PORA* and *PORB*, *PORC* has a low expression level in darkness, which is caused by a complicated network of transcription factors both dependent on and independent of phytochromes. The expression of the genes of the c2 cluster, including *PORC*, is suppressed by phytochrome interacting factor 3 (PIF3) associated with histone deacetylase 1 (HDA1).[40] Scarecrow-like proteins (SCLs) have a similar effect on *PORC* expression.[41] It was shown that in the dark gibberellic acid (GA) biosynthesis is not inhibited, resulting in a decrease in the level of DELLA proteins. A low concentration of DELLAs cannot block the activity of SCLs, so that active SCLs inhibit the expression of *PORC*. At the same time, SCLs promote the expression of miR171, which blocks the expression of SCLs. This negative feedback loop keeps the concentration of SCLs under control.[41] One of the positive regulators of *PORC* expression is the long hypocotyl 5 (HY5) transcription factor, but in darkness, it is blocked by the COP1/SPA1 complex (constitutive photomorphogenic 1/suppressor of phytochrome A 1).[42] In the dark, the low level of *PORC* expression is maintained by PIF1, which binds to the G-box DNA sequence element of the *PORC* promoter, but not to the E-box sequence present in the promoters of *PORA* and *PORB*.[43] After illumination, light strongly upregulates *PORC* expression in multiple ways. After PhyA activation and its translocation to the nucleus, it promotes PIF3 phosporylation and deactivation, leading to the acetylation of histone H4 that initiates gene expression.[40] At the same time, PhyB interacts with the COP1/SPA1 complex, releasing HY5, which upregulates *PORC* transcription.[42] Recently, it was shown that both phytochromes A and B can deactivate the COP1/SPA1 complex.[44] HY5 activity can also be modified by temperature cues leading to changes in *PORC* expression.[45] Moreover, light activates protein kinase CK2, which phosphorylates PIF1 promoting its degradation in the 26S proteasome.[46] PIF1 is responsible for weak *PORC* expression in the dark,[43] but it also inhibits the chlorophyll biosynthetic pathway by suppressing the expression of ALA dehydratase.[47] Furthermore, light exposure inhibits GA biosynthesis, allowing DELLA proteins to inhibit SCL activity, which stimulates *PORC* expression. At the same time, light promotes the expression of miR171, which inhibits SCL genes transcription, enhancing *PORC* expression.[41]

It is clear that the ethylene transduction mechanism regulates the expression of *PORA* and *PORB*, while COP1 regulates the expression of *PORC*; those two mechanisms, however, are not independent. It was shown that COP1 inhibits the expression of EIN3.[48] On the other hand, after the application of ethylene, EIN3 is responsible for the translocation of COP1 from the cytoplasm to the nucleus in the light, where COP1 blocks the activity of HY5.[49] In the light, in the absence of ethylene, COP1 localizes mainly in the cytoplasm, allowing HY5 to initiate *PORC* transcription.[42] Recently, it was shown that calcium-dependent calmodulin 7 regulates HY5 expression, which indicates the important role of calcium ions in photomorphogenesis.[50] The simplified scheme illustrating the influence of light and ethylene on the expression of *PORA*, *PORB* and *PORC* is presented in Figure 2.

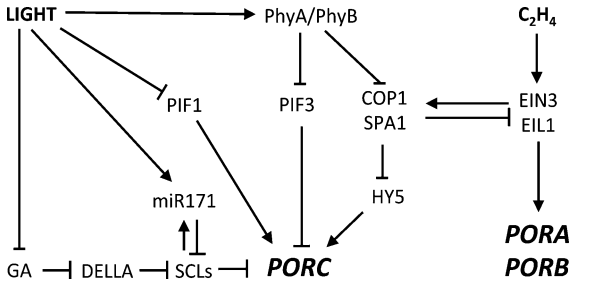

Figure 2. Overall, simplified scheme of the network regulating the expression of *PORA*, *PORB*, and *PORC* genes in *A. thaliana*. Only two main factors influencing *POR* expression are included: light and ethylene. Arrows indicate positive interactions, and blunt-ended lines represent negative interactions.

Some studies suggest that chlorophyll intermediates act as signal molecules influencing gene expression after translocation from the etioplast or chloroplast to the nucleus. Such an effect was shown for Mg-protoporphyrin IX (Mg-protoIX), which purportedly affected the expression of more than 300 genes, including *PORB*,[51] although these findings are not generally accepted.[52,53]

REGULATION OF POR ACTIVITY

There are no known allosteric regulators of POR, although recently it was shown that oligomerization of the enzyme increases the efficiency of the reaction,[54] suggesting that it is possible that the plant can modify the activity of the enzyme by precise control of its oligomerization level. Light intensity is another factor influencing the reaction rate, although the plant cannot fully control it. Substrate concentrations, which can be easily controlled, are regulated strictly.

