Chapter 5

Biosynthesis of Chlorophyll and Bilins in Algae

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I. Introduction

Chlorophylls and bilins are tetrapyrrole pigments that are synthesized from the universal five carbon precursor aminolevulinic acid (ALA). Chlorophylls are used as light harvesting pigments but are also essential components for energy transduction within the reaction centres of photosystem I (PSI) and photosystem II (PSII), with chlorophyll a (Fig. 5.1) being the main chlorophyll found in algae and cyanobacteria. Like the chlorophylls, bilins are also used as light harvesting pigments for photosynthesis and they are also used in light sensing. As light harvesting pigments, bilins are usually covalently attached to proteins, known generally as phycobiliproteins, with phycocyanobilin (Fig. 5.1) being one of the most common bilins covalently attached through the $C3^2$ to the protein. These phycobiliproteins are subunits of a large light harvesting protein complex called a phycobilisome (PB) that is associated with PSII. In contrast light sensing bilins are covalently attached to proteins known generally as phytochromes that are used to sense light and produce chemical signals in response to both light quality and light intensity. The spectral properties of both phycobiliproteins and phytochromes are dependent both on the protein environment as well as the type of bilin pigment bound.

All algae and cyanobacteria make chlorophylls, but they don't all make phycobilisomes. Phycobilisomes appear to be restricted to the cyanobacteria, glaucophytes, red algae and the secondary endo-

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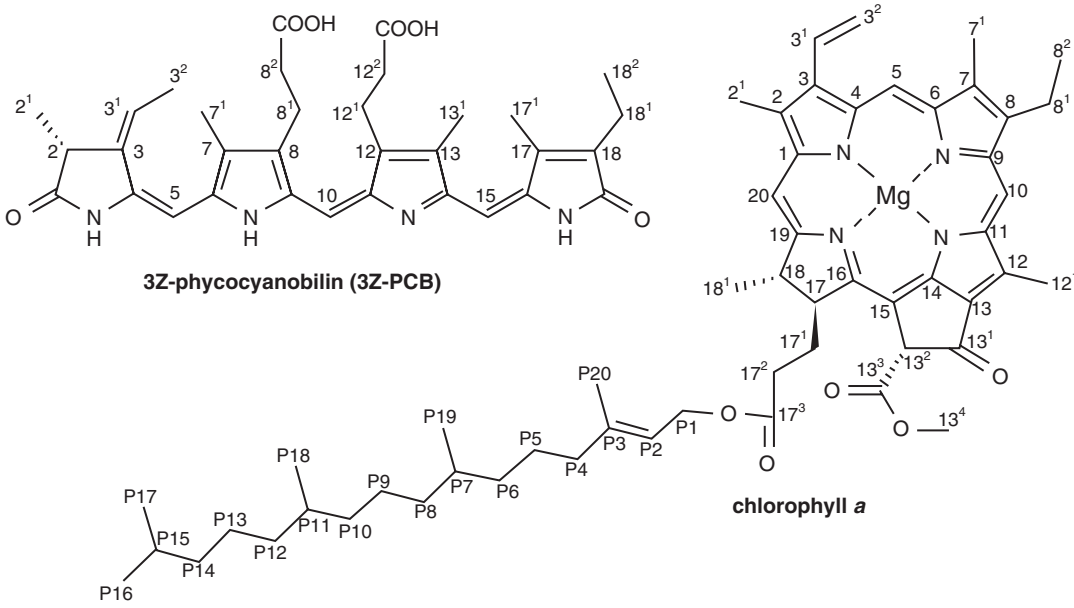


Fig. 5.1. Structure and numbering of a representative bilin and chlorophyll. 3Z-phycocyanobilin and chlorophyll *a*

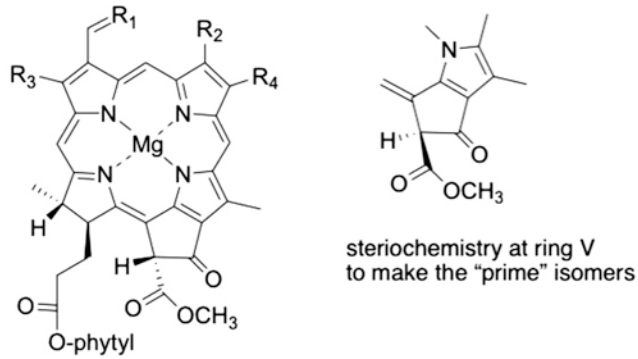
symbiotic ancestors of the red algae such as the cryptophytes (Bernstein and Miller 1989; Stiller et al. 2014). While most cyanobacteria make phycobilisomes, the well known exceptions are the "Prochlorophytes" *Prochloron*, *Prochlorothrix*, and *Prochlorococcus* (Hess et al. 2001). These organisms have a small genome and are the most abundant primary producers on the planet. One of the possible reasons for the lack of phycobilisomes in these organisms is related to evolutionary pressure on nitrogen utilisation. In terms of pigment content chlorophyll dependent light harvesting complexes (LHCs) are more efficient in per pigment molecule in terms of nitrogen usage than PB's as LHCs utilise 1/3rd of the N per pigment molecule bound. The prochlorophytes are thought to be the ancestors of the green algal lineage, which also do not contain PB's, although they do make bilins and in some cases they have the genes to make certain phycobiliproteins and the corresponding bilin (Hess et al. 2001).

