Photocatalysis as the 'master switch' of photomorphogenesis in early plant development

DOI:
10.1038/s41477-021-00866-5

Document Version
Accepted author manuscript

The role of photocatalysis as the 'master switch' of photomorphogenesis in early plant development.

Derren J. Heyes*, Shaowei Zhang, Aoife Taylor, Linus O. Johannissen, Samantha J. O. Hardman, Sam Hay, and Nigel S. Scrutton*

[1]Manchester Institute of Biotechnology, Department of Chemistry, School of Natural Sciences, The University of Manchester, 131 Princess Street, Manchester M1 7DN, UK

*Correspondence: derren.heyes@manchester.ac.uk; nigel.scrutton@manchester.ac.uk

Enzymatic photocatalysis is seldom used in biology. Photocatalysis by protochlorophyllide oxidoreductase (LPOR) – one of only a few natural light-dependent enzymes – is an exception, and is responsible for the conversion of protochlorophyllide to chlorophyllide in chlorophyll biosynthesis. Photocatalysis by LPOR not only regulates biosynthesis of the most abundant pigment on Earth, it is also a 'master switch' in photomorphogenesis in early plant development. Upon illumination, LPOR promotes chlorophyll production, plastid membranes are transformed and the photosynthetic apparatus is established. Given these remarkable, light-induced pigment and morphological changes, the LPOR-catalyzed reaction has been studied extensively from catalytic, physiological and plant development perspectives, highlighting vital, and multiple, cellular roles of this intriguing enzyme. Here, we offer a perspective in which the link between LPOR photocatalysis and plant photomorphogenesis is explored. Notable have been breakthroughs in LPOR structural biology that have uncovered the structural-mechanistic basis of photocatalysis. These studies have clarified how photon absorption by the pigment protochlorophyllide – bound in a ternary LPOR–protochlorophyllide–NADPH complex – triggers photocatalysis, and a cascade of complex molecular and cellular events that lead to plant morphological change. Photocatalysis is therefore the 'master switch' responsible for early stage plant development, and ultimately life on Earth.

Main

As the cofactor responsible for harvesting and converting sunlight into photochemical energy in plants and other photosynthetic organisms, chlorophyll is the most abundant pigment and is crucial for life on Earth.[1] Reflecting this abundance, the rise and fall of chlorophyll levels across the globe is the only biochemical process visible from outer space.[2] The chlorophyll biosynthetic pathway consists of at least 12 different enzymatic steps (Figure 1). It is tightly regulated by a complex network of transcription factors, protein-protein interactions and putative allosteric interactions to prevent the accumulation of photoreactive intermediates.[3-9] Many of the genes that encode the enzymes of the pathway are involved in a large coexpression network and several are controlled by photoreceptor (e.g. phyotochrome, cryptochrome) mediated light-responsive signaling cascades that are also coupled to the expression of downstream genes required for photosynthesis.[9] In plants, chlorophyll biosynthesis occurs in the chloroplast and starts from 5-aminolevulinic acid, a precursor for the biosynthesis of all tetrapyrroles.[4,5] A series of enzymatic steps, common to the synthesis of most tetrapyrroles, then leads to the formation of a large, complex molecule, protoporphyrin IX, comprised of four pyrrole rings.[4,5] Protoporphyrin IX lies at the branch-point of heme and chlorophyll biosynthesis, where either ferrochelatase inserts Fe^{2+} into the porphyrin macrocycle to form heme, or – along the chlorophyll branch – Mg^{2+} is incorporated through the action of ATP-dependent Mg-chelatase.[4,5,10,11] This Mg-protoporphyrin IX complex is converted to chlorophyll by enzyme-catalyzed methylation,[11-13] formation of an additional isocyclic ring E,[11,14,15] two subsequent reductions[11,16] and a final esterification of the long phytyl tail.[11,17] Reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is the penultimate step in this biosynthesis pathway.[18-22] Control of this reaction is vital to regulated biosynthesis of chlorophyll biosynthesis and, importantly, early plant development.

In nature, two routes have evolved to reduce Pchlide, specifically at the C17-C18 double bond, to form Chlide (Figure 1).[23] In evolutionary terms, a light-independent (or dark-operative) Pchlide oxidoreductase (DPOR) is the older enzyme.[24,25] It is a multi-subunit enzyme comprising three separate subunits.[26-28] DPOR closely resembles the enzyme nitrogenase. It catalyzes Pchlide reduction in an ATP-dependent manner and is sensitive to oxygen.[26-28] DPOR arose in anoxygenic

photosynthetic bacteria and can also be found in non-flowering land plants, algae and cyanobacteria.[24,25] Light-dependent protochlorophyllide oxidoreductase (LPOR) is distinct from DPOR. LPOR also catalyzes reduction of Pchlide and is found in all plants, algae and cyanobacteria. Phylogenetic analyses indicate that LPOR appeared first in cyanobacteria at the onset of oxygenic photosynthesis.[24,25,29] However, LPOR enzymes have also recently been discovered in anoxygenic phototrophic bacteria, which most likely obtained the enzyme by horizontal gene transfer from cyanobacteria, and suggests that LPORs are more widespread than first thought.[29-31] Some organisms, including non-flowering land plants, algae and cyanobacteria, possess DPOR and LPOR and, although the role of both enzymes in these species is still not fully understood, it is proposed that they enable optimal production of chlorophyll under variable conditions.[24,25] As the DPOR enzyme is highly sensitive to oxygen the presence of both enzymes may help the photosynthetic organism to adapt to changes in the light and oxygen conditions in the environment.[25] However, LPOR is the only enzyme responsible for Pchlide reduction in angiosperms and has a vital regulatory role. It is known to interact with several upstream proteins involved in the import of LPOR to the plastid (*e.g.* Pchlide *a* oxygenase, tyrosine aminotransferase, chaperone-like protein of POR1),[32,33] in addition to a number of regulatory proteins (*e.g.* FLU protein, CHL27, phycocyanobilin:ferredoxin oxidoreductase) and the neighbouring cyclase enzyme to control flux through the entire chlorophyll biosynthetic pathway.[7,34,35] Moreover, LPOR also interacts with downstream proteins, such as light-harvesting like proteins (LILs) and the POR-interacting TPR protein, involved in the assembly pathway for the formation of photosynthetic complexes.[7,36,37] Hence, it acts as a light-activated trigger to control the chlorophyll content and subsequent development of the photosynthetic apparatus in plant cells.[18-22] Photocatalysis by LPOR is therefore a 'master switch' in early plant development triggering formation of the light-dependent photosynthetic apparatus. These plant development changes occur on timescales many orders of magnitude slower than LPOR photocatalysis. Across these timescales, advances in physiological, mechanistic and structural appreciation of LPOR function are leading to a new understanding of the biological importance of LPOR. Further exploration of these interconnected functional relationships is therefore warranted.

Photocatalysis is the 'master switch' in early plant development

Active LPOR complexes were first discovered in etiolated leaves over 70 years ago.[38] This has led to a long, intensive research history, initially on dark-drown seedlings and leaves, followed by isolated membranes, cell fragments and more recently purified protein samples, to establish the pivotal role of LPOR in plant physiology and development (reviewed in detail in [18,39]). In the dark (*e.g.* under fallen leaves or stones) angiosperm seedlings undergo skotomorphogenesis, attributed to the accumulation of Pchlide by the chlorophyll biosynthesis pathway.[40] In this scenario, development of a proplastid into a chloroplast is stalled at the etioplast – plastids that develop in darkness.[39] In such etiolated plants and seedlings, LPOR and Pchlide accumulate as a ternary complex with NADPH to form highly organized, paracrystalline structures, known as prolamellar bodies (PLBs) (Figure 2).[41-44] These PLBs are located within the membranes of etioplasts and are surrounded by prothylakoid membranes, which develop ultimately into the thylakoids of the chloroplast. PLBs are rich in lipid – mainly monogalactosyldiacylglycerol and digalactosyldiacylglycerol – and these are essential to membrane structure organization and subsequent development of the etioplast.[44-47] The mechanism of PLB assembly is still not fully understood and it is likely that several regulatory pathways are required to control PLB formation.[39,41] Any impairment in the production of Pchlide is known to prevent PLB formation, which, in turn, is also thought to be coupled to the import pathway of LPOR into the plastid.[41,48] The incorporation pathway of lipids into PLBs remains unclear but it is suggested that the interaction between LPOR and monogalactosyldiacylglycerol is essential for the assembly of the PLB.[39,41,44-46]

PLBs contain a range of spectrally distinct forms of Pchlide, attributed to the formation of aggregates of different sizes with LPOR and NADPH.[49,50] These aggregates account for approximately 95 % of total protein in the PLBs.[39] Diverse interactions of LPOR-Pchlide complexes with PLB lipids are thought also to influence the spectral properties of the pigment.[44,50] Interactions with PLB lipids regulate LPOR activity in plants and might even be required to form efficient photoactive LPOR-Pchlide-NADPH complexes.[44,45] Non-photoactive forms of Pchlide, representing unbound forms of the pigment, are also found in etioplasts but these are mainly restricted to the prothylakoids.[43,50] Characteristic PLB structures

have been restored in a LPOR knockout strain of *Arabidopis thaliana* by replacing the native plant LPOR with those from cyanobacteria.[51] However, it should be noted that although the size and structure of PLBs were normal, there was a lower ratio of photoactive to non-photoactive Pchlide. PLB-like ultrastructures also form in etiolated cyanobacteria by overexpressing LPOR in DPOR-deficient cells.[52] It seems therefore that LPOR enzymes, irrespective of their origin, are sufficient to trigger PLB formation in photosynthetic organisms.