Pchlide Availability. The maintenance of an optimal Pchlide:POR ratio is essential to prevent photooxidative stress; thus, the chlorophyll biosynthetic pathway is regulated precisely. Pchlide accumulation negatively regulates its own biosynthesis (Figure 1A). Both PORB and PORC can interact with the FLU and CHL27 proteins after Pchlide binding, initiating the inhibition of glutamyl-tRNA reductase (GluTR).[55] In this way, the whole pathway of Pchlide biosynthesis is blocked at its initial step. It is similar to the heme molecule, which is an allosteric inhibitor of GluTR.[56] These two negative feedback loops protect plants from overproducing highly phototoxic intermediates of chlorophyll. An extensive amount of Pchlide accumulates in an *A. thaliana* mutant lacking FLU protein in the darkness because the negative feedback inhibiting GluTR activity is affected.[57]

NADPH Availability. The main source of NADPH in chloroplasts is the light stage of photosynthesis at which $NADP^+$ is reduced by ferredoxin/$NADP^+$ reductase, although the enzyme responsible for NADPH/$NADP^+$ *de novo* synthesis is a special kinase (NADK). In *A. thaliana*, there are three isoforms of this enzyme. NADK1 and NADK2 prefer NAD^+ as a substrate, while NADK3 is NADH kinase. These enzymes differ in subcellular and tissue localization;[58] however, only NADK2 localizes in the plastid, more precisely in the stroma.[58] Although the catalytic motifs of NADKs are highly conserved, NADK2 possesses a large N-terminal extension responsible for interaction with calmodulins (CaM).[59] The calcium- and CaM-

DOI: 10.1021/acs.biochem.5b00704
Biochemistry 2015, 54, 5255−5262

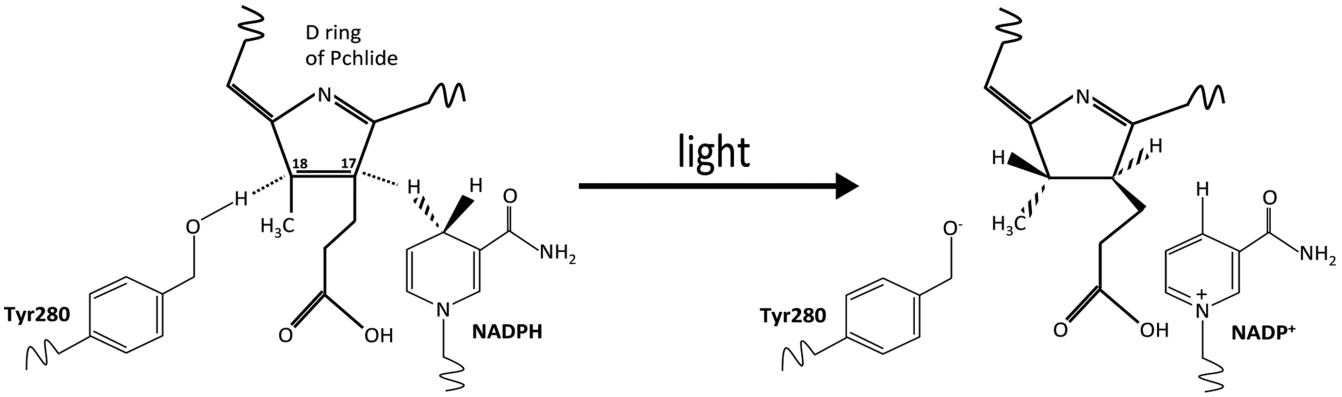

Figure 3. Mechanism of the light-dependent reduction of Pchlide by POR proposed by Wilks and Timko.[87]

dependent activity of NADK has not been verified using purified recombinant enzymes. However, such a dependency can be observed for NADKs purified from leaf extract.[59] A *NADK2* knockout mutant showed a reduced chlorophyll content caused by the inhibition of *PORB* and *PORC* expression.[60] At the same time, Mg-protoIX accumulated, which may trigger a repression of *POR* gene expression in the *nadk2* mutant.[53,60] This crosstalk between NADPH and the chlorophyll biosynthetic pathways shows the level of complexity of the regulatory machinery of the plant metabolism.