This chapter will concentrate on examining the diversity and biosynthesis of both bilins and chlorophylls which are used in light harvesting for photosynthesis and will not discuss in any detail the use of bilins in light sensing or signalling.

II. Diversity of Chlorophylls in Algae

The most common chlorophyll found in algae is chlorophyll *a*. Chlorophyll *a* is found in the reaction centres of photosystem I (PSI) and photosytem II (PSII) as well as a major component of the light harvesting complexes of both PSI and PSII. All algae make chlorophyll *a* except for a number of *Prochlorococcus* species which do not reduce the 8-vinyl group and so make and utilise DV-chlorophyll *a*.

Chlorophyll *a'* where the stereoisomer is inverted at position 13^2 in ring V (Fig. 5.2), is found in the PSI reaction centre complex



	R_1	R_2	R_3	R_4
chlorophyll *a*	CH_2	CH_3	CH_3	ethyl
chlorophyll *b*	CH_2	CHO	CH_3	ethyl
chlorophyll *d*	O	CH_3	CH_3	ethyl
chlorophyll *f*	CH_3	CH_3	CHO	ethyl
DV-chlorophyll *a*	CH_2	CH_3	CH_3	vinyl
DV-chlorophyll *b*	CH_2	CHO	CH_3	vinyl

	R_4	R_2
chlorophyll c_1	ethyl	CH_3
chlorophyll c_2	vinyl	CH_3
chlorophyll c_3	vinyl	CO_2CH_3

Fig. 5.2. Structure of chlorophylls found in algae

(Kobayashi et al. 1988; Jordan et al. 2001) as the chlorophyll responsible for the P700 absorbance. However, the mechanism of synthesis of chlorophyll *a'* presumably from chlorophyll a has not been determined. Chlorophyll *b* is the next most common chlorophyll in algae and is found in the LHC's and as accessory pigments of PSII of land plants, green algae as well as some of the prochlorophyta cyanobacteria. Other cyanobacteria as well as Rhodophyta and Glaucocystophyta algae in which chlorophyll *a* is the dominant pigment do not contain any chlorophyll *b* but instead contain phycobilins as accessory pigments (Tomitani et al. 1999).

Brown seaweeds, diatoms, chrysomonads, dinoflagellates, and cryptomonads contain one or more chlorophyll *c* pigments (Fig. 5.2) (Jeffrey 1968, 1969) in addition to chlorophyll *a*. Chlorophyll *c* pigments are accessory light harvesting pigments and have not been reported in the reaction centre. The general structure of chlorophyll *c* pigments was first deduced by Granick in 1949 as being similar to protochlorophyllide (Granick 1949). The complete structures of chlorophylls *c1* and *c2* shown in Fig. 5.2 with an acrylate at position C17 and a double bond between C17 and C18, were determined in 1971 (Budzikiewicz and Taraz 1971). A third member of the family, chlorophyll *c3*, was determined in 1989 (Fookes and Jeffrey 1989). Chlorophyll *c* pigments are usually found with the 17-acrylic acid unesterified, but isoprenylate esterified forms have been reported (Nelson and Wakeham 1989; Zapata and Garrido 1997). Chlorophylls *c* are porphyrins rather than chlorins and are thus more similar to protochlorophyllides than chlorophylls. Surprisingly, although chlorophyll *c* pigments are widely utilized by photosynthetic algae and appear to be important in chromatic adaptation (Garrido et al. 2016) almost nothing is known about how they are synthesized.