LPOR is the basis of the light-dependent switch between skotomorphogenesis and photomorphogenesis following irradiation of etioplasts with visible light. Illumination triggers profound changes in the physiological development of plants that are manifest in a reorganization and modification of plastid membranes.[18-20,39,41-43] Illumination results in rapid photoreduction of Pchlide to form Chlide, followed by conversion of LPOR-Chlide-$NADP^+$ aggregates into LPOR-Chlide-NADPH complexes, which are proposed to play a photoprotective role in PLBs as they act as a strong energy quencher.[39] Photocatalysis ultimately leads to dispersion of PLBs as the LPOR–Chlide complexes dissociate,[9,39,41] aided by the presence of the galactolipid digalactosyldiacylglycerol (Figure 2).[47] Following dispersal of PLBs, chlorophyll levels increase and photosynthetic proteins are expressed as the etioplasts are transformed into mature chloroplasts to establish the photosynthetic apparatus.[39,41,43] Morphologically, the tubular structure of PLBs transforms into a linear system of parallel lamellae that develop into the photosynthetically active thylakoid membranes of the grana in a process that occurs in a number of stages over several hours / days.[39,43] Initially, the dispersed PLBs flow outward into the stroma (1-2 hours) but until recently, it was unclear whether the PLB transforms into thylakoids continuously or through the formation of vesicles.[39,41-43,53] Recent three dimensional reconstruction of the etioplast to chloroplast transformation show direct conversion of PLBs to flat slats and the appearance of the first grana as overlapping thylakoids (<8 hours).[43] During these early stages partial reformation of PLBs, connected to the developing thylakoids, is also possible due to the accumulation of photoactive LPOR-Pchlide-NADPH ternary complexes.[43,54] More complex grana structures with a helical character are slowly generated over 2-3 days, concomitant with the formation of ordered supercomplexes of light-harvesting complex II and photosystem II that are proposed to stabilize the structure of the grana stacks.[43,55] What is clear is that

photocatalysis by LPOR is crucial for this entire process, triggering multiple events across a hierarchy of timescales. These include photon capture (sub-picoseconds), catalysis (micro – milliseconds), product release (millisecond – seconds), and dispersal of PLBs (minutes-hours). These timescales are extended further (hours-days) as the chlorophyll-binding proteins accumulate and mature chloroplasts form.

Angiosperms can contain multiple isoforms of LPOR. These have different patterns of light and developmental regulation and allow the plant to optimize levels of chlorophyll production at different stages of the greening process.[56-58] One isoform of LPOR is only expressed in etiolated seedlings or plants, whereas another isoform is thought to be necessary for maintaining chlorophyll synthesis throughout the entire life cycle of a plant. A third isoform has been identified in *Arabidopsis*.[56-58] A recent phylogenetic analysis of LPOR sequences revealed that all LPORs diverged from a single common ancestor 1.36 billion years ago.[59] The LPOR gene has since been duplicated at least 10 times in angiosperms, leading to the appearance of two or more LPOR isoforms in several species. A new classification of LPOR enzymes was proposed based on their evolutionary origin and biochemical properties. Z-type LPORs are present in bacteria, whereas S-type and L-type isoforms are found in plants, but differ in their interactions with lipids.[59] Two of the isoforms, originally termed named LPORA and LPORB – have been shown to form high molecular weight, putative light-harvesting complexes in the PLBs of barley.[20] These complexes have been reconstituted *in vitro* using synthesized Pchlide forms (Pchlide *a* and Pchlide *b*) and might allow rapid chlorophyll synthesis under low light intensities as well as offering a photoprotective role during greening.[20,60] This, however, remains a controversial hypothesis. Whether such complexes exist in plants has been hotly debated,[61] and a similar LPOR light-harvesting complex does not form in *Arabidopsis* due to a different geometry of the LPOR proteins.[62] A role for LPOR in regulating oxidative stress and vitamin E levels has also been suggested,[63,64] extending further the physiological reach of LPOR.

Structural biology shines new light on LPOR function

The size, shape and architecture of PLBs is known to be determined by the structural properties of the LPOR protein. These include its ability to form oligomers,[62,65-71] the

association of LPOR with the membrane[71,72] and the interaction of the enzyme with lipids.[44,59,71] The propensity of LPOR to form large, substrate-bound, PLB aggregates in the etioplast – and potentially during the greening process – was first observed over 30 years ago[65] and has generated much interest in understanding this process *in vitro*.[62,66-71] LPORs from different organisms can adopt a variety of soluble oligomeric forms in the absence of substrates, ranging from monomers in cyanobacterial LPORs[66,67,70] through to higher order oligomers in plant enzymes.[62,68,69] In all cases, formation of a LPOR–Pchlide–NADPH ternary complex leads to formation of larger multimeric complexes (Figure 3).[66-71] In plant LPORs, binding of Pchlide is surmised to alter the oligomeric structure. This triggers further coupling of oligomers to form higher molecular weight aggregates.[62,68,69,71] Recent cryoelectron microscopy studies show that these LPOR oligomers form helical filaments with the lipid bilayer, allowing efficient exchange of the Pchlide substrate and Chlide product from the membrane.[71] Such LPOR filaments also directly shape the membranes into the characteristic curved tubules found in the PLB and may provide a source of lipids upon light-driven dissociation for the subsequent assembly of the thylakoids during biogenesis of the photosynthetic apparatus.[71]

The binding of Pchlide to LPOR elicits structural change in the protein, which in turn induces LPOR dimer formation and subsequent oligomerization (Figure 3).[66,70] Conformational changes in two highly conserved loop regions, which fold over the Pchlide and form a 'lid' upon binding, are proposed to trigger oligomer formation.[70] The protein conformational changes linked to Pchlide binding are rate-limiting in the overall LPOR photocatalytic cycle and also lead to a red-shift in the electronic absorption spectrum of Pchlide.[73] One suggestion is that oligomerization allows neighboring Pchlide molecules to interact, leading to higher reaction efficiency.[68,69] This idea is supported by excited state energy transfer between neighboring pigment molecules in LPOR–Pchlide complexes,[74] and between Pchlide and chlorophyll binding proteins during assembly of the photosynthetic membranes assembly.[75] The helical array of Pchlide molecules observed in the recent structure of the LPOR filaments suggest a distance of ~25 - 78 Å between adjacent Pchlides, allowing efficient energy transfer upon illumination.[71] Also, pre-illumination of the LPOR-Pchlide-NADPH ternary complex with low levels of light has been reported to lead to

progressive protein rigidification and increased enzyme activity.[76,77] This is an intriguing finding but molecular details of this process remain unclear.

Structural understanding of LPOR function has, for many years, been restricted to modelling studies.[60,68,78,79] X-ray crystal structures of cyanobacterial LPORs in their free form, and in complex with NADPH, have recently been solved and these offer new insights into LPOR function (Figure 3).[67,80] LPOR has a typical dinucleotide binding Rossmann-fold with a central β-sheet comprised of 8 β-strands surrounded by 6 α-helices. [67,80] There are multiple flexible loops, one of which extends from the central β-sheet and becomes ordered on binding NADPH. This loop acts as a lid, covering the nicotinamide group of the coenzyme in the enzyme active site.[67] Other residues are also implicated in binding NADPH.[67] There is still no crystal structure of the LPOR–Pchlide–NADPH complex from which to visualize how Pchlide is bound.[67,80] Molecular docking and dynamics simulations,[67] together with recent cryoelectron microscopy studies,[71] have highlighted important and multiple interactions between residues in the active site and Pchlide (Figure 3). Many of these interactions have been validated in functional–mutagenesis studies of photocatalysis and binding.[67,78] Pchlide polar functional groups include the keto, methylester and carboxylic acid groups, and these sit deep in a hydrophilic active site pocket where they form hydrogen bonding interactions with active site residues. The hydrophobic edge of Pchlide is located in a hydrophobic region of the enzyme, which includes the aforementioned 'lid' regions that close over Pchlide and are also crucial to optimally position the Pchlide for efficient photocatalysis.[46,70] Changes in the structure of these hydrophobic regions upon Pchlide binding are also proposed to be important for association of the protein with the membrane.[71] The spatial organization of the active site offers some insight into the structural rearrangements triggered by the binding of Pchlide. These recent structural advances are leading to a more detailed understanding of how conformational changes in the LPOR–Pchlide–NADPH complexes enable monomers of the complex to nucleate and initiate the formation of oligomers, as well as interact with the membranes of the PLB.[67,70,71,80]

Determinants of photocatalysis identified from enzyme structures

Photocatalysis by the LPOR 'master switch' triggers chemical and structural change at molecular and cellular levels that result in changes in plant morphology over many hours / days (Figure 4). This cascade of events occurs over timescales that span an unprecedented 10^{15}-10^{18} orders of magnitude. LPOR is one of only a few enzymes that are known to have a direct, natural requirement for light, the others being photosynthetic reaction centres, including the related chlorophyll f synthase,[81] and the flavoenzymes DNA photolyase[82] and fatty acid photodecarboxylase.[83] As a consequence, LPOR is an experimental system from which we can learn how to harness photocatalysis.[19,21,84] There are unprecedented opportunities to advance mechanistic understanding of enzymes with photo-initiation. Because most enzyme reactions are thermally activated, experimental studies are often limited to slower timescales (milliseconds and longer), due to the necessity of bringing together, through rapid mixing, a substrate and enzyme. The limitations of mixing are side-stepped in photocatalysis because the enzyme-substrate complex can be pre-formed in the dark. Photocatalysis can then be initiated, typically using a laser pulse or LED, and the photocatalytic cycle studied over a wide range of timescales (femtoseconds to seconds, or longer) and across a wide range of temperatures, including cryogenic, to identify short-lived reaction intermediates.[19,21] All this combines to make the chemistry of photocatalysis accessible in ways that are not possible with conventional diffusion-controlled enzymes.