IMPORT OF POR INTO PLASTIDS

POR is a nucleus-encoded protein; thus, the import of the enzyme into plastids is guided by a transit peptide located on its N-terminal end. This phenomenon was most extensively studied a few years ago in *Hordeum vulgare* (barley), which has two POR isoforms, PORA and PORB. In the case of PORA, the five-amino acid motif, TTSPG, from the transit peptide was shown to be crucial for the proper import of the enzyme into etioplasts.[61] The lack of the TTSPG motif within the transit peptide of PORB indicated that PORB did not require Pchlide for plastid import.[61] The glycine residue from this motif was proposed to be responsible for Pchlide binding by the transit peptide of PORA.[61] It was hypothesized that Pchlide binding by the transit peptide of PORA is required for the translocation of the enzyme, which might be the first identified case of substrate-dependent import of protein into organelles. However, it remains unclear how pigment molecules leak from the etioplast into the cytoplasm. Moreover, some studies suggest that the TTSPG motif is not located within the transit peptide but is a part of mature PORA protein.[62]. Very recently, it was shown that OEP16-1[63] and cell growth defect factor 1[64] are crucial for the import of PORA in *A. thaliana*.

POR AND PROLAMELLAR BODIES

During etiolation, POR accumulates inside etioplasts in prolamellar bodies (PLBs). PLBs consist of both lipids (mainly DGDG and MGDG[7,65]) and proteins (mainly POR[66]) and have a semicrystalline structure of tetrahedrally branched tubular membranes forming large complexes ~1 μm in diameter.[67,68] After illumination, PLB starts to swell and the semicrystalline structure gradually disappears, while small vesicles form and detach from the surface of the whole body.[68] It is commonly accepted that PLB dispersion is induced by Pchlide photoreduction, although there is no clear molecular evidence of this conjecture. The activation volume measured for Pchlide reduction in isolated PLBs may be associated with the process of macrodomain rearrangement, protein−protein dissociation, or the dissociation of peripheric membrane protein.[69] There are some indications suggesting that macrodomain reorganization is responsible for changes in the fluorescence spectrum of PLBs after illumination.[69] Experiments with fluorescence quenchers located in the lipid membrane and performed on isolated PLBs indicate that POR dissociates from the membrane after photoreduction.[70] However, quenching of the protein fluorescence is not sufficient in itself to conclude that POR is associated with the membrane at all.

It was shown that the characteristic structure of PLBs can be restored in the *A. thaliana PORA* knockout mutant expressing *POR* originating from *Synechocystis* or *Gloeobacter violaceus*.[71] In these plants, the size and structure of PLBs were normal, although the ratio of photoactive to nonphotoactive Pchlide was decreased but similar to that of *PORA* knockout. These data indicate that the formation of PLBs is irrelevant to the assembly of photoactive Pchlide:POR complexes.[71] Surprisingly, even the main lipid component of PLBs is not essential for its proper structure. In the *A. thaliana* mutant *mgd1-1*, the inhibited activity of MGDG synthase leads to a decrease in the MGDG level of ~50% (in comparison to that of the WT plant).[72] The mutated plants had normal etioplasts, but they showed abnormal chloroplasts at later stages of development. Unexpectedly, the *mgd1-1* mutant had more photoactive Pchlide:POR complexes that WT plants, even though the expression level of *POR* was not affected.[73]

It was proposed that Pchlide:POR:NADPH complexes that accumulated in PLB have a photoprotective role.[8,74] The energy absorbed by one pigment molecule can be transferred to another because of the arrangement of the Pchlide molecules within the complex.[75] The process minimizes the chances of photooxidation of the pigment molecules, proteins, and lipids.

Etiolated leaves and the PLB itself contain carotenoids, mainly lutein.[76] Inhibition of the lutein biosynthetic pathway leads to an accumulation of *cis* intermediates and results in the complete loss of PLB.[77] In the case of the *ccr2* mutant of *A. thaliana*, accumulation of tetra-*cis*-lycopene disturbs the formation of PLB but not of Pchlide:POR complexes, although the level of photoactive Pchlide is decreased compared to that of WT plants.[77] It was also shown that low pH and heavy metals interrupt the structure of the PLB and Pchlide:POR complexes.[68,78−80]

DOI: 10.1021/acs.biochem.5b00704
Biochemistry 2015, 54, 5255−5262

SUBSTRATES BINDING AND REACTION MECHANISM

POR, in spite of its unique features, exhibits the characteristic sequence patterns of the SDR family,[33] namely, nucleotide binding and catalytic motifs. NADPH, but not NADH,[81] effectively binds to the enzyme thanks to the *G-rich* and *NAA motifs*.[82] It was shown that a mutation of Cys286 (numbering of *A. thaliana* PORA) dramatically alters the catalytic mechanism of the enzyme,[83] which indicates that the Pchlide binding site is close to this residue.