The far-red absorbing chlorophyll *d* was first discovered in small amounts in extracts in several red macroalgae (Manning and Strain 1943). More recently it has been found that chlorophyll *d* can be produced chemically from chlorophyll *a* under mild condi-

tions in the presence of thiols (Loughlin et al. 2014, 2015; Fukusumi et al. 2012; Oba et al. 2011). Thus it is possible that these first reports of chlorophyll *d* may have arisen due to the extraction method employed. However, in 1996, a novel cyanobacterium was isolated from colonial ascidians which contained chlorophyll *d* as the major chlorophyll constituting >95% of the total chlorophyll with most of the remainder being chlorophyll *a* (Miyashita et al. 1997). This organism, *Acaryochloris marina*, has chlorophyll *d′* in its PSI reaction centre complex that has a 740 nm absorbance maxima (Akiyama et al. 2001). It is worth noting that the primary electron acceptors in *A. marina* are chlorophyll *a* in PS I and the magnesium free chlorophyll *a* derivative, pheophytin *a*, in PS II, respectively (Akiyama et al. 2001, 2002a, b), which is true of all algal PSI and PSII reaction centres described so far except for the DV containing *Prochlorococcus* species.

In 2010 a new chlorophyll, chlorophyll *f* was discovered which has a formyl group at C2 (Chen et al. 2010) as shown in Fig. 5.2. The organism which made this chlorophyll *f*, *Halamicronema hongdechloris* is a filamentous cyanobacteria isolated from stomatolites from Shark Bay in Western Australia (Chen et al. 2010, 2012; Li et al. 2012) and has up to 20% of its content as chlorophyll *f* when grown under far red light. Subsequently, other organisms have been identified which make chlorophyll *f* under what has been termed FarLiP chromatic adaptation (Gan et al. 2014a, b; Akutsu et al. 2011). Most of these organisms also make some chlorophyll *d* although no chlorophyll *d* is made by *H. hongdechloris*.

III. Diversity of Bilins in Algae

Bilin diversity in algae is slightly more complicated than chlorophyll diversity as the bilins are usually, although not always, covalently attached as prosthetic groups to protein. In addition they are utilised in both light harvesting when bound to phycobiliproteins and associated with PBs, as well as in signalling when attached to phytochromes or more recently as freely diffusible signalling molecules (Wittkopp et al. 2017). In addition a number of the bilin reductions and isomerisations occur after or during covalent attachment of the pigments to the phycobiliproteins. Given this complexity, I will concentrate on the main types of bilins involved in light harvesting attached to phycobiliproteins, which are usually subunits of a functional PBs.

PBs are found mainly in cyanobacteria, red algae and the glaucophytes and are made up of large numbers of different phycobiliproteins to which bilins are attached. The variation of the spectral property of phycobiliproteins are mainly dictated by their prosthetic groups, which are linear tetrapyrroles known as phycobilins. The free phycobilins including phycocyanobilin, phycoerythrobilin and occasionally dihydrobiliverdin, shown in Fig. 5.3 (Frankenberg and Lagarias 2003; Glazer 1989), are covalently attached to the phycobiliproteins through cysteine residues on the proteins as shown in Fig. 5.4.

The core of the phycobilisome is made from allophycocyanin subunits, each containing the bilin phycocyanobilin covalently attached. From the core there are several outwardly oriented rods which are made from stacked disks of the phycobiliprotein, phycocyanin, that primarily has the pigment phycocyanobilin covalently bound. These disks may also contain other types of phycobiliproteins, such as phycoerythrin or phycoerythrocyanin. Phycoerythrin contains the pigments, phycoerythrobilin or sometimes phycourobilin, while phycoerythrocyanin contains a mixture of phycoviolobilin and phycocyanobilin. These pigments can be bound through one or two cysteine residues on the proteins as shown in Fig. 5.4.

