Dual role of the active site ‘lid’ regions of protochlorophyllide oxidoreductase in photocatalysis and plant development

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Protochlorophyllide oxidoreductase (POR) catalyses reduction of protochlorophyllide (Pchlide) to chlorophyllide, a light-dependent reaction of chlorophyll biosynthesis. POR is also important in plant development as it is the main constituent of prolamellar bodies in etioplast membranes. Prolamellar bodies are highly organised, paracrystalline structures comprising aggregated oligomeric structures of POR–Pchlide–NADPH complexes. How these oligomeric structures are formed and the role of Pchlide in oligomerisation remains unclear. POR crystal structures highlight two peptide regions that form a ‘lid’ to the active site, and undergo conformational change on binding Pchlide. Here, we show that Pchlide binding triggers formation of large oligomers of POR using size exclusion chromatography. A POR ‘octamer’ has been isolated and its structure investigated by cryo-electron microscopy at 7.7 Å resolution. This structure shows that oligomer formation is most likely driven by the interaction of amino acid residues in the highly conserved lid regions. Computational modelling indicates that Pchlide binding stabilises exposure of hydrophobic surfaces formed by the lid regions, which supports POR dimerisation and ultimately oligomer formation. Studies with variant PORs demonstrate that lid residues are involved in substrate binding and photocatalysis. These highly conserved lid regions therefore have a dual function. The lid residues position Pchlide optimally to enable photocatalysis. Following Pchlide binding, they also enable POR oligomerisation – a process that is reversed through subsequent photocatalysis in the early stages of chloroplast development.

Introduction

Photosynthesis harnesses sunlight and converts it to chemical energy that drives most living processes on Earth [1]. Chlorophylls are the main functional pigments used in photosynthetic pathways [1,2]. Protochlorophyllide oxidoreductase (POR) catalyses the reduction of protochlorophyllide (Pchlide) to
chlorophyllide (Chlide), which is the penultimate reaction of chlorophyll biosynthesis [3,4]. Photosynthetic organisms have developed two different strategies for Pchlide reduction: one light-dependent and the other light-independent [5–8]. In photosynthetic bacteria and cyanobacteria, a light-independent nitrogenase-like protochlorophyllide oxidoreductase (DPOR) catalyses Pchlide reduction in an ATP-dependent manner [7,9,10]. Light-dependent POR is found in cyanobacteria, algae and plants, and is one of only three natural photoenzymes [11]. POR regulates plant development, in particular the formation of prolamellar bodies (PLBs) in plant etioplasts [12]. Conversion of these PLBs into mature chloroplasts is also triggered by light. PLBs contain both lipids and proteins, and 95% of the total protein content is comprised of POR [13]. The paracrystalline nature of PLBs forms through oligomerisation of the POR–Pchlide–NADPH ternary complex [13,14]. Overexpression of light-dependent POR leads to formation of PLB-like ultrastructures in etiolated cyanobacterial cells [15]. In plants, PLBs degrade following illumination and they transform directly to flat slats. This is the natural pathway by which etioplasts develop into mature chloroplasts [14]. Degradation of paracrystalline PLBs has been attributed to the photoreduction of Pchlide, which releases the Chlide product from the POR ternary complex [16]. Despite its importance to plant morphological development, how Pchlide binding and photocatalysis affects the oligomerisation/dissociation at the molecular level is currently unknown.

Protochlorophyllide oxidoreductase transfers a hydride equivalent – in a stepwise electron-H atom transfer reaction – from NADPH to the C17 atom of Pchlide in a step that is driven by light [17]. This is followed by proton transfer to the C18 position of Pchlide [18–22]. The stepwise ‘hydride’ transfer is facilitated by interactions of the excited-state Pchlide with active site residues (Fig. 1A) [18]. Although several residues important to photocatalysis are known [23–25], a structural basis for POR photocatalysis has emerged only recently. Crystal structures of cyanobacterial POR as the apo-enzyme and a NADPH-bound complex are known [26,27]. A crystal structure of the ternary complex is not yet available, which has been attributed to the oligomerisation of POR when Pchlide is bound. Oligomerisation has also been observed by mass spectrometry and small-angle X-ray scattering [28,29].

A structural model of the POR–Pchlide–NADPH ternary complex has been reported and validated experimentally [26]. This model has highlighted the potential importance of hydrogen-bonding networks involving active site residues and Pchlide. It has also provided some structural insights into POR photocatalysis. Two active site regions distal to the Pchlide C17–C18 atoms have been identified. These incorporate a peptide loop and helix that undergo major conformational change on binding of Pchlide. These so-called ‘lid’ regions (named here ‘lid1’ and ‘lid2’) line the upper regions of – and form a predominantly hydrophobic opening to – the enzyme active site (Fig. 1B). These conformational changes could limit the rate of photocatalysis by POR [30]. They might also be responsible for nucleating POR oligomer formation [26]. Some have argued that oligomerisation of POR ternary complexes could increase catalytic efficiency because neighbouring Pchlide molecules can interact [29,31]. Following laser excitation, excited-state energy transfer occurs between neighbouring Pchlide molecules in POR ternary complexes, which suggests a close association of the pigment molecules [32].