The precise molecular mechanism of Pchlide reduction is still the subject of extensive studies. The crystallization of DPOR with Pchlide made it possible to establish the principle of a light-independent reaction.[84] However, the lack of the crystal structure of POR hinders any explanation of its catalytic mechanism. In spite of this obstacle, a few years ago it seemed that the principle of the reaction could be assumed and the first intermediate, called I675*, was described.[6] On this basis, it was shown that in fact two photons are required for a single-molecule reduction,[4] and the picosecond scale dynamics of the reaction was determined.[85] However, very recently, the same group of authors showed that I675* is not an intermediate at all but that it reflects excited-state energy transfer between neighboring pigment molecules in Pchlide−Chlide dimers.[86]

In light of new findings, the mechanism of Pchlide reduction consists of at least three major steps: light absorption, subsequent hydride transfer from the *pro-S* face of the nicotinamide ring of NADPH to C17 of the Pchlide molecule, and finally proton transfer probably from Tyr280 to C18[86,87] (Figure 3). However, the role of Tyr280 remains unclear. It is considered the most important residue of the catalytic motif,[33,82] despite the fact that POR variants with mutated Tyr280 remain weakly enzymatically active.[88] It was suggested that quantum tunnelling may play an important role in the physics of the reaction[89] and that light absorption promotes intramolecular charge transfer along the C17−C18 bond, which allows subsequent events.[86]

An important factor influencing reaction efficiency is enzyme oligomerization.[54] *In vivo*, POR forms oligomers with a characteristic fluorescence emission of 655 nm (hereafter F655) (measured at 77K).[90] Recently, all three isoforms of POR from *A. thaliana* were shown to form photoactive oligomeric complexes F655 in a lipid-free buffer with Pchlide and NADPH only.[54] Previously, such complexes were reconstructed using a mixture of PORA and PORB from *H. vulgare*, a mixture of zinc derivatives of two Pchlide forms (Pchlide *a* and Pchlide *b*) in the presence of liposomes.[91] Those results formed the basis of the hypothesis of Pchlide:POR light-harvesting complexes summarized in ref 92. However, this idea is rather controversial.[93] Some studies indicate that the mechanism of Pchlide reduction differs in *A. thaliana* and *H. vulgare* because of different arrangements of cysteine residues.[94]

SUMMARY AND PERSPECTIVES

POR has been extensively studied for decades because of its importance for plant physiology and unique features: the mechanism of the reaction, the structure of PLB and the probable role of POR in its assembly, and the substrate-dependent import of the enzyme into plastids.

We already know a lot about the Pchlide reduction mechanism, although there are still many unanswered questions about the influence of the pigment binding pocket on the reaction and the role of enzyme oligomerization. Moreover, the interaction of POR with the lipid membrane is still uncertain. Questions about the effect of POR on PLB formation and about the role of the PLB itself remain open. It is important to verify the hypothesis about Pchlide-dependent import of protein into plastids and to confirm binding of Pchlide to the transit peptide of the enzyme. POR crystallization will certainly shed new light on the structure of the enzyme, providing new information about its function. We are hopeful that such data will allow the design of specific inhibitors of POR, potential new herbicides and tools in plant physiology studies.

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Funding

This work was financially supported by Grant 2013/09/N/NZ1/00200 from the National Science Centre of Poland (NCN). The Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Pchlide, protochlorophyllide; HGT, horizontal gene transfer; Chlide, chlorophyllide; GluTR, glutamyl-tRNA reductase; EIN3, ethylene insensitive 3; EIL1, EIN3-like 1; PhyA, phytochrome A; CIPK14, CBL-interacting protein kinase 14; PIF3, phytochrome interacting factor 3; SCLs, scarecrow-like proteins; COP1, constitutive photomorphogenic 1; SPA1, suppressor of phytochrome A 1; GA, gibberellic acid; HY5, long hypocotyl 5; CaM, calmodulins; NADK, NADPH/NADP$^+$ kinase; Mg-protoIX, Mg-protoporphyrin IX; PLBs, prolamellar bodies; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

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