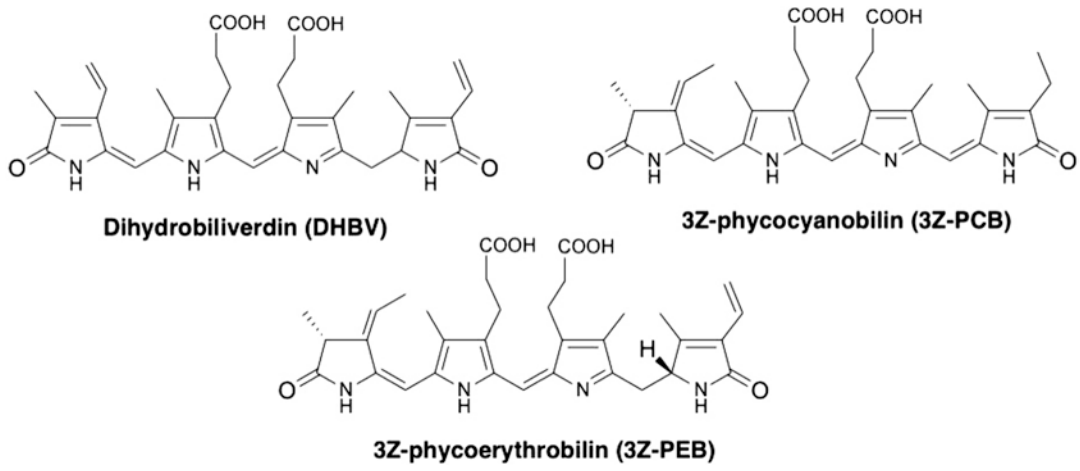


Fig. 5.3. Structures of free bilin pigments DHBV, PEB and PCB

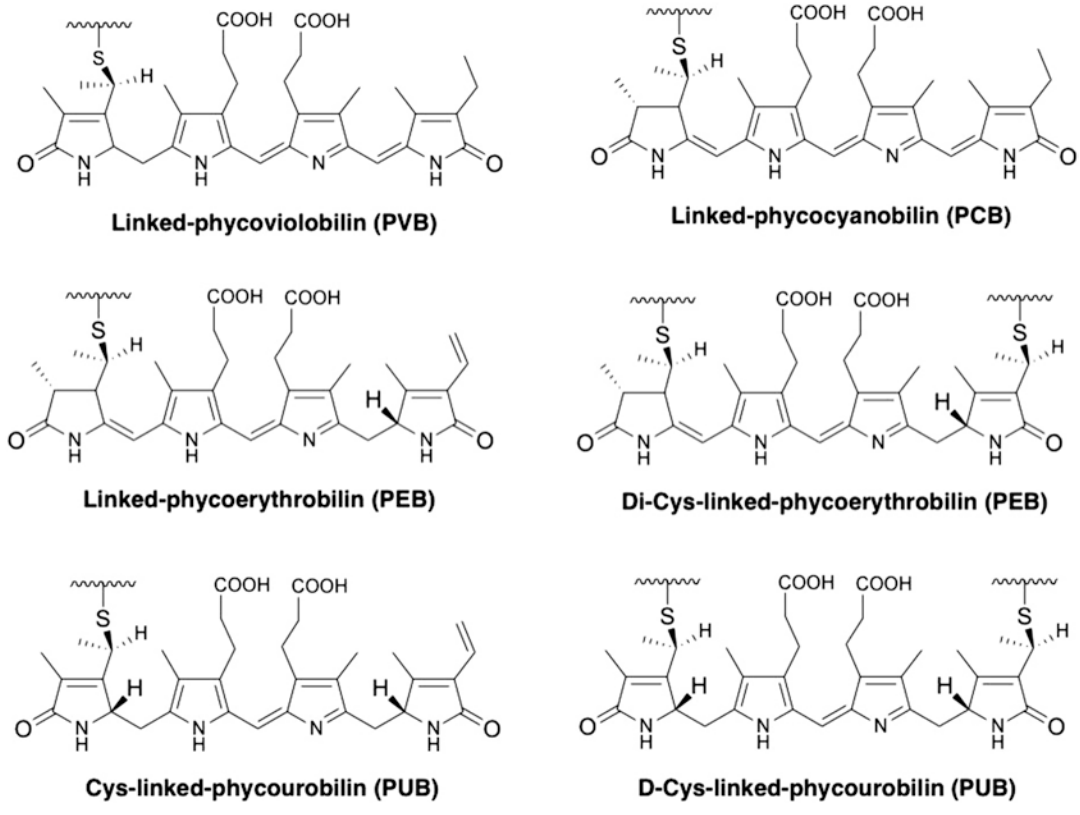


Fig. 5.4. Structures and attachments of bilins commonly found in phycobiliproteins. Cys-PVB, Cys-PCB, Cys-PEB, diCys-PEB, Cys PUB, diCys-PUB

As shown in Fig. 5.4 these bilins are attached through a cysteine residue to ring A and also sometimes attached to two cysteine residues through both ring A and ring D. The isomerisation to produce alternative bilins with different spectral properties occurs during pigment attachment using bilin lyases as discussed later.

IV. Overview of Biosynthesis of Bilins and Chlorophylls

Chlorophylls and bilins have common biosynthetic intermediates from aminolevulinate (ALA) up to and including the intermediate protoporphyrin IX (Fig. 5.5), which is the first coloured intermediate in