Here, we have investigated POR oligomer formation and photocatalysis to gain molecular level understanding of the role of the lid1 and lid2 regions. We have combined biochemical and biophysical approaches to discover the function of the lid regions in photocatalysis and oligomerisation, including size exclusion chromatography (SEC), cryo-electron microscopy (cryo-EM), cryo-trapping and time-resolved spectroscopy, and computational analyses with wild-type and POR variants. Our work highlights dual roles for these regions, which has implications for both photocatalysis and early stages of plant development.

**Results**

**Substrate binding and POR oligomer formation**

We used SEC to investigate the effects of Pchlide binding in POR oligomer formation. Both apo–POR and the POR–NADPH binary complexes elute as monomers at approximately 17.3 mL in SEC [26]. In the presence of Pchlide, however, oligomers of the POR ternary complex are formed. This is evidenced by the appearance of new peaks at smaller elution volumes (between 8 and 15 mL), reflecting a number of different oligomer forms, ranging in size from ~ 200 kDa to > 1 MDa (Fig. 2; Fig. S1a–c). By using multiple absorbance detection wavelengths in the SEC experiments, POR-bound pigment was found to elute at approximately 8 and 13 mL (Fig. 2D), consistent with large oligomeric forms of the protein. Oligomers formed either in the presence or absence of ambient light, and also in the presence of the reaction product, Chlide (Fig. 2A,B and Fig. S1d). Negative staining
Transmission electron microscopy (TEM) images of samples prior to SEC purification also contained a mixture of very different oligomeric states, ranging from long tube-like complexes to smaller particles (Fig. 2E). TEM images of samples after SEC purification showed it was possible to produce a more homogeneous sample but still indicated a mixture of oligomers of variable size (Fig. 2F). Oligomers are also formed on binding the substrate analogue mesoporphyrin IX dihydrochloride (MPCl) (Fig. 2C), suggesting that the binding of porphyrin analogues, and not Pchlide only, is sufficient to drive oligomer formation. Long tube-like oligomers were observed in TEM images of the POR–MPCl–NADPH ternary complex. These are distinct from TEM images of the globular oligomers formed with POR–Pchlide–NADPH (following light illumination) or POR–Chlide–NADPH complexes. Combined, these data indicate that binding of Pchlide, Chlide and analogue binding triggers POR oligomerisation, and that a range of different aggregation states are formed rather than a unique multimeric structure.

To understand the molecular basis of POR oligomerisation, we isolated one of the oligomeric states and analysed its structure by cryo-electron microscopy (cryo-EM). High-resolution purification by SEC–multi-angle light scattering (MALS) analysis was used to improve the homogeneity of the sample, resulting in cryo-EM grids that were prepared with a partially turned-over POR–Pchlide–NADPH ternary complex (Fig. S2). Ternary complex samples were imaged by cryo-TEM on a Titan Krios cryo-electron microscope and 9882 movies were collected and processed in RELION [33]. Picked particles were ‘3D classified’ before being refined using 3D autorefine, resulting in an oligomeric POR 3D structure at a resolution of 7.7 Å (Fig. 3 and Fig. S3). Sample heterogeneity caused by different oligomeric states, and intra- and interdomain flexibility from the dissociating Chlide molecule likely contribute to the limitations in structure determination. The EM structure allowed seven distinct densities to be resolved, arranged around a hollow centre (Fig. 3A,B); six of these densities have a similar shape and size that is consistent with a POR monomer, whereas one has a more elongated shape which could represent two POR molecules in closer contact (Fig. 3A,B). To determine how the POR–Chlide–NADPH ternary complex is arranged in the oligomer, an atomic model of POR was docked into the density using the ‘fit in map’ tool in UCSF CHIMERA (University of California, San Francisco, CA, USA) at the same resolution as the cryo-EM structure. Eight POR molecules could be docked into the density, with the highest correlating position for each density in the oligomer, except one, having the lid region facing towards the centre (Fig. 3C,D). The density which could accommodate two POR molecules had one of the lid regions pointing outwards.
Protein docking was used to investigate how the POR oligomers may form upon Pchlide binding (Figs 4 and 5). The interaction of POR monomers was investigated using the CLUSPRO 2.0 software [34], which scores the most likely structures based on hydrophobic, electrostatic or Van der Waals (VdW) plus electrostatic interactions. A range of ternary complex dimers was produced using each of the docking modes (Fig. 4).