Pchlide is both the substrate and the light-sensing chromophore for LPOR photocatalysis. It has excited state properties that allow it to capture sunlight and initiate reduction of the C17–C18 double bond when bound in the active site. Time-resolved spectroscopy has been used to investigate these excited state properties, and studies have shown that Pchlide is highly reactive.[85-92] Excitation brings about charge separation across Pchlide.[85-91] This process is highly dependent on solvent polarity[85,86,90] and is attributed to a number of polar groups attached to Pchlide, including an electron-withdrawing carbonyl group on ring E.[91] The excited state of Pchlide is quenched through solvation of this intramolecular charge-transfer species,[85-91] and a triplet state is then formed on the nanosecond timescale with a quantum yield of approximately 30 %.[89-92] Excited state charge separation across Pchlide has also been confirmed computationally, and these calculations suggest that this leads to site-specific solvation of photoexcited Pchlide through strengthening of

H-bonding interactions.[91,93] Photochemical studies of the mechanism in the ternary enzyme-substrate complex are challenging[74,76] but it appears that the dipolar character of excited state Pchlide is also important in LPOR photocatalysis (Figure 4).[94] Structural modelling of the LPOR–Pchlide–NADPH complex has highlighted how the LPOR active site stabilizes excited state charge separation in Pchlide by strengthening an extensive hydrogen bonding network between active site residues and Pchlide (Figure 3). These excited state interactions generate an electron-deficient site at the C17-C18 region, activating it for subsequent reduction.[67,94]

Reduction of the C17-C18 bond of Pchlide has been studied using a combination of time-resolved spectroscopy[78,94-101] and cryogenic spectroscopy techniques with purified LPOR protein.[97,98,102-107] Crucially, the mechanism of photocatalysis is identical *in vivo* as the same reaction intermediates are also observed in low temperature analyses of plants.[18,54,108] After initial excitation of the Pchlide substrate, a hydride equivalent is transferred – sequentially as 2 electrons and a proton – from NADPH to the C17 position of Pchlide.[95,105] This is followed by proton transfer to C18, either directly from an active site tyrosine residue, or mediated by solvent (Figure 4).[95,97] Both H-transfer reactions proceed by quantum mechanical tunnelling.[95] In biology, enzymatic hydride transfers are generally concerted, involving hydride anion transfer in a single reaction step. This is not so, however, in LPOR which represents – to the best of our knowledge – the only known example of stepwise hydride transfer in biology.[98,101] Resolution of fast sequential reaction steps highlights the benefits of using short laser pulses to initiate enzyme reactions – a clear advantage of working with photocatalysts to study enzyme mechanisms.[101]

The chemistry of ‘hydride’ transfer is conserved across many LPOR enzymes, from plants, to algae and cyanobacteria. Hydride transfer leads to the formation of a deprotonated Chlide anion, which has no detectable fluorescence and has a characteristic broad absorbance band at 696 nm.[95,99,100,102,104,107,108] Hydride transfer occurs well below 200 K in studies with many LPOR enzymes. This indicates that hydride transfer progresses at temperatures lower than the ‘glass transition’ temperature of proteins, below which most protein motions, other than bond vibrations, are frozen.[102,105-108] Hydride transfer is not influenced by bulk solvent viscosity. This further implies that large-scale protein motions are not involved in, or required for, stepwise ‘hydride’ transfer.[96,99,100] Structural models of the LPOR–

Pchlide–NADPH complex have confirmed that the *pro-S* hydrogen of NADPH[95] is transferred, consistent with experimental findings.[67]

Protonation of the C18 atom of the hydride-reduced Pchlide leads to formation of the final Chlide product (Figure 4).[95,97] Proton transfer is different across LPOR enzymes from cyanobacteria compared to plants.[99,100,107] In cyanobacterial LPORs, proton transfer occurs above 200 K and is coupled to solvent dynamics, suggesting involvement of a complex, extended network of protein motions that accompany proton transfer on the microsecond timescale.[96,99,100,103,106] In plant LPORs, proton transfer is faster, can occur below the 'glass transition' temperature, and is not dependent on an extended solvent-coupled dynamic network.[99,100,107] This implies that the proton donor (presumed to be an active site tyrosine) is positioned optimally in plant LPORs, close to C18 of Pchlide, whereas extended solvent-coupled dynamics are needed to optimize donor-acceptor proton transfer distances in cyanobacterial enzymes.[99,100,107] An equilibrium donor-acceptor distance of about 5 Å is observed between the active site tyrosine (the presumed donor) and C18 of Pchlide in structural models of cyanobacterial LPOR, although this may decrease following hydride transfer.[67] A potential and extensive proton-relay pathway from solvent to this tyrosine residue is observed, comprising a water network coordinated by several active site residues.[67,80] However, as noted from the recent cryoelectron microscopy structure of plant LPOR it is possible that an alternative proton donor may exist.[71] Following proton transfer to C18, a series of ordered product release and substrate binding events, then complete the catalytic cycle.[104,109] In plants, these steps lead to a blue shift in the Chlide absorbance spectrum as the LPOR-Chlide complexes breakdown and the Chlide is esterified to produce chlorophyll,[54] causing dispersal of the PLBs (Figure 4).[39,41-43] Significant conformational changes in the protein are proposed to accompany Chlide release, which also disrupt interactions with the lipid bilayer and lead to the disassembly of the LPOR filaments.[71]

Outlook and future perspectives

Chemical and molecular level understanding of photocatalysis by LPOR, and implications for the dissolution of PLBs, is beginning to emerge. What is clear is that photocatalysis is the 'master switch' for plant photomorphogenesis, and that this leads

to maturation of the chloroplast correlated with chlorophyll pigment supply (Figure 4). However, important questions remain. In terms of chlorophyll biosynthesis it is still not fully understood why some photosynthetic organisms have retained two separate enzymes for this step and it is possible that DPOR might be involved in developmental functions that have yet to be established.[25] The function of LPOR in anoxygenic phototrophic bacteria is also still an open question and it will be interesting to ascertain if they show the same structural and biochemical properties as their plant and cyanobacterial counterparts.[29-31] It will also be important to build on recent pioneering advances in understanding how LPOR interacts with other enzymes and regulatory components to control the levels of chlorophyll production and coordinate this with the assembly of the chlorophyll-binding proteins required for the photosynthetic apparatus.[7]

Despite a number of recent breakthroughs about the role of PLBs in the greening process we still do not fully understand how PLBs are formed and what their function is in the development of the chloroplast.[39-53] PLB-like structures can form in cyanobacteria and this offers an attractive opportunity to study their formation in an amenable model system.[52] It has been suggested that PLBs may play an important role in the storage of lipids for the formation of thylakoids but the pathway for delivering galactolipids to the PLBs remains poorly understood.[39] It is also unclear how LPOR interacts with the lipid membranes to form highly organized networks in PLBs, although new hypotheses for this process are beginning to emerge with the recent advances in cryoelectron microscopy.[71] Models for how Pchlide binding drives oligomer formation to promote PLB formation are also now being developed and involve protein structural reorganization.[67,70,71,80] Further molecular insights into LPOR oligomerization are now likely to be developed and will be important for a complete understanding of how PLBs are formed. Developments in 3D reconstruction techniques will also be crucial for deriving mechanisms for the disassembly of the PLBs and subsequent formation of the photosynthetic apparatus.

Crystal structures[67,80] are now being used to interpret a large body of biophysical information within a structural context.[73,74,76-78,85-109] Atomic level insight into how excited state chemistry, bond making / breaking and the dynamics of photocatalysis are controlled by protein structure is within reach. Continued work in these areas will furnish a more complete understanding of how biology harnesses light energy to

derive catalytic power, to biosynthesize chlorophyll and to install the ‘master switch’ of early plant development. Ultimately, photoactive crystals of the LPOR–Pchlide–NADPH complex may give access to time-resolved visualization of early phases of the photocatalytic cycle. Spatial and temporal resolution of photochemistry using X-FEL methods[110] would add eye-watering details to atomic level cinematics of photocatalysis. Coupling this information to macromolecular depictions of PLB disassembly – visualized, for example, by photo-initiated cryoelectron microscopy, mass spectrometry and X-ray scattering – would bridge the crucial knowledge gap of how the photocatalysis marshals chloroplast maturation. These are exciting times to shine a light on the many roles of LPOR.