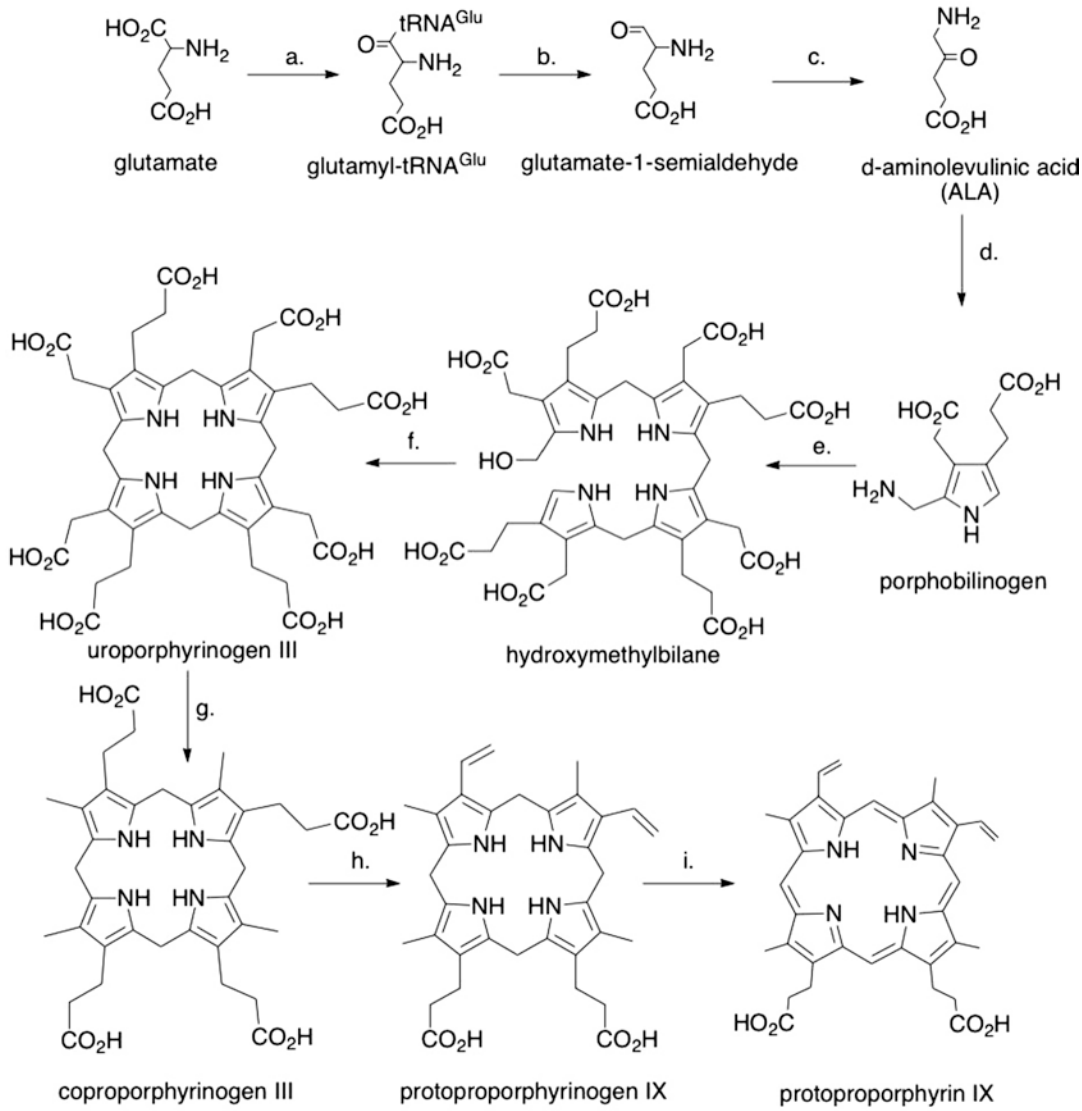


Fig. 5.5. Enzymatic steps and encoding genes from glutamate to protoporphyrin IX: (**a**) Glutamyl tRNA-synthetase, *gluRS*; (**b**) Glutamyl-tRNA reductase, *gluTR* or *hemA*; (**c**) glutamate-1-semialdehyde aminotransferase, *GSAT* or *hemL*; (**d**) porphobilinogen synthase or ALA-dehydratase, *PBGS* or *ALAD* or *hemB*; (**e**) Porphobilinogen deaminase or hydroxymethbilane synthase, *PBGD* or *HMBS* or *hemC*; (**f**) uroporphyrinogen III synthase, *UROS* or *hemD*; (**g**) uroporphyrinogen decarboxylase, *UROD* or *hemE;* (**h**) coproporphyrinogen III oxidase, Oxygen independent encoded by *hemN* and oxygen dependent enzyme encoded by *hemF* or *CPO*; (**i**) protoporphyrinogen IX oxidase, *PPO* or *hemG*

the synthesis of these pigments. From protoporphyrin IX the pathways branch (Figs. 5.6 and 5.7) with either magnesium inserted in the chlorophyll branch to make magnesium protoporphyrin or iron inserted to make heme on the pathway for bilin synthesis.