For the ‘closed’ POR conformation, the strong similarity between the ‘balanced’ and ‘hydrophobic’ homodimer models suggest that hydrophobic interactions are dominant (Fig. 4A). Only the balanced and hydrophobic-favoured models, all of which predominantly interact through the lid regions, can fit into the envelope of the oligomeric structure obtained from the cryo-EM data (Fig. 5D). It was suggested recently that the lid regions change from highly flexible structures to more rigid, stabilised forms upon Pchlide binding [26]. Hydrophobicity surface analysis suggests that in the closed conformation, the lid regions form a large hydrophobic patch (Fig. 5B), in addition to the hydrophobic edge of the Pchlide molecule, consistent with lid closure causing dimerisation through hydrophobic interactions. As the lid regions adopt multiple conformations in the absence of substrate [26], we hypothesise that the hydrophobic surface on the POR protein is also likely to undergo significant transient changes in the apo-enzyme and that this prevents dimer formation. Although the high degree of mobility of the lid regions is likely to be the main

**Fig. 2.** SEC analysis and TEM images of ternary complex samples. (A) POR-Pchlide-NADPH under ambient light. (B) POR-Chlide-NADPH. (C) POR-MPCl-NADPH. (D) SEC analysis of POR-Pchlide-NADPH monitored at multiple detection wavelengths to detect the Pchlide and protein concentration of the various peaks. (E) TEM image of POR-Pchlide-NADPH before SEC purification. (F) TEM image of the 200 kDa (according to column calibration) fraction from SEC purification of POR-Pchlide-NADPH. Samples contained ~10 mg of protein, ~100 mg of NADPH and ~100 mg of ligand (Pchlide, Chlide or MPCl). The samples were incubated overnight at room temperature in the dark and exposed to ambient light when loaded on the SEC column. TEM grids were negatively stained by 5% uranyl acetate.

**Lid regions and POR oligomer formation**

Protein docking was used to investigate how the POR oligomers may form upon Pchlide binding (Figs 4 and 5). The interaction of POR monomers was investigated using the CLUSPRO 2.0 software [34], which scores the most likely structures based on hydrophobic, electrostatic or Van der Waals (VdW) plus electrostatic interactions. A range of ternary complex dimers was produced using each of the docking modes (Fig. 4). For the ‘closed’ POR conformation, the strong similarity between the ‘balanced’ and ‘hydrophobic’ homodimer models suggest that hydrophobic interactions are dominant (Fig. 4A). Only the balanced and hydrophobic-favoured models, all of which predominantly interact through the lid regions, can fit into the envelope of the oligomeric structure obtained from the cryo-EM data (Fig. 5D). It was suggested recently that the lid regions change from highly flexible structures to more rigid, stabilised forms upon Pchlide binding [26]. Hydrophobicity surface analysis suggests that in the closed conformation, the lid regions form a large hydrophobic patch (Fig. 5B), in addition to the hydrophobic edge of the Pchlide molecule, consistent with lid closure causing dimerisation through hydrophobic interactions. As the lid regions adopt multiple conformations in the absence of substrate [26], we hypothesise that the hydrophobic surface on the POR protein is also likely to undergo significant transient changes in the apo-enzyme and that this prevents dimer formation. Although the high degree of mobility of the lid regions is likely to be the main
reason that dimerisation is disfavoured in the apo-enzyme, we have also explored how dimers might be formed if a single, ‘open’ conformation of the lid was possible. In this case, the initial dimerisation process appears to be driven by a combination of interactions, rather than predominantly by hydrophobic interactions. The dimers acquired through docking POR in this conformation do not have the lid regions at the dimer interface, and do not fit into the cryo-EM model (Fig. 4B), suggesting that lid closure is required for the observed oligomerisation.

The interaction of ternary complex dimers is necessary to generate larger oligomeric (more than three subunits) structures. It appears that other intramolecular forces, such as VdW interactions, are important for the interaction of POR dimers and are responsible for the wide range of multimeric states that can be formed by the ternary complex. For example, VdW-favoured structures lead to a globular oligomer, similar to the octameric structure observed in the cryo-EM studies (Fig. 5C,D). In contrast, hydrophobic-favoured structures result in a chain-like oligomer (Fig. 4C), similar to the tube-like structures observed in TEM data (Fig. 2E). It is likely that multiple interaction pathways are possible, leading to the wide range of multimeric forms of the ternary complex that were observed in the experimental studies. The docking studies have identified a plausible octameric model from the cryo-EM data (Fig. 5A,D), although it is clear that the cryo-EM model is one of many potential structures.