References

1. Hörtensteiner, S. Update on the biochemistry of chlorophyll breakdown. *Plant Mol. Biol.* **82**, 505−517 (2013).
2. Rudiger, W. Chlorophyll metabolism: From outer space down to the molecular level. *Phytochem.* **46,** 1151-1167 (1997).
3. Masuda, T. & Fujita, Y. Regulation and evolution of chlorophyll metabolism. *Photochem. Photobiol. Sci.* **7**, 1131−1149 (2008).
4. Stenbaek, A. & Jensen, P. E. Redox regulation of chlorophyll biosynthesis. *Phytochem.* **71**, 853−859 (2010).
5. Mochizuki, N., Tanaka, R., Grimm, B., Masuda, T., Moulin, M., Smith, A. G., Tanaka, A. & Terry, M. J. The cell biology of tetrapyrroles: A life and death struggle. *Trends Plant Sci.* **15**, 488−498 (2010).
6. Czarnecki, O. & Grimm, B. Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. *J. Exp. Bot.* **63**, 1675−1687 (2012).
7. Wang, P. & Grimm, B. Organization of chlorophyll biosynthesis and insertion of chlorophyll into the chlorophyll-binding proteins in chloroplasts. *Photosynth. Res.* **126**, 189−202 (2015).
8. Kobayashi, K. & Masuda, T. Transcriptional Regulation of Tetrapyrrole Biosynthesis in *Arabidopsis thaliana*. *Front. Plant Sci.* **7**, 1811 (2016).
9. Armarego-Marriott, T., Sandoval-Ibanez, O. & Kowalewska, L. Beyond the darkness: recent lessons from etiolation and de-etiolation studies. *J. Exp. Bot.* 71, 1215-1225 (2020).
10. Chidgey, J. W., Jackson, P. J., Dickman, M. J. & Hunter C. N. PufQ regulates porphyrin flux at the haem/bacteriochlorophyll branchpoint of tetrapyrrole biosynthesis via interactions with ferrochelatase. *Mol. Microbiol.* **106**, 961-975 (2017).
11. Chen, G. E., Canniffe, D. P., Barnett, S. F. H., Hollingshead, S., Brindley, A. A., Vasilev, C., Bryant, D. A. & Hunter, C. N. Complete enzyme set for **chlorophyll** biosynthesis in *Escherichia coli*. Sci. Adv. 4, eaaq1407 (2018).

12. Chen, X., Wang, X., Feng, J., Chen, Y., Fang, Y., Zhao, S., Zhao, A., Zhang, M. & Liu, L. Structural insights into the catalytic mechanism of Synechocystis magnesium protoporphyrin IX O-methyltransferase (ChlM). *J. Biol. Chem.* **289**, 25690-25698 (2014).

13. Richter, A. S., Wang, P. & Grimm, B. Arabidopsis Mg-Protoporphyrin IX Methyltransferase Activity and Redox Regulation Depend on Conserved Cysteines. *Plant Cell Physiol.* **57**, 519-527 (2016).

14. Hollingshead, S., Bliss, S., Baker, P. J. & Hunter C. N. Conserved residues in Ycf54 are required for protochlorophyllide formation in *Synechocystis* sp. PCC 6803. *Biochem. J.* **474**, 667-681 (2017).

15. Chen, G. E., Canniffe, D. P. & Hunter, C. N. Three classes of oxygen-dependent cyclase involved in chlorophyll and bacteriochlorophyll biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 6280-6285 (2017).

16. Heyes, D. J., Kruk, J. & Hunter, C. N. Spectroscopic and kinetic characterization of the light-dependent enzyme protochlorophyllide oxidoreductase (POR) using monovinyl and divinyl substrates. *Biochem. J.* **394**, 243-248 (2006).

17. Hitchcock, A., Jackson, P. J., Chidgey, J. W., Dickman, M. J., Hunter, C. N. & Canniffe, D. P. Biosynthesis of Chlorophyll a in a Purple Bacterial Phototroph and Assembly into a Plant Chlorophyll-Protein Complex. ACS Synth Biol. 5, 948-954 (2016).

18. Schoefs, B. & Franck, F. Protochlorophyllide Reduction: Mechanisms and Evolution. *Photochem. Photobiol.* **78**, 543–557 (2003).

19. Heyes, D. J. & Hunter, C. N. Making light work of enzyme catalysis: protochlorophyllide oxidoreductase. *Trends Biochem. Sci.* **30**, 642-649 (2005).

20. Reinbothe, C., El Bakkouri, M., Buhr, F., Muraki, N., Nomata, J., Kurisu, G., Fujita, Y. & Reinbothe, S. Chlorophyll biosynthesis: spotlight on protochlorophyllide reduction. *Trends Plant Sci.* **15**, 614-624 (2010).

21. Scrutton, N.S., Groot, M.L., Heyes, D.J. Excited state dynamics and catalytic mechanism of the light-driven enzyme protochlorophyllide oxidoreductase. *Phys Chem Chem Phys* **14**, 8818-8824 (2012).

22. Gabruk, M. & Mysliwa-Kurdziel, B. Light-dependent protochlorophyllide oxidoreductase: phylogeny, regulation, and catalytic properties. *Biochemistry* **54**, 5255-5262 (2015).

23. Yang, J. & Cheng, Q. Origin and evolution of the light-dependent protochlorophyllide oxidase (LPOR) genes. *Plant Biol.* **6**, 537-544 (2004).

24. Armstrong, G. A. Greening in the dark: Light independent chlorophyll biosynthesis from anoxygenic photosynthetic bacteria to gymnosperms. J. *Photochem. Photobiol. B* **43**, 87-100 (1998).

25. Vedalankar, P. & Tripathy, B. C. Evolution of light-independent protochlorophyllide oxidoreductase. *Protoplasma* **256**, 293-312 (2019).

26. Sarma, R., Barney, B. M., Hamilton, T. L., Jones, A., Seefeldt, L. C. & Peters, J. W. Crystal structure of the L protein of *Rhodobacter sphaeroides* light-independent protochlorophyllide reductase with MgADP bound: A homologue of the nitrogenase Fe protein. *Biochemistry* **47**, 13004-13015 (2008).

27. Bröcker, M. J., Schomburg, S., Heinz, D. W., Jahn, D., Schubert, W. D. & Moser, J. Crystal structure of the nitrogenase-like dark operative protochlorophyllide oxidoreductase catalytic complex $(ChlN/ChlB)_2$. *J. Biol. Chem.* **285**, 27336-27345 (2010).

28. Yamamoto, H., Kurumiya, S., Ohashi, R. & Fujita, Y. Oxygen sensitivity of a nitrogenase-like protochlorophyllide reductase from the cyanobacterium *Leptolyngbya boryana*. *Plant Cell Physiol.* **50**, 1663-1673 (2009).

29. Kaschner, M., Loeschcke, A., Krause, J., Minh, B. Q., Heck, A., Endres, S., Svensson, V., Wirtz, A., von Haeseler, A., Jaeger, K. E., Drepper, T. & Krauss, U. Discovery of the first light dependent protochlorophyllide oxidoreductase in anoxygenic phototrophic bacteria. *Mol. Microbiol.* **93**, 1066-1078 (2014).

30. Kasalicky, V., Zeng, Y., Piwosz, K., Šimek, K., Kratochvilová, H. & Koblížek, M. Aerobic Anoxygenic Photosynthesis Is Commonly Present within the Genus *Limnohabitans*. *Appl. Env. Microbiol.* **84**, e02116-17 (2018).

31. Chernomor, O., Peters, L., Schneidewind, J., Loeschcke, A., Knieps-Grünhagen, E., Schmitz, F., von Lieres, E., Kutta, R. J., Svensson, V., Jaeger, K. E., Drepper, T., von Haeseler, A. & Krauss, U. Complex evolution of light-dependent

protochlorophyllide oxidoreductases in aerobic anoxygenic phototrophs: origin, phylogeny and function. *Mol. Biol. Evol.* in press (2020). DOI: 10.1093/molbev/msaa234

32. Reinbothe, S., Quigley, F., Gray, J., Schemenewitz, A., Reinbothe, C. Identification of plastid envelope proteins required for import of protochlorophyllide oxidoreductase A into the chloroplast of barley. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2197–2202 (2004).

33. Reinbothe, S., Gray, J., Rustgi, S., von Wettstein, D. & Reinbothe, C. Cell growth defect factor 1 is crucial for the plastid import of NADPH:protochlorophyllide oxidoreductase A in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5838–5843 (2015).

34. Kauss, D., Bischof, S., Steiner, S., Apel, K. & Meskauskiene, R. FLU, a negative feedback regulator of tetrapyrrole biosynthesis, is physically linked to the final steps of the Mg(++)-branch of this pathway. *FEBS Lett* **586**, 211–216 (2012).

35. Zhang, W., Zhong, H., Lu, H., Zhang, Y., Deng, X., Huang, K. & Duanmu, D. Characterization of Ferredoxin-Dependent Biliverdin Reductase PCYA1 Reveals the Dual Function in Retrograde Bilin Biosynthesis and Interaction With Light-Dependent Protochlorophyllide Oxidoreductase LPOR in *Chlamydomonas reinhardtii*. *Front. Plant Sci.* **9**, 676 (2018).

36. Schottkowski, M., Ratke, J., Oster, U., Nowaczyk, M. & Nickelsen, J. Pitt, a novel tetratricopeptide repeat protein involved in light-dependent chlorophyll biosynthesis and thylakoid membrane biogenesis in *Synechocystis* sp. PCC 6803. *Mol. Plant* **2**, 1289–1297 (2009).

37. Hey, D., Rothbart, M., Herbst, J., Wang, P., Müller, J., Wittmann, D., Gruhl, K. & Grimm, B. LIL3, a Light-Harvesting Complex Protein, Links Terpenoid and Tetrapyrrole Biosynthesis in *Arabidopsis thaliana*. *Plant Physiol.* **174**, 1037–1050 (2017).