While the spectroscopic and physicochemical properties of chlorophylls, in particular, make them ideal for light harvesting and energy transduction, these properties also makes them reactive in the presence of oxygen and light. Thus when chlorophylls and their coloured intermediates are not positioned to allow dissipation of absorbed light energy either by Förster energy transfer to another pigment or transduction within the reaction centre, the excited state may react with molecular oxygen to form singlet oxygen. This makes chlorophyll synthesis a challenge in the presence of light as accumulation of non-protein bound chlorophyll or its coloured intermediates can cause oxidative stress and potentially cell death. In contrast to the chlorophylls, the bilins are not as photoreactive in their non-protein bound state and are even suggested to be antioxidants.

Thus regulation of chlorophyll biosynthesis in particular is essential in order to prevent accumulation of free pigment molecules that could result in cell death. The regulatory mechanisms involve both transcriptional and post-transcriptional controls with the key regulatory steps being: (1) aminolevulinate biosynthesis from glutamate; (2) The branch point for metal insertion to make either heme or Mg-protoporphyrin; (3) The reduction of protochlorophyllide to chlorophyllide. As discussed in more detail later, the primary regulatory factors involved in regulating one or more of these steps include: light, oxygen or reactive oxygen species, heme, and protochlorophyllide.

V. Biosynthesis of Protoporphyrin IX

The primary control point for both chlorophyll and heme/bilin synthesis is at the level of ALA synthesis. The enzymes involved in this regulatory process are glutamyl-tRNA reductase, GluTR or HemA, and glutamate-1-semialdehyde aminotransferase, GSAT or HemL (Fig. 5.5 reactions b and c). GluTR catalyses the NADPH dependent reduction of glutamyl-tRNA^{glu} while GSAT catalyses the isomerisation of glutamate-1-semialdehyde, produced by GluTR, to ALA. The x-ray crystal structure of GSAT from *Synechococcus* has been determined (Hennig et al. 1994, 1997) and it was suggested, based on the structure of an archeal GluTR (Moser et al. 2001), that the two enzymes form a stable complex *in vivo* to allow channeling of the unstable glutamate-1-semialdehyde directly from GluTR to GSAT. This interaction has been confirmed using the enzymes from *Chlamydomonas* by co-immunoprecipitation and kinetic analysis (Nogaj and Beale 2005). In addition the GluTR from *Chlamydomonas* was shown to form a stable complex with the glutamyl-tRNA synthetase (Jahn 1992) suggesting substrate channeling from glutamate through to ALA occurs without release of intermediates. Blue light is a transcriptional regulator of GSAT in *Chlamydomonas reinhardtii* (Im et al. 1996) and this regulation is modulated by nitrogen and carbon availability. Similarly GluTR transcription is regulated by light with both transcripts increasing significantly in the light, however, the quantity of GluTR and GSAT proteins and activity levels remain constant despite changes in transcript levels of up to sixfold, indicating that transcription does not regulate the activity (Nogaj et al. 2005; Srivastava et al. 2005).

When organisms are fed ALA they accumulate various tetrapyrroles depending on the relative activities of the enzymes in the pathway and when feeding occurs in the dark both protoporphyrin IX and protochlorophyllide often accumulate, suggesting some regulation of these steps which will be discussed later. This also indicates that ALA synthesis is a key control point and regulation of ALA synthesis by end products such as heme and intermediates such as protochlorophyllide have been reported for plants