**Lid regions and Pchlide binding**

Given the inferred importance of the lid regions in oligomer assembly, we set out to investigate the extent of sequence conservation in these regions of POR. It has recently been shown that POR enzymes from cyanobacteria can also form PLBs [15], which implies that plant and cyanobacterial PORs must share common features that trigger the oligomerisation process. A multiple sequence alignment of POR enzymes from cyanobacteria through to plants was used to investigate whether these regions are conserved between PORs of different organisms. Lid1 consists of a conserved GXXP motif at the N-terminal side of the loop, but there are clear sequence differences in the rest of the loop between cyanobacteria and plant PORs (Fig. 6A). In plant PORs, lid1 is highly conserved with...
a common GNVPPKANL sequence motif. However, in cyanobacterial PORs there is more variation, with some (including Thermosynechococcus elongatus, studied here) containing two extra residues. The lid2 region is highly conserved in all organisms. This region is comprised mainly of hydrophobic residues with a number of highly conserved Phe residues found in all POR enzymes (Fig. 6). This suggests that the lid2 region provides hydrophobic residues that are important to the interaction of POR monomers. Several site-directed variants were investigated to ascertain the importance of the lid1 and lid2 regions to POR photocatalysis. Lid1 variants included K156N, I157V, P158A, P158F and K156N/I157V. Binding and steady-state kinetic assays showed little change to overall reaction parameters, with only minor changes observed in the $K_m$ values for NADPH and Pchlide (Table 1 and Figs S4 and S5). A ‘lid swap’ variant in which lid1 residues in T. elongatus POR were replaced by those found in plant PORs (lidP variant) had an elevated $K_m$ value for Pchlide (Table 2). A ~10-fold increase in the $K_d$ value for the POR–Pchlide complex was also observed (Fig. S6), indicating a reduced affinity for Pchlide in the lidP variant. The highly conserved Phe residues of the lid2 region (Phe233, Phe237, Phe240, Phe244 and Phe247) were also exchanged for Tyr. With variants F240Y, F244Y and F247Y, there is a major loss of catalytic activity and reduced binding affinity for Pchlide, whereas with the F237Y variant wild-type levels of activity are retained (Table 2 and
Figs S6–S8). In the wild-type POR ternary complex, the visible spectrum of Pchlide is red-shifted (by ~12 nm) compared to free Pchlide [21] (Fig. 7A). This spectral shift is observed in F237Y, but not the F240Y, F244Y and F247Y variants (Fig. 7A). This implies different geometries for Pchlide binding in the F240Y, F244Y and F247Y variants, consistent with kinetic and binding data. The potential importance of π-π stacking interactions between Phe233 and Pchlide has been emphasised in models of the POR–Pchlide–NADPH complex [26]. We isolated five variants to explore the importance of this interaction, F233A/H/W/L/Y. All had reduced catalytic efficiency and impaired Pchlide binding compared to the wild-type POR (Table 1 and Figs S6–S8). The F233L and F233Y variants were least affected, retaining around 40% of wild-type activity.

**Lid regions and POR photochemistry**

To investigate how lid residues/regions affect POR light-activated H-transfer, we have used single turnover laser photoexcitation measurements to measure the kinetics of these reactions in wild-type and variant PORs. The rate of hydride transfer is unaffected in the lidP variant, but the subsequent proton transfer rate is modestly increased by factor of 2 (Table 2 and Figs S9 and S10). In the lid2 region, most variants have decreased reaction quantum yields; this is not the case, however, for the conservative substitutions made in the F237Y and F240Y variants (Tables 1 and 2 and Figs S11–S13). The impaired photochemistry/H-transfer reactions of many of the variant enzymes explains the poor levels of activity measured in steady-state multiple turnover assays. This is likely attributed to poor binding geometries of the Pchlide in these variants, which are less compatible with light-activated H-transfer (Fig. 7A). Variants F233A and F233W had very low activity in steady-state turnover measurements. For these same variants, it was not possible to accurately measure the $k_{\text{hydride}}$ and $k_{\text{proton}}$ rates because of very low quantum yields (Table 1 and Figs S14–S17). Most other variants retained hydride transfer rates that were similar to...
wild-type POR (Tables 1 and 2 and Figs S14–S17), with the F233L and F233Y variants showing slowest rates (approximately 2- and 80-fold slower, respectively, compared to wild-type) (Tables 1 and 2 and Fig. S15). The proton transfer step was also slowed in these variants (10- and 4-fold, respectively). Interestingly, in one variant (F233H) the rate of both hydride and proton transfer was increased (3-fold) compared to wild-type POR (Table 1).

Given that hydride and proton transfer rates were affected to a major extent in the F233Y variant, we used time-resolved and low-temperature spectroscopy to investigate any potential changes to mechanism. It is known that reaction intermediates in the POR catalytic cycle can be trapped at low temperatures [20,21]. The cryo-trapped intermediates observed for F233Y are similar to those seen for wild-type POR. These intermediates have very similar temperature dependencies, suggesting that the reaction mechanisms of F233Y and wild-type are the same (Fig. S18). Transient absorption data were collected across the ps to μs timescale (Figs S19–S26). For nonreactive samples (in which NADPH was replaced by NADP⁺), F233Y and wild-type POR have similar photochemistry.
Rearrangement of solvent around excited-state Pchlide is slowed slightly in F233Y, reflecting a change in Pchlide environment (Figs S21–S23). In the active NADPH-bound ternary complex, the wild-type and F233Y enzymes have similar excited-state species spectra (Fig. 7B,C, Figs S19 and S20). However, the hydride transfer rate for F233Y variant is reduced ~40 times, and the proton transfer rate by ~2-fold compared to wild-type. This correlates well with single wavelength laser flash photolysis data (Table 2).