38. Smith, J. H. C. & Kupke, D. W. (1956) Some Properties of Extracted Protochlorophyll Holochrome. *Nature* **178**, 751−752.

39. Solymosi, K. & Schoefs, B. Etioplast and etiochloroplast formation under natural conditions: the dark side of chlorophyll biosynthesis in angiosperms. *Photosynth. Res.* **105**, 143-166 (2010).

40. Josse, E-M. & Halliday, K. J. Skotomorphogenesis: The dark side of light signalling. *Current Biol.* **18**, R1144-R1146 (2008).

41. Adam, Z., Charuvi, D., Tsabari, O., Knopf, R. R. & Reich, Z. Biogenesis of thylakoid networks in angiosperms: knowns and unknowns. *Plant Mol. Biol.* **76**, 221–234 (2011).

42. Grzyb, J. M., Solymosi, K., Strzalka, K. & Mysliwa-Kurdziel, B. Visualization and characterization of prolamellar bodies with atomic force microscopy. *J. Plant Physiol.* **170,** 1217-1227 (2013).

43. Kowalewska, L., Mazur, R., Suski, S., Garstka, M. & Mostowska, A. Three-Dimensional Visualization of the Tubular-Lamellar Transformation of the Internal Plastid Membrane Network during Runner Bean Chloroplast Biogenesis. *Plant Cell* **28**, 875-891 (2016).

44. Gabruk, M., Mysliwa-Kurdziel, B. & Kruk, J. MGDG, PG and SQDG regulate the activity of light-dependent protochlorophyllide oxidoreductase. *Biochem. J.* **474,** 1307-1320 (2017).

45. Fujii, S., Kobayashi, K., Nagata, N., Masuda, T. & Wada, H. Monogalactosyldiacylglycerol facilitates synthesis of photoactive protochlorophyllide in etioplasts. *Plant Physiol.* **174,** 2183-2198 (2017).

46. Fujii, S., Kobayashi, K., Nagata, N., Masuda, T. & Wada, H. Digalactosyldiacylglycerol is essential for organization of the membrane structure in etioplasts. *Plant Physiol.* **177,** 1487-1497 (2018).

47. Fujii, S., Nagata, N., Masuda, T., Wada, H. & Kobayashi, K. Galactolipids are essential for internal membrane transformation during etioplast-to-chloroplast differentiation. *Plant Cell Physiol.* **60,** 1224-1238 (2019).

48. Aronsson, H., Sundqvist, C. & Dahlin, C. POR hits the road: import and assembly of a plastid protein. *Plant Mol. Biol.* **51**, 1–7 (2003).

49. Selstam, E., Schelin, J., Brain, T. &Williams, W. P. The effects of low pH on the properties of protochlorophyllide oxidoreductase and the organization of

prolamellar bodies of maize (*Zea mays*). *Eur. J. Biochem.* **269**: 2336–2346 (2002).

50. Selstam, E., Brain, A. P. R. & Williams, W. P. The relationship between different spectral forms of the protochlorophyllide oxidoreductase complex and the structural organisation of prolamellar bodies isolated from *Zea mays*. *Photosynth. Res.* **108**, 47-59 (2011).

51. Masuda, S., Ikeda, R., Masuda, T., Hashimoto, H., Tsuchiya, T., Kojima, H., Nomata, J., Fujita, Y., Mimuro, M., Ohta, H. & Takamiya, K. I. Prolamellar bodies formed by cyanobacterial protochlorophyllide oxidoreductase in *Arabidopsis*. *Plant J.* **58**, 952-960 (2009).

52. Yamamoto, H., Kojima-Ando, H., Ohki, K. & Fujita, Y. Formation of prolamellar-body-like ultrastructures in etiolated cyanobacterial cells overexpressing light-dependent protochlorophyllide oxidoreductase in *Leptolyngbya boryana*. *J. Gen. Appl. Microbiol.* **66**, 129-139 (2020).

53. Pribil, M., Labs, M. & Leister, D. Structure and dynamics of thylakoids in land plants. *J. Exp. Bot.* **65**, 1955–1972 (2014).

54. Schoefs, B. & Franck, F. The photoenzymatic cycle of NADPH: protochlorophyllide oxidoreductase in primary bean leaves (*Phaseolus vulgaris*) during the first days of photoperiodic growth. *Photosyn. Res.* **96**, 15–26 (2008).

55. Rumak, I., Gieczewska, K., Kierdaszuk, B., Gruszecki, W. I., Mostowska, A., Mazur, R. & Garstka, M. 3-D modelling of chloroplast structure under (Mg^{2+}) magnesium ion treatment. Relationship between thylakoid membrane arrangement and stacking. *Biochim. Biophys. Acta* **1797**, 1736–1748 (2010).

56. Holtorf, H., Reinbothe, S., Reinbothe, C. & Bereza, B. Two routes of chlorophyllide synthesis that are differentially regulated by light in barley. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3254–3258 (1995).

57. Su, Q., Frick, G., Armstrong, G. & Apel, K. POR C of *Arabidopsis thaliana*: a third light- and NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by light. *Plant Mol. Biol.* **47**, 805–813 (2001).

58. Masuda, T. & Takamiya, K. Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. *Photosyn. Res.* **81,** 1-29 (2004).

59. Gabruk, M. & Mysliwa-Kurdziel, B. The origin, evolution and diversification of multiple isoforms of light-dependent protochlorophyllide oxidoreductase (LPOR): focus on angiosperms. *Biochem. J.* **477**, 2221–2236 (2020).

60. Buhr, F., El Bakkouri, M., Valdez, O., Pollmann, S., Lebedev, N., Reinbothe, S. & Reinbothe, C. Photoprotective role of NADPH:protochlorophyllide oxidoreductase A. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12629−12634 (2008).

61. Armstrong, G. A., Apel, K. & Rüdiger, W. Does a light-harvesting protochlorophyllide a/b-binding protein complex exist? *Trends Plant Sci.* **5**, 40−44 (2000).

62. Yuan, M., Zhang, D. W., Zhang, Z. W., Chen, Y. E., Yuan, S., Guo, Y. R. & Lin, H. H. Assembly of NADPH:protochlorophyllide oxidoreductase complex is needed for effective greening of barley seedlings. *J. Plant Physiol.* **169**, 1311–1316 (2012).

63. Pattanayak, G. K. & Tripathy, B. C. Overexpression of Protochlorophyllide Oxidoreductase C Regulates Oxidative Stress in Arabidopsis. *Plos One* **6**, e26532 (2011).

64. Zhan, W., Liu, J., Pan, Q., Wang, H., Yan, S., Li, K., Deng, M., Li, W., Liu, N., Kong, Q., Fernie, A. R. & Yan, J. An allele of ZmPORB2 encoding a protochlorophyllide oxidoreductase promotes tocopherol accumulation in both leaves and kernels of maize. *Plant J.* **100**, 114-127 (2019).

65. Boddi, B., Lindsten, A., Ryberg, M. & Sundqvist, C. On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. *Physiol. Plant.* **76**, 135–143 (1989).

66. Schneidewind, J., Krause, F., Bocola, M., Stadler, A. M., Davari, M. D., Schwaneberg, U., Jaeger, K. E. & Krauss, U. Consensus model of a cyanobacterial light-dependent protochlorophyllide oxidoreductase in its pigment-free apo-form and photoactive ternary complex. *Comm. Biol.* **2**, 351 (2019).

67. Zhang, S., Heyes, D. J., Feng, L., Sun, W., Johannissen, L. O., Liu, H., Levy, C. W., Li, X., Yang, J., Yu, X., Lin, M., Hardman, S. J. O., Hoeven, R., Sakuma, M., Hay, S., Leys, D., Rao, Z., Zhou, A., Cheng, Q. & Scrutton, N. S. Structural basis for enzymatic photocatalysis in chlorophyll biosynthesis. *Nature* **574**, 722-725 (2019).

68. Gabruk, M., Stecka, A., Strzałka, W., Kruk, J., Strzałka, K. & Mysliwa-Kurdziel, B. Photoactive protochlorophyllide-enzyme complexes reconstituted with PORA, PORB and PORC proteins of *A. thaliana*: fluorescence and catalytic properties. *PLoS One* **10**, e0116990 (2015).

69. Gabruk, M., Nowakowska, Z., Skupien-Rabian, B., Kedracka-Krok, S., Mysliwa-Kurdziel, B. & Kruk, J. Insight into the oligomeric structure of PORA from *A. thaliana. Biochim. Biophys. Acta - Proteins Proteom.* **1864**, 1757-1764 (2016).

70. Zhang, S., Godwin, A. R. F., Taylor, A., Hardman, S. J. O., Jowitt, T. A., Johannissen, L. O., Hay, S., Baldock, C., Heyes, D. J. & Scrutton, N. S. Dual role of the active site 'lid' regions of protochlorophyllide oxidoreductase in photocatalysis and plant development. *FEBS J.* in press (2020). DOI: 10.111/febs.15542

71. Nguyen, H. C., Melo, A. A Kruk, J., Frost, A. & Gabruk, M. Photocatalytic plant LPOR forms helical lattices that shape membranes for chlorophyll synthesis. bioRxiv (pre-print)**.** DOI: 10.1101/2020.08.19.257774

72. Aronsson, H., Sundqvist, C. & Dahlin, C. POR-import and membrane association of a key element in chloroplast development. *Physiol. Plant.* **118**, 1–9 (2003).