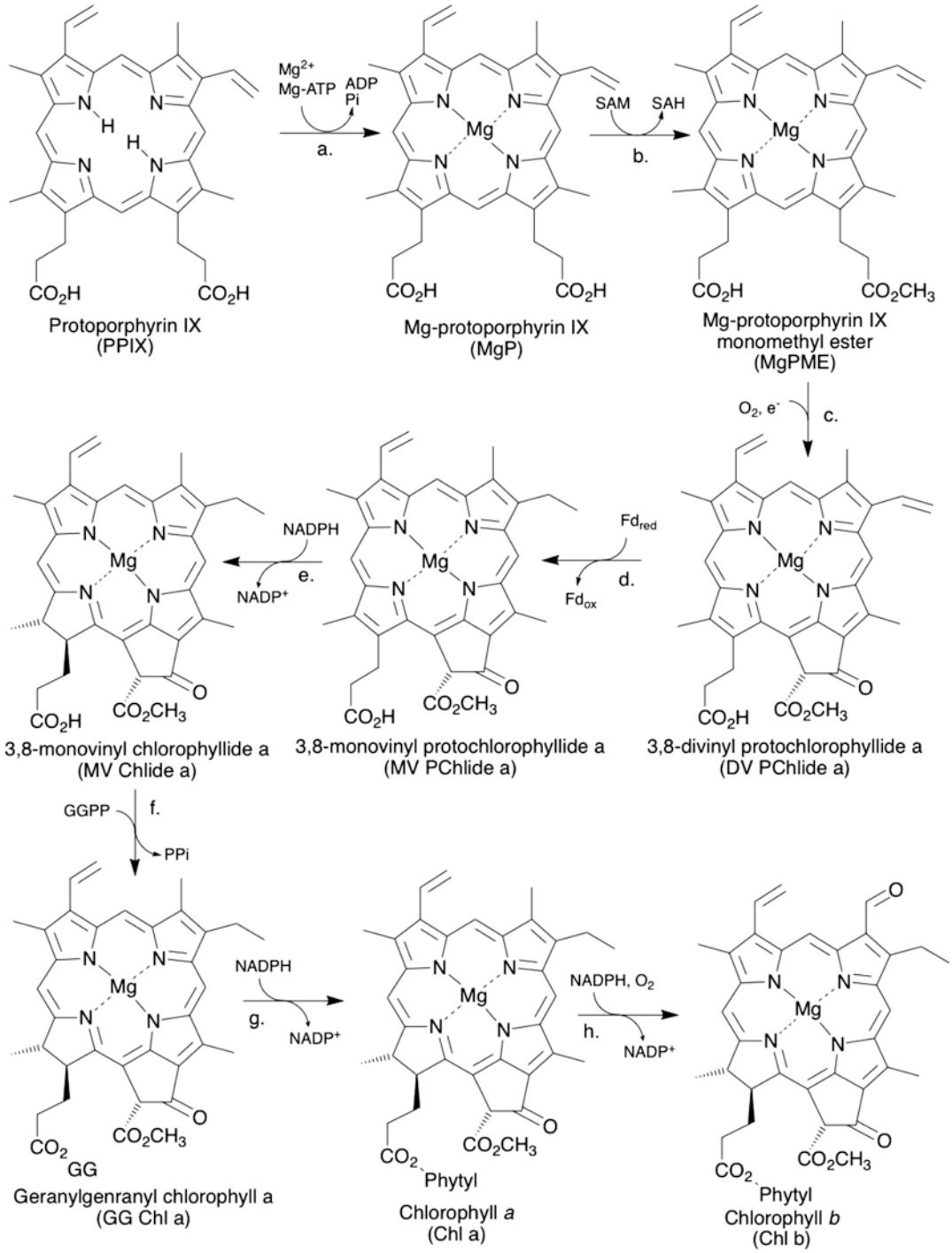


Fig. 5.6. Enzymatic steps and encoding genes from protoporphyrin IX to chlorophyll *b*: (**a**) Magnesium chelatase, *bchH/chlH, bchD/chlD, bchI/chlI* and *gun4*; (**b**) S-adenosylmethionone Mg- protoporphyrin IX monomethylester transferase, *chlM*; (**c**) MgPME oxidative cyclase, *acsF, bchE,* or *CTH1/ycf54*; (**d**) protochlorophyllide oxido-reductase. Light dependent enzyme encoded by *por* and light independent enzyme encoded by *bchB/chlB, bchN/chlN,* and *bchL/chlL*; (**e**) Divinyl reductase, *DVR*; (**f**) Chlorophyll synthase, *chlG*; (**g**) Geranylgeranyl reductase/Phytyl synthase, *chlP*; (**h**) Chlorophyll *a* oxygenase, *COA*