**Discussion**

In plants and seedlings, POR and Pchlide accumulate as a ternary complex with NADPH, and from large multimeric complexes that make up the highly organised networks of the PLBs. Understanding the basis of this oligomerisation process is important. Small-angle X-ray scattering measurements have shown that Pchlide binding leads to a structural reorganisation in the POR enzyme, which in turn induces an interaction of POR monomers [28]. Here, we have confirmed that Pchlide binding leads to oligomer assembly and demonstrated that a range of different oligomeric states are formed. Our docking studies show that the initial dimerisation of the ternary complex is likely to occur through the interaction of hydrophobic regions of the protein, and possibly the hydrophobic edge of the Pchlide molecule itself. The subsequent formation of larger oligomers can proceed via a range of different intramolecular interactions that are responsible for the variation in oligomeric structures observed experimentally. These multimeric structures are likely to be formed in a similar manner in plant PORs. Here, Pchlide binding is believed to trigger coupling of POR oligomerisation to higher molecular weight aggregate formation [29,31,36,37].

We have shown that two lid regions located in the distal region of the active site are important for oligomerisation and photocatalysis. These lid regions are inherently flexible and adopt multiple conformations in the absence of substrate [26,27]. This mobility in the apo-enzyme likely disfavours interaction with lid regions of other POR monomers. Dimer and oligomer formation is therefore unfavourable. On binding Pchlide, however, these loops become more ordered. This allows interaction between lid regions, and we suggest this initiates the formation of POR oligomers. The lid2 region is highly conserved in all POR enzymes. It comprises a number of conserved hydrophobic phenylalanine residues that interact with the hydrophobic edge of Pchlide [26]. Pchlide binding stabilises lid conformations that expose a large hydrophobic surface.

**Table 1. Steady-state kinetic parameters and relative quantum yield of T. elongatus POR lid regions variants.**

| Enzyme | K<sub>m</sub>NADPH (µM) | K<sub>m</sub>Pchlide (µM) | k<sub>cat</sub> (µM<sup>-1</sup>s<sup>-1</sup>) | K<sub>c</sub>(Chlide) (µM<sup>-1</sup>) | Quantum yield | Reaction
|--------|---------------------|---------------------|-----------------|-----------------|--------------|------------------------|
| WT     | 0.02 ± 0.02         | 0.035 ± 0.002      | 0.002 ± 0.002   | 0.001 ± 0.002   | 1.5 ± 0.1    | 0.1 ± 0.1               |
| F233W  | 0.9 ± 0.1           | 2.9 ± 0.4           | 2.2 ± 0.2       | 3.3 ± 0.4       | 10.5 ± 0.1   | 0.1 ± 0.1               |
| F233H  | 7.8 ± 1.4           | 15.1 ± 1.6          | 10.7 ± 1.5      | 16.6 ± 1.9      | 13.5 ± 3.3   | 1.0 ± 0.1               |
| F233L  | 7.9 ± 1.0           | 2.4 ± 0.3           | 2.2 ± 0.1       | 2.4 ± 0.2       | 1.5 ± 0.1    | 0.1 ± 0.1               |
| F233F  | 2.2 ± 0.4           | 2.5 ± 0.1           | 2.7 ± 0.1       | 2.9 ± 0.5       | 1.5 ± 0.1    | 0.1 ± 0.1               |
| F233A  | 1.5 ± 0.1           | 1.4 ± 0.1           | 1.3 ± 0.1       | 1.4 ± 0.1       | 1.3 ± 0.1    | 0.1 ± 0.1               |

*<sup>a</sup*K<sub>m</sub> and K<sub>c</sub> values are apparent measured at 15 µM Pchlide, varying NADPH up to 100 µM. *<sup>b</sup>K<sub>k</sub> values are apparent measured at 30 µM Pchlide, varying NADPH up to 30 µM. *<sup>c</sup>*<sup>−</sup>1µM NADPH, varying Pchlide up to 30 µM. *<sup>d</sup>*<sup>−</sup>1µM Pchlide, varying NADPH up to 100 µM. *<sup>e</sup>Hydride transfer rate for F233Y variant is reduced ~40 times, and proton transfer rate by ~2-fold compared to wild-type. This correlates well with single wavelength laser flash photolysis data (Table 2).
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Table 2. Steady-state kinetics parameters and relative quantum yield of T. elongatus POR lid region variants. lidP*: lid1 region residues of T. elongatus POR changed from bacterial to plants (KIPIPAPPD to NVP-PKAN).

| Enzyme          | WT         | lidP*       | F233Y   | F237Y   | F240Y   | F244Y   | F247Y   |
|-----------------|------------|-------------|---------|---------|---------|---------|---------|
| $k_{\text{cat}}$ [s$^{-1}$] | 0.027 ± 0.002 | 0.028 ± 0.02 | 0.013 ± 0.001 | 0.030 ± 0.001 | –       | –       | –       |
| $K_m$[NADPH] [µM] | 0.9 ± 0.1  | 0.8 ± 0.2  | 1.0 ± 0.1 | 2.5 ± 0.6 | –       | –       | –       |
| $K_m$[Pchlide] [µM] | 7.8 ± 1.4  | 99.8 ± 78.0 | 8.1 ± 1.5 | 6.9 ± 0.7 | –       | –       | –       |
| $k_{\text{Pchlide}}$ [(x10$^5$ s$^{-1}$)] | 8.0 ± 1.0  | 63.7 ± 14.5 | –       | –       | 154 ± 76.0 | 94.7 ± 39.1 | 49.2 ± 4.6 |
| $k_{\text{hydride}}$ [(x10$^4$ s$^{-1}$)] | 2.2 ± 0.3  | 2.2 ± 0.1  | 0.04 ± 0.01 | 2.2 ± 0.1 | 2.2 ± 0.2 | 2.0 ± 0.3 | ND      |
| $k_{\text{proton}}$ [(x10$^4$ s$^{-1}$)] | 2.2 ± 0.4  | 4.9 ± 0.4  | 0.5 ± 0.3 | 1.6 ± 0.03 | 0.9 ± 0.1 | ND      | ND      |
| Quantum yield µM Chlide/Laser flash | 1.5 ± 0.1  | 1.6 ± 0.1  | 0.8 ± 0.02 | 1.3 ± 0.1 | 1.6 ± 0.1 | 0.26 ± 0.01 | 0.31 ± 0.01 |