73. Heyes, D. J., Menon, B. R. K., Sakuma, M. & Scrutton, N. S. Conformational events during ternary enzyme-substrate complex formation are rate limiting in the catalytic cycle of the light-driven enzyme protochlorophyllide oxidoreductase. *Biochemistry* **47**, 10991–10998 (2008).

74. Heyes, D. J., Hardman, S. J. O., Mansell, D., Gardiner, J. M. & Scrutton, N. S. Mechanistic reappraisal of early stage photochemistry in the light-driven enzyme protochlorophyllide oxidoreductase. *PLoS One*, **7**, e45642 (2012).

75. Kolossov, V. L., Kopetz, K. J. & Rebeiz, C. A. Chloroplast biogenesis 87: Evidence of resonance excitation energy transfer between tetrapyrrole

intermediates of the chlorophyll biosynthetic pathway and chlorophyll a. *Photochem. Photobiol.* **78**, 184-96 (2003).

76. Sytina, O. A., Heyes, D. J., Hunter, C. N., Alexandre, M. T., van Stokkum, I. H. M., van Grondelle, R., & Groot, M. L. Conformational changes in an ultrafast light-driven enzyme determine catalytic activity. *Nature*, **456**, 1001-1004 (2008).

77. Stadler, A. M., Schneidewind, J., Zamponi, M., Knieps-Grünhagen, E., Gholami, S., Schwaneberg, U., Rivalta, I., Garavelli, M., Davari, M. D., Jaeger, K. E. & Krauss, U. Ternary Complex Formation and Photoactivation of a Photoenzyme Results in Altered Protein Dynamics. *J. Phys. Chem. B* **123**, 7372-7384 (2019).

78. Menon, B. R., Hardman, S. J., Scrutton, N. S. & Heyes, D. J. Multiple active site residues are important for photochemical efficiency in the light-activated enzyme protochlorophyllide oxidoreductase (POR). *J. Photochem. Photobiol. B.* **161**, 236–243 (2016).

79. Gholami, S., Nenov, A., Rivalta, I., Bocola, M., Bordbar, A. K., Schwaneberg, U., Davari, M. D. & Garavelli, M. Theoretical Model of the Protochlorophyllide Oxidoreductase from a Hierarchy of Protocols. *J. Phys. Chem. B* **122**, 7668–7681 (2018).

80. Dong, C. S., Zhang, W. L., Wang, Q., Li, Y. S., Wang, X., Zhang, M. & Liu, L. Crystal structures of cyanobacterial light-dependent protochlorophyllide oxidoreductase. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 8455-8461 (2020).

81. Ho, M. Y., Shen, G., Canniffe, D. P., Zhao, C. & Bryant, D. A. Light-dependent chlorophyll f synthase is a highly divergent paralog of PsbA of photosystem II. *Science* **353**, aaf9178 (2016)

82. Aubert, C., Vos, M. H., Mathis, P., Eker, A. P. & Brettel, K. Intraprotein radical transfer during photoactivation of DNA photolyase. *Nature* **405**, 586-590 (2000).

83. Sorigue, D., Légeret, B., Cuiné, S., Blangy, S., Moulin, S., Billon, E., Richaud, P., Brugière, S., Couté, Y., Nurizzo, D., Müller, P., Brettel, K., Pignol, D., Arnoux, P., Li-Beisson, Y., Peltier, G. & Beisson, F. An algal photoenzyme converts fatty acids to hydrocarbons. *Science* **357**, 903-907 (2017).

84. Schmermund, L., Bierbaumer, S., Schein, V. K., Winkler, C. K., Kara, S. & Kroutil, W. Extending the Library of Light-Dependent Protochlorophyllide

Oxidoreductases and their Solvent Tolerance, Stability in Light and Cofactor Flexibility. *ChemCatChem* **12**, 4044–4051 (2020).

85. Dietzek, B., Maksimenka, R., Siebert, T., Birckner, E., Kiefer, W., Popp, J., Hermann, G. & Schmitt, M. Excited-state processes in protochlorophyllide a a femtosecond time-resolved absorption study. *Chem. Phys. Lett.* **397**, 110-115 (2004).

86. Dietzek, B., Kiefer, W., Hermann, G., Popp, J. & Schmitt, M. Solvent effects on the excited-state processes of protochlorophyllide: a femtosecond time-resolved absorption study. *J. Phys. Chem. B* **110**, 4399-4406 (2006).

87. Dietzek, B., Kiefer, W., Yartsev, A., Sundstrom, V., Schellenberg, P., Grigaravicius, P., Hermann, G., Popp, J. & Schmitt, M. The excited-state chemistry of protochlorophyllide a: a time-resolved fluorescence study. *ChemPhysChem* **7**, 1727-1733 (2006).

88. Dietzek, B., Tschierlei, S., Hermann, G., Yartsev, A., Pascher, T., Sundstrom, V., Schmitt, M. & Popp, J. Protochlorophyllide a: A comprehensive photophysical picture. *ChemPhysChem* **10**, 144-150 (2009).

89. Dietzek, B., Tschierlei, S., Hanf, R., Seidel, S., Yartsev, A., Schmitt, M., Hermann, G. & Popp, J. Dynamics of charge separation in the excited-state chemistry of protochlorophyllide. *Chem. Phys. Lett.* **492**, 157-163 (2010).

90. Sytina, O. A., van Stokkum, I. H. M., Heyes, D. J., Hunter, C. N., van Grondelle, R. & Groot, M. L. Protochlorophyllide excited-state dynamics in organic solvents studied by time-resolved visible and mid-infrared spectroscopy. *J. Phys. Chem. B* **114**, 4335-4344 (2010).

91. Heyes, D. J., Hardman, S. J., Mansell, D., Ní Cheallaigh, A., Gardiner, J. M., Johannissen, L. O., Greetham, G. M., Towrie, M. & Scrutton, N. S. Excited-State Properties of Protochlorophyllide Analogues and Implications for Light-Driven Synthesis of Chlorophyll. *J. Phys. Chem. B* **121**, 1312-1320 (2017).

92. Brandariz-de-Pedro, G., Heyes, D. J., Hardman, S. J., Shanmugam, M., Jones, A. R., Weber, S., Nohr, D., Scrutton, N. S. & Fielding, A. J. Direct Evidence of an Excited-State Triplet Species upon Photoactivation of the Chlorophyll Precursor Protochlorophyllide. *J. Phys. Chem. Lett.* **8**, 1219-81223 (2017).

93. Zhao, G.J. & Han, K.L. Site-specific solvation of the photoexcited protochlorophyllide a in methanol: formation of the hydrogen-bonded intermediate state induced by hydrogen-bond strengthening. *Biophys. J.* **94**, 38-46 (2008).

94. Heyes, D. J. Hardman, S. J. O., Hedison, T. M., Hoeven, R., Greetham, G. M., Towrie, M. & Scrutton, N. S. Excited state charge separation in the photochemical mechanism of the light-driven enzyme protochlorophyllide oxidoreductase. *Angewandte Chemie Intl. Ed.* **54**, 1512-1515 (2015).

95. Heyes, D. J., Sakuma, M., de Visser, S. & Scrutton, N. S. Nuclear quantum tunneling in the light-activated enzyme protochlorophyllide oxidoreductase. *J. Biol. Chem.* **284**, 3762-3767 (2009).

96. Heyes, D. J., Sakuma, M. & Scrutton, N. S. Solvent-slaved protein motions accompany proton but not hydride tunneling in light-activated protochlorophyllide oxidoreductase, *Angew. Chem. Int. Edit.* **48**, 3850-3853 (2009).

97. Menon, B. R. K., Waltho, J. P., Scrutton, N. S. & Heyes, D. J. Cryogenic and laser photoexcitation studies identify multiple roles for active site residues in the light-driven enzyme protochlorophyllide oxidoreductase. *J. Biol. Chem.* **284**, 18160-18166 (2009).

98. Menon, B. R., Davison, P. A., Hunter, C. N., Scrutton, N. S. & Heyes, D. J. Mutagenesis alters the catalytic mechanism of the light-driven enzyme protochlorophyllide oxidoreductase. *J. Biol. Chem.* **285**, 2113-2119 (2010).

99. Heyes, D. J., Levy, C., Sakuma, M., Robertson, D. L. & Scrutton, N. S. A Twin-track Approach Has Optimized Proton and Hydride Transfer by Dynamically Coupled Tunneling during the Evolution of Protochlorophyllide Oxidoreductase. *J. Biol. Chem.* **286**, 11849-11854 (2011).

100. Hoeven, R., Hardman, S. J. O., Heyes, D. J. & Scrutton, N. S. Cross-species analysis of protein dynamics associated with hydride and proton transfer in the catalytic cycle of the light-driven enzyme protochlorophyllide oxidoreductase, *Biochemistry* **55**, 903-913 (2016).

101. Archipowa, N., Kutta, R. J., Heyes, D. J. & Scrutton, N. S. Stepwise Hydride Transfer in a Biological System: Insights into the Reaction Mechanism of the Light-Dependent Protochlorophyllide Oxidoreductase. *Angew. Chem. Int. Ed.* **57**, 2682-2686 (2018).