$a k_{\text{cat}}$ values are apparent measured at 200 µM NADPH, varying Pchlide up to 30 µM; $b K_m$ NADPH is an apparent value measured at 15 µM Pchlide, varying NADPH up to 100 µM; $c K_m$ Pchlide is an apparent value measured at 200 µM NADPH, varying Pchlide up to 30 µM.

Associated with the lid region, Hydrophobic interactions between different POR monomers can then initiate oligomerisation. This oligomerisation model is in broad agreement with a small-angle X-ray scattering study of the POR ternary complex, which showed that structural changes on binding Pchlide induce dimersisation [28]. However, in the absence of crystal structures, the authors presented a model of the structural change involving rigidification of a C-terminal extension of the protein. A recent crystal structure of POR suggests that the lid regions formed an interface between two POR monomers, although it is not clear if this was an artefact of the crystallisation process [27]. The studies described here support the idea that Pchlide binding leads to a more rigid protein. Stabilisation of the lid regions then exposes a hydrophobic surface, which becomes a nucleating point for dimer formation.

Modifications of the lid regions also affect Pchlide binding and photocatalysis. Poor binding is observed in selected variants. Altered binding also correlates with changes in the absorption maximum of bound Pchlide and photochemistry, that is decreases in the quantum yield of the reaction. We infer that the lid regions are key to setting up optimal binding geometries for photoactivation and subsequent catalytic steps. Substitution of Phe233 by a Tyr leads to a ~ 50% reduction in the quantum yield. We infer therefore that π–π stacking of Phe233 and Pchlide is an important interaction in setting up the electronic configuration of Pchlide for photoexcitation. Effects of these localised structural changes on rates of hydride and proton transfer are relatively modest.

To conclude, our work points to a dual role for the conserved lid regions in all POR enzymes. We have shown that Pchlide binding initiates oligomer assembly and have provided plausible models based on cryo-EM and modelling studies for involvement of the lid regions in nucleating higher order assembly. The lid regions are also crucial to setting up an optimal binding configuration for Pchlide that presents the substrate optimally for photoactivation and for subsequent reduction of the C17-C18 double bond. The distal region of the active site therefore has a dual function, with major implications not only for POR photocatalysis but also early stage plant development.

Experimental procedures

Site-directed mutagenesis of T. elongatus POR

All chemicals were obtained from Sigma-Aldrich (St. Louis, MI, USA). The genes for the T. elongatus POR variants were made using PCR. The wild-type (WT) T. elongatus POR gene with a 4× His tag in a pET21a vector was used as template. All primers were designed using the online QuikChange Primer Design program and are shown in Table S1. PCR reactions were performed according to conditions in Table S2. PCR products were purified (gel extraction; Qiagen, Hilden, Germany), incubated with Dpn I (New England Biolabs Inc., Ipswich, MA, USA) and transformed into Escherichia coli NEB5α. Transformed cells were selected on Luria-Bertani (LB) agar plates (ampicillin 100 µg·mL$^{-1}$) and incubated overnight at 37 °C. Single colonies were selected, and grown in LB medium (ampicillin 50 µg·mL$^{-1}$) and plasmid purified (miniprep kit Qiagen, Hilden, Germany). Mutations were confirmed by DNA sequencing (Eurofins Genomics, Ebersberg, Germany).

Expression and purification of T. elongatus POR proteins

Recombinant protein samples were prepared from transformed E. coli BL21(DE3) grown at 25 °C overnight in 2 L Erlenmeyer flasks with 500 mL auto induction LB medium (Formedium, Hunstanton,
Norfolk, UK, glucose/lactose ratio 1:4) containing 50 μg/mL ampicillin. After harvesting, cells were resuspended [25 mM imidazole, 0.1 % (v/v) 2-mercaptoethanol, 150 mM NaCl, 20 mM HEPES, pH 7.5] and lysed by sonication (30 × 15 s). After centrifugation (44 800 g; 30 min; 4 °C) the supernatant was loaded onto a 5-mL HisTrap HF column and washed with different concentrations of imidazole in the washing buffer [0.1 % (v/v) 2-mercaptoethanol, 150 mM NaCl, 20 mM HEPES, pH 7.5]. POR protein was eluted using elution buffer [250 mM imidazole, 0.1 % (v/v) 2-mercaptoethanol, 150 mM NaCl, 20 mM HEPES, pH 7.5] and concentrated in a Vivaspin concentrator (Generon Ltd, Slough, UK). Imidazole was removed by desalting (emp Biotech GmbH, Berlin, Germany). Protein concentration was determined by absorbance at 280 nm (extinction coefficient of 35.41 mM−1 cm−1; ProtParam tool on the Expasy web server) and shown to be pure by SDS/PAGE.

Sample preparation for negative-stain transmission electron microscopy

Purified POR complex (3 μL) was applied to carbon-coated 300 mesh copper grids (Agar Scientific Ltd, Essex, UK), which had been glow discharged for 30 s at 25 mA before being stained with 5% uranyl acetate. Grids were imaged using a FEI Tecnai Biotwin transmission electron microscope (Hillsboro, OR, USA) operating at an accelerating voltage of 120 keV with a Gatan Orius SC1000 CCD camera (Pleasanton, CA, USA). Images were recorded with a 1-s exposure.

Protein and protein docking

Protein-protein docking was carried out with the CLUSPRO 2.0 application (available online at https://cluspro.bu.edu/home.php) [34,38,39]. Homodimers were assessed using the four scoring methods mentioned, and then, larger oligomeric structures were docked in order to build an octamer model as shown in Fig. 5A. The starting monomer for the octamer model was the TePOR ternary complex structure from previous study [26]. The homodimers of both the closed and open lid structure derived from the ternary complex were analysed to identify the importance of the lid region and its conformation for binding preference.

Sample preparation and data processing for cryo-electron microscopy

A 3 μL sample of purified TePOR at a concentration of ~ 0.25 mg·mL−1 was adsorbed onto 300 mesh copper R2/2 holey carbon grids (Quantifoil, Essex, UK), which had been glow discharged for 30 s at 25 mA. The grids were blotted for between 2 and 6 s at 90%
humidity at 4 °C before plunge freezing in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific, Waltham, MA, USA). Grids were stored under liquid nitrogen and screened for optimal ice on a Titan Krios electron microscope (Thermo Fisher Scientific) at the Astbury Biostructure Laboratory, University of Leeds. Grids with optimal ice were imaged at the UK national electron bio-imaging centre (eBIC) on a Titan Krios operating at an accelerating voltage 300 keV, through a Gatan energy filter and equipped with a K3 direct electron detector, using SERIALEM software [40]. 9882 Movies comprising 40 frames with a total dose of 40 e− Å−2 were collected in counting mode at a nominal magnification of 81 000 × which gave a corresponding pixel size of 1.078 Å.

Movies were motion corrected and dose weighted using MOTIONCOR2 [41]. Motion corrected images were imported into Warp [42] where 935 838 particles were picked using the Warp box net. Patch-based CTF estimation in Warp was used to calculate local CTF values for the particles. An initial model was created in RELION 3.1 [33] and was refined using an initial data set of 100 000 particles, and this initial model was then used as a starting structure for a 3D classification of the full data set. The best 3D class was then selected which contained 340 024 particles and these particles were then used for a final 3D refinement. The resolution of the final model was calculated in RELION 3.1 using the Fourier shell correlation (FSC) of the two half maps from the final refinement and taken at the 0.143 cut off.

**Steady-state kinetic assays**

All samples for steady-state kinetic measurements were prepared under low-intensity green light. Substrate or product concentrations in the reaction buffer [0.5–100 μM NADPH, 1–30 μM Pchlide, 0.1% Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 150 mM NaCl, 20 mM HEPES, pH 7.5] were determined using the following extinction coefficients in aqueous solution: NADPH, 6.22 mm−1·cm−1 at 340 nm; Pchlide, 24.95 mm−1·cm−1 at 630 nm; and Chlide, 69.95 mm−1·cm−1 at 670 nm. Steady-state activity measurements were carried out at 40 °C using a Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA). 1 mL of samples containing 10 μM Pchlide, 250 μM NADPH and 50 μM POR was incubated at the corresponding temperature using an Optistat DN liquid nitrogen cryostat (Oxford Instruments Inc., Abingdon, UK). The POR reaction was initiated by the same LED light used in the activity measurements. For each temperature point, the sample was illuminated for 10 min, or for the dark steps, the sample was illuminated at 190 K for 10 min, and then incubated in the dark at the corresponding temperature for 10 min. All absorbance spectra were recorded at 77 K by using a Cary 50 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

**Pchlide binding assays**

The spectral shift in the Pchlide absorption on formation of the POR ternary complex was used to follow Pchlide binding [21]. Binding was measured by following the red shift in absorbance at 642 nm. Binding assays comprised 0.5 μM Pchlide, 100 μM NADPH and 0.5–100 μM POR. Apparent $K_d$ values were obtained by fitting the absorbance ratio changes ($\Delta A = A_{642 nm}/A_{630 nm}$) against the concentration of POR using the equation ($A_o$ is the initial ratio of the absorbance at $A_{642 nm}/A_{630 nm}$):

$$\Delta A = \frac{A_{max}[POR]}{K_d + [POR]} + A_o.$$  

**Low-temperature absorbance spectroscopy**

Low-temperature absorbance spectra were measured in reaction buffer with 44% glycerol, 20% sucrose and 0.1% Genapol in a Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA). 1 mL of samples containing 10 μM Pchlide, 250 μM NADPH and 50 μM POR was incubated at the corresponding temperature using an Optistat DN liquid nitrogen cryostat (Oxford Instruments Inc., Abingdon, UK). The POR reaction was initiated by the same LED light used in the activity measurements. For each temperature point, the sample was illuminated for 10 min, or for the dark steps, the sample was illuminated at 190 K for 10 min, and then incubated in the dark at the corresponding temperature for 10 min. All absorbance spectra were recorded at 77 K by using a Cary 50 spectrophotometer.