102. Heyes, D. J., Ruban, A. V., Wilks, H. M. & Hunter, C. N. Enzymology Below 200 K: the Kinetics and Thermodynamics of the Photochemistry Catalyzed by Protochlorophyllide Oxidoreductase. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11145−11150 (2002).

103. Heyes, D. J., Ruban, A. V. & Hunter, C. N. Protochlorophyllide Oxidoreductase: "Dark" Reactions of a Light-Driven Enzyme. *Biochemistry* **42**, 523−528 (2003).

104. Heyes, D. J. & Hunter, C. N. Identification and Characterization of the Product Release Steps Within the Catalytic Cycle of Protochlorophyllide Oxidoreductase. *Biochemistry* **43**, 8265−8271 (2004).

105. Heyes, D. J., Heathcote, P., Rigby, S. E., Palacios, M. A., van Grondelle, R. & Hunter, C. N. The First Catalytic Step of the Light driven Enzyme Protochlorophyllide Oxidoreductase Proceeds via a Charge Transfer Complex. *J. Biol. Chem.* **281**, 26847−26853 (2006).

106. Durin, G., Delaunay, A., Darnault, C., Heyes**,** D. J., Royant, A., Vernede, X., Hunter, C. N., Weik, M. & Bourgeois, D. Simultaneous measurements of solvent dynamics and functional kinetics in a light-activated enzyme. *Biophys J.* **96**, 1902-1910 (2009).

107. Garrone, A., Archipowa, N., Zipfel, P. F., Hermann, G. & Dietzek, B. Plant Protochlorophyllide Oxidoreductases A and B: Catalytic efficiency and initial reaction steps. *J. Biol. Chem.* **290**, 28530-28539 (2015).

108. Raskin, V. I. & Schwartz, A. The charge-transfer complex between protochlorophyllide and NADPH: an intermediate in protochlorophyllide photoreduction. *Photosyn. Res.* **74,** 181–186 (2002).

109. Heyes, D. J., Sakuma, M. & Scrutton, N. S. Laser excitation studies of the product release steps in the catalytic cycle of the light-driven enzyme, protochlorophyllide oxidoreductase. *J. Biol. Chem.* **282**, 32015-32020 (2007).

110. Levantino, M., Yorke, B. A., Monteiro, D. C., Cammarata, M. & Pearson, A. R. Using synchrotrons and XFELs for time-resolved X-ray crystallography and solution scattering experiments on biomolecules. *Curr. Op. Struct. Biol.* **35**, 41-48 (2015).

Correspondence

Correspondence can be addressed to derren.heyes@manchester.ac.uk and nigel.scrutton@manchester.ac.uk

Author contributions

D.J.H. and N.S.S. conceived and led on the writing of the manuscript. S.Z., A.T., L.O.J., S.J.O.H. and S.H. contributed to the writing of specific sections, critical reading of the manuscript and reviewing of appropriate references. S.Z., D.J.H and A.T. prepared figures.

Competing interests

The authors declare no competing interests

Figure legends

Figure 1. Overview of chlorophyll biosynthetic pathway. The enzymatic steps involved in the synthesis of chlorophyll starting from 5-aminolevulinic acid. The steps up to the formation of protoporphyrin IX are also common to heme biosynthesis and involve following enzymes: ALAD, aminolevulinic acid dehydratase; PBGD, porphobilinogen deaminase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen III decarboxylase; CPOX, coproporphyrinogen oxidase; PPOX, protoporphyrinogen IX oxidase. Insertion of Mg^{2+} by Mg chelatase leads to synthesis of chlorophyll. The penultimate step, which involves the reduction of protochlorophyllide, can be catalyzed by two different enzymes, a light-dependent and a light-independent protochlorophyllide oxidoreductase.

Figure 2. The role of LPOR in chloroplast development. The steps involved in the conversion of the protoplast to the mature chloroplast *via* the etioplast are shown. Galactolipids, anionic lipids that are characteristic for plastids, are important for prolamellar bodies (PLBs) formation and during later greening stages. LPOR and Pchlide build up on the lipid bilayer to form the PLBs within the etioplast. Illumination triggers LPOR photocatalysis, leading to chlorophyll accumulation and the change from skotomorphogenesis to photomorphogenesis. PLBs disintegrate and chlorophyll-containing thylakoids are formed. These then fuse together to form the grana and stoma of the mature chlorophyll as the photosynthetic complexes are generated. Timescales for each process are estimated from recent 3D reconstruction studies.[43]

Figure 3. Overview of the structural organization of LPOR. The crystal structures of apo-LPOR and a LPOR-NADPH binary complex solved for cyanobacterial LPORs.[67,80] A structural model of the LPOR–Pchlide–NADPH ternary complex highlights conformational change on Pchlide binding and active site residues that interact with the Pchlide molecule.[67] Structural changes induced by Pchlide binding lead to dimerization of LPOR–Pchlide–NADPH ternary complexes[66] and further interactions lead to larger oligomers required for PLB formation.

Figure 4: Timeline of chemical and structural changes triggered by illumination of LPOR–Pchlide–NADPH complexes. The initial absorption of light by bound Pchlide induces chemical and structural change at the molecular, cellular and whole organism levels. The Pchlide excited state leads to LPOR photocatalysis, involving

sequential hydride and proton transfer steps, to form Chlide and subsequent conversion to chlorophyll. At the cellular level, PLBs disintegrate to form thylakoids in the mature chloroplast as the plant begins to develop.

Figure 1.

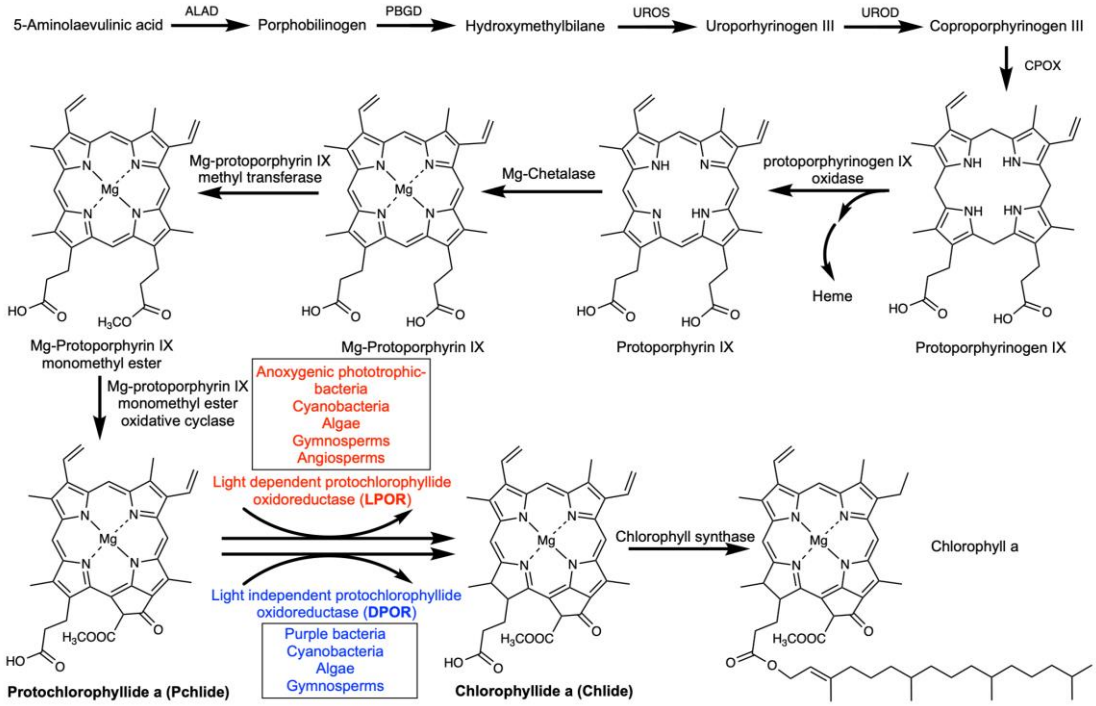

5-Aminolaevulinic acid
ALAD
Porphobilinogen
PBGD
Hydroxymethylbilane
UROS
Uroporhyrinogen III
UROD
Coproporphyrinogen III
CPOX
Protoporphyrinogen IX
protoporphyrinogen IX oxidase
Heme
Protoporphyrin IX
Mg-Chetalase
Mg-Protoporphyrin IX
Mg-protoporphyrin IX methyl transferase
Mg-Protoporphyrin IX monomethyl ester
Mg-protoporphyrin IX monomethyl ester oxidative cyclase
Anoxygenic phototrophic-bacteria
Cyanobacteria
Algae
Gymnosperms
Angiosperms
Light dependent protochlorophyllide oxidoreductase (LPOR)
Light independent protochlorophyllide oxidoreductase (DPOR)
Purple bacteria
Cyanobacteria
Algae
Gymnosperms
Protochlorophyllide a (Pchlide)
Chlorophyllide a (Chlide)
Chlorophyll synthase
Chlorophyll a

Figure 2.

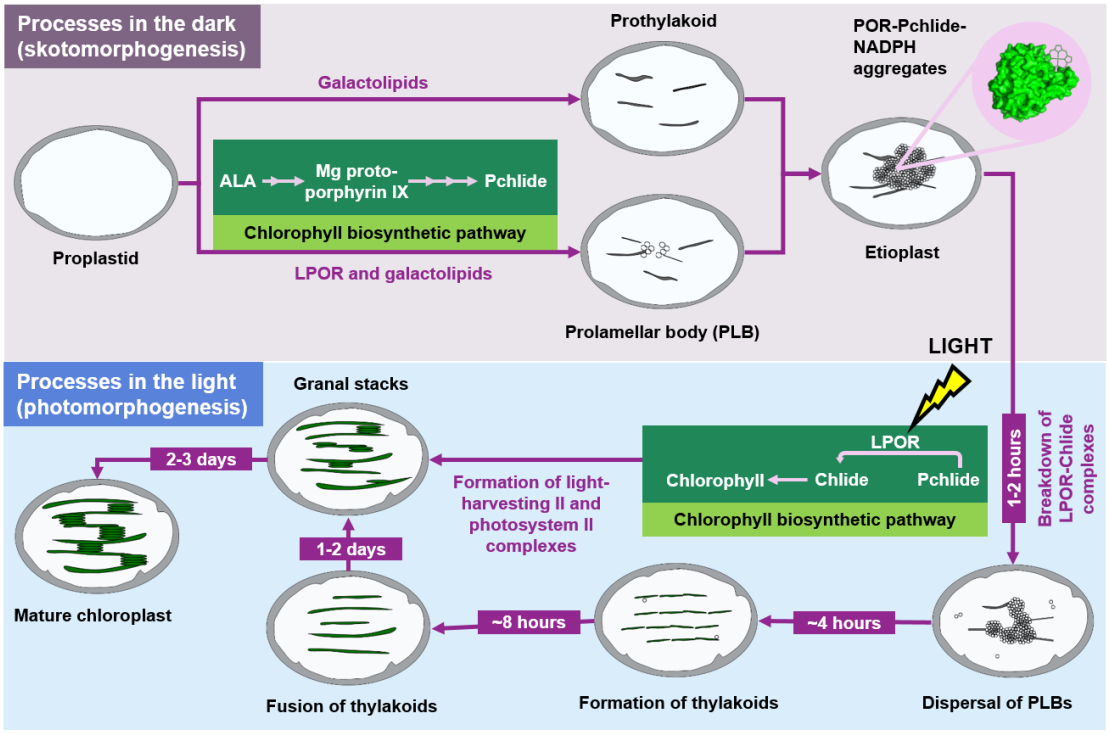

Processes in the dark (skotomorphogenesis)
Prothylakoid
POR-Pchlide-NADPH aggregates
Galactolipids
ALA
Mg proto-porphyrin IX
Pchlide
Chlorophyll biosynthetic pathway
Proplastid
LPOR and galactolipids
Etioplast
Prolamellar body (PLB)
LIGHT
Processes in the light (photomorphogenesis)
Granal stacks
LPOR
Chlorophyll
Chlide
Pchlide
Chlorophyll biosynthetic pathway
1-2 hours
Breakdown of LPOR-Chlide complexes
2-3 days
Formation of light-harvesting II and photosystem II complexes
1-2 days
Mature chloroplast
~8 hours
~4 hours
Fusion of thylakoids
Formation of thylakoids
Dispersal of PLBs

Figure 3.

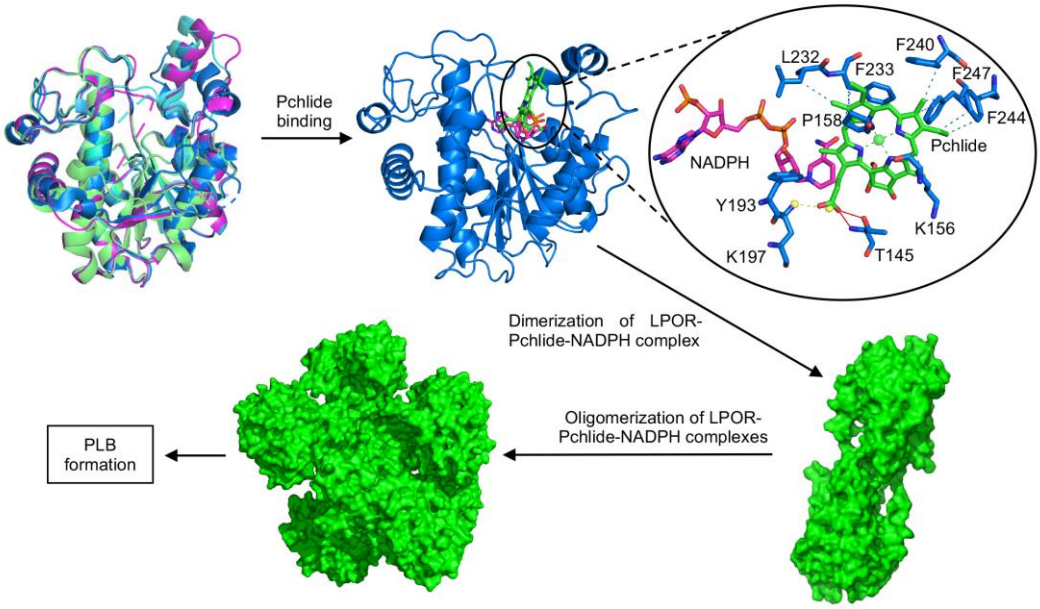
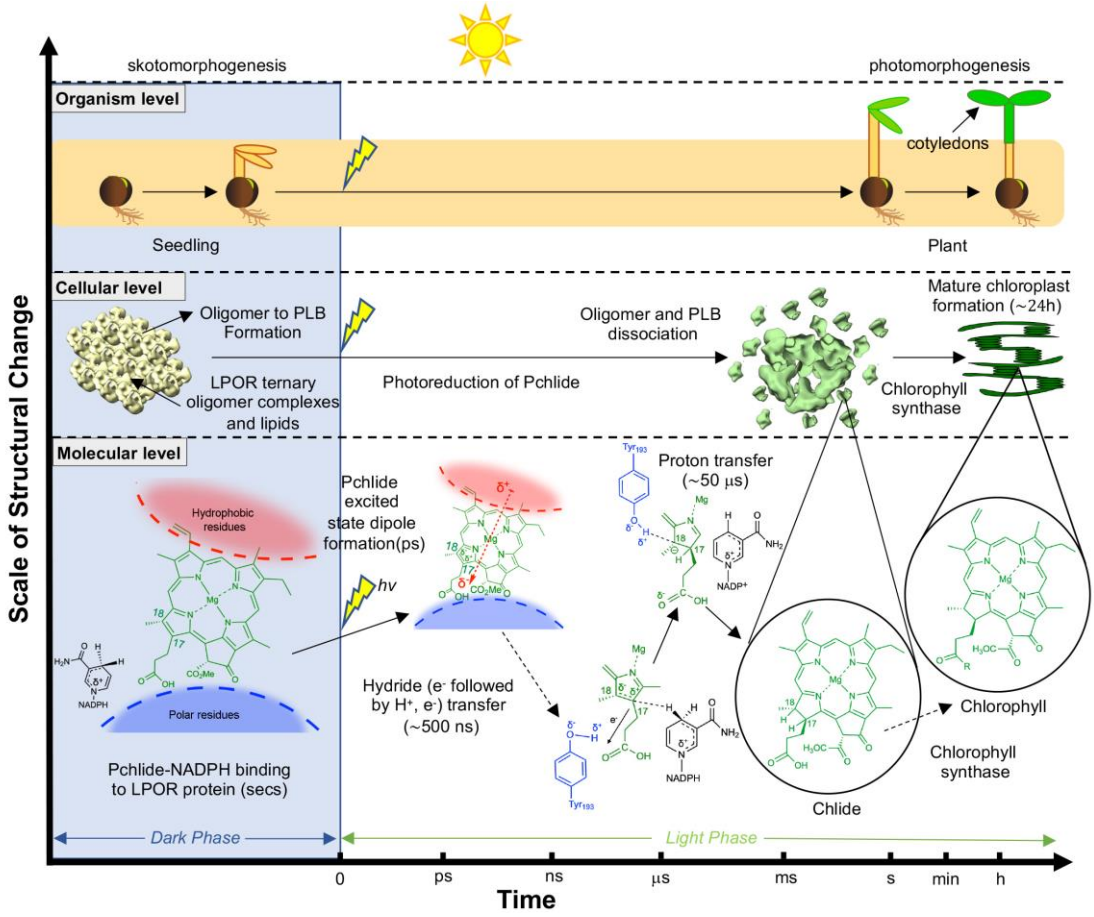

Pchlide
binding
L232
F233
F240
F247
F244
P158
Pchlide
NADPH
Y193
K156
K197
T145
Dimerization of LPOR-
Pchlide-NADPH complex
Oligomerization of LPOR-
Pchlide-NADPH complexes
PLB
formation

Figure 4.


skotomorphogenesis
photomorphogenesis
Organism level
cotyledons
Seedling
Plant
Cellular level
Oligomer to PLB Formation
LPOR ternary oligomer complexes and lipids
Photoreduction of Pchlide
Oligomer and PLB dissociation
Mature chloroplast formation (~24h)
Chlorophyll synthase
Molecular level
Hydrophobic residues
Polar residues
Pchlide excited state dipole formation(ps)
hv
Proton transfer (~50 µs)
Hydride (e⁻ followed by H⁺, e⁻) transfer (~500 ns)
Pchlide-NADPH binding to LPOR protein (secs)
Chlide
Chlorophyll
Chlorophyll synthase
Scale of Structural Change
Dark Phase
Light Phase
0
ps
ns
µs
ms
s
min
h
Time