**Laser flash photolysis**

A Nd:YAG laser and OPO system (Surelite, Continuum) was used to generate a 450 nm, 5 ns duration, laser pulse (~ 25 mJ). Upon excitation of the sample, absorbance changes at 696 nm were monitored by an LKS-60 flash photolysis instrument (Applied Photonics Ltd., Leatherhead, UK). Rate constants were acquired by fitting the data to a single or double exponential function, as appropriate. Each measurement was repeated 5 times to obtain an average rate. The laser flash photolysis sample contained 15 μM Pchlide, 250 μM
NADPH, 0.1 % Triton X-100, 0.1 % (v/v) 2-mercaptoethanol, 150 mM NaCl and 20 mM HEPES, pH 7.5. A circulating water bath (Fisher-brand, Leicestershire, UK) was used to maintain the temperature at 25 °C.

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**Conflicts of interest**

The authors declare no conflict of interest.

**Author contributions**

 NSS, DJH and CB initiated and coordinated the project. SZ, NSS, DJH and CB designed experiments, analysed data and wrote the manuscript with contributions from other authors. SZ characterised all POR variants and prepared samples for electron microscopy. ARFG prepared cryo-EM sample grids, collected and processed cryo-EM data. AT, SH and LOJ performed and analysed protein docking studies. SJOH and DJH performed laser photoexcitation measurements. TAJ ran the MALS-SEC purification of the cryo-EM samples. All authors discussed the results and commented on the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. SEC results for the POR ternary complex.
Fig. S2. Absorbance spectra of POR ternary complex sample after SEC-MALS analysis.
Fig. S3. The Fourier shell correlation (FSC) of the cryo-TEM POR oligomer structure.
Fig. S4. Steady-state activity of wild type and lid1 variants of T. elongatus POR.
Fig. S5. Steady-state activity of wild type and lid1 variants of T. elongatus POR.
Fig. S6. Pchlide binding affinity of T. elongatus POR variants.
Fig. S7. Steady-state activity of wild type and lid2 variants of T. elongatus POR.
Fig. S8. Steady-state activity of wild type and lid2 variants of T. elongatus POR.
Fig. S9. Kinetic transients of hydride transfer for variants of T. elongatus POR in lid1.
Fig. S10. Kinetic transients of proton transfer for wild type and variants of T. elongatus POR in lid1.

Fig. S11. Absorbance spectra of ternary complex samples of T. elongatus POR lid2 variants after increasing number of laser pulses.
Fig. S12. Absorbance spectra of T. elongatus POR ternary complex samples of Phe233 variants after increasing number of laser pulses.
Fig. S13. Relative quantum yield (Φ) calculation of T. elongatus POR variants.
Fig. S14. Kinetic transients of hydride transfer for wild type and variants of T. elongatus POR in lid2 region.
Fig. S15. Kinetic transients of hydride transfer for Phe233 variants of T. elongatus POR.
Fig. S16. Kinetic transients of proton transfer for wild type and variants of T. elongatus POR in lid2 region.
Fig. S17. Kinetic transients of proton transfer for Phe233 variants of T. elongatus POR.
Fig. S18. Cryogenic absorbance measurements of T. elongatus POR F233Y variant.
Fig. S19. Time-resolved visible spectroscopy data for TePOR WT ternary complex sample.
Fig. S20. Time-resolved visible spectroscopy data for TePOR F233Y variant ternary complex sample.
Fig. S21. Species associated difference spectra (SADS) resulting from a global analysis of the time-resolved visible data. Data was collected from ps to μs for Pchlide only sample after excitation at 450 nm.
Fig. S22. Species associated difference spectra (SADS) resulting from a global analysis of the time-resolved visible data. Data was collected from ps to μs for wild type non-reactive ternary complex sample after excitation at 450 nm.
Fig. S23. Species associated difference spectra (SADS) resulting from a global analysis of the time-resolved visible data. Data was collected from ps to μs for T. elongatus POR F233Y variant non-reactive ternary complex sample after excitation at 450 nm.
Fig. S24. Time-resolved visible spectroscopy data for Pchlide only sample.
Fig. S25. Time-resolved visible spectroscopy data for TePOR wild type ternary complex sample.
Fig. S26. Time-resolved visible spectroscopy data for TePOR F233Y variant ternary complex sample.

Table S1. Primer list.
Table S2. PCR reaction conditions.