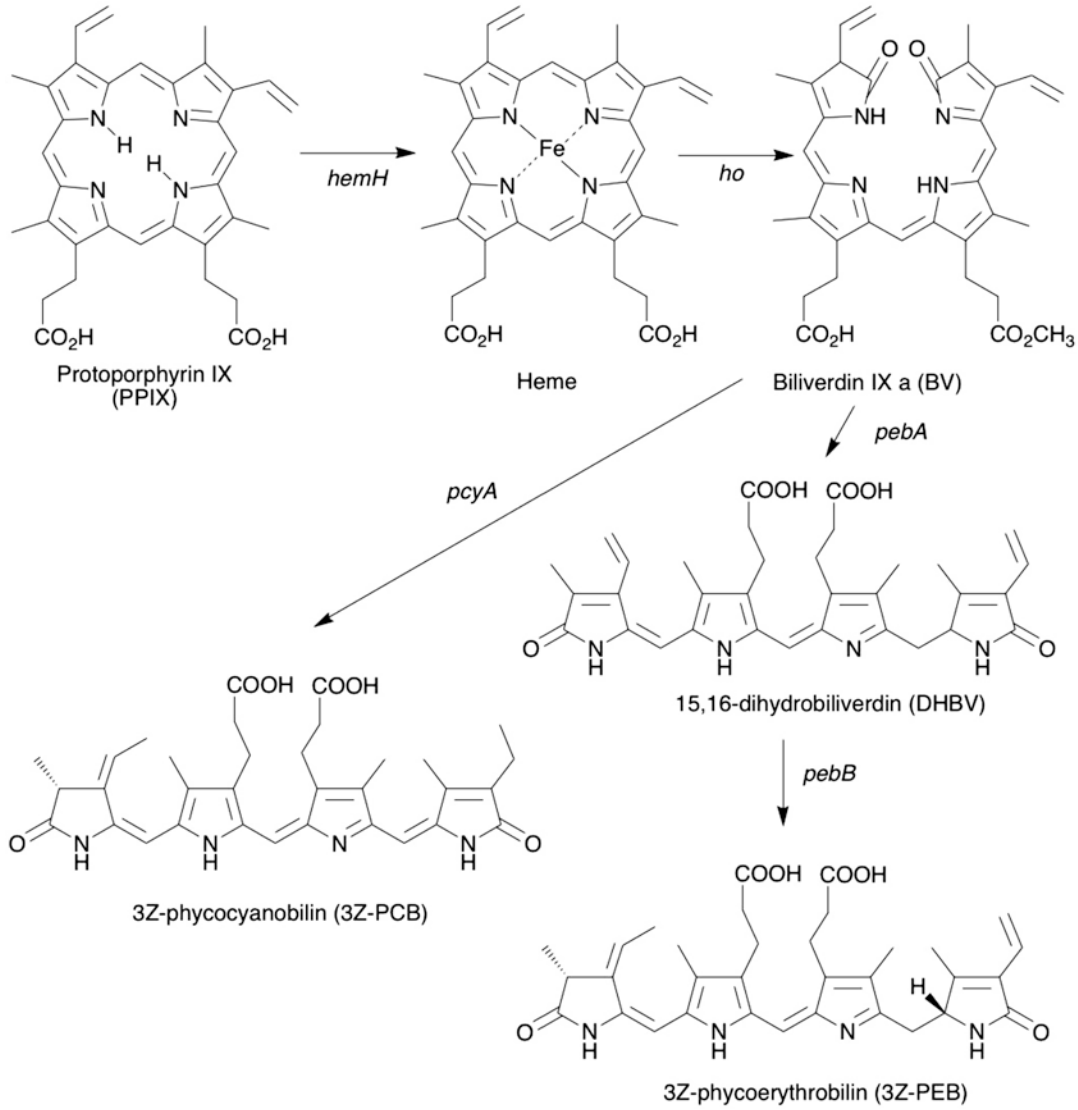


Fig. 5.7. Enzymatic steps and encoding genes from protoporphyrin to free bilins

and algae. Although heme has been reported to regulate barley GluTR activity through an N-terminal extension (Vothknecht et al. 1998), this may not occur in algae as heme and protoporphyrin IX have not been reported to regulate algal GluTR although they have been reported to regulate transcription of the GluTR (Vasileuskaya et al. 2005). Feedback regulation of ALA synthesis by protochlorophyllide or later intermediates has been long suspected due to the occurrence of brown mutants of *Chlamydomonas* which are unable to make chlorophyll but accumulate MgPPIX or PPIX. These mutants suggest the presence of a feedback regulatory mechanism on ALA synthesis involving intermediates after these enzymatic steps (Wang et al. 1974; Meinecke et al. 2010; Chekounova et al. 2001). In contrast plants that are mutated at the same biosynthetic steps as the brown *Chlamydomonas* mutants are yellow with no significant accumulation

of MgPPIX or PPIX, unless they are fed ALA (Henningsen et al. 1993). This suggests that heme may be a key regulator of ALA synthesis in plants but not in *Chlamydomonas* or perhaps in other algae.

Protochlorophyllide dependent feedback regulation of ALA synthesis is mediated by a protein called FLU which was first identified in *Arabidopsis thaliana* (Meskauskiene et al. 2001; Meskauskiene and Apel 2002). Unicellular algae such as *Chlamydomonas* also contain FLU, also called FLP, and this protein is alternatively spliced and was found to also be important in regulating ALA synthesis (Falciatore et al. 2005), presumably by interacting with protochlorophyllide.

All of the genes for steps from ALA to protoporphyrin IX have been identified in *Chlamydomonas* and in many cyanobacteria, including *Synechocystis* (Lohr et al. 2005; Merchant et al. 2007; Kaneko et al. 1996). One key difference between eukaryotic algae like *Chlamydomonas* and cyanobacteria is in in the later steps in protoporphyrin IX synthesis. Most cyanobacteria have oxygen-independent as well as an oxygen-dependent enzyme for coproporphyrinogen oxidase CPO and possibly protoporphyrinogen oxidase PPO, while eukaryotic algae, like *Chlamydomonas,* appear to only contain genes for the oxygen dependent enzymes (Lohr et al. 2005).

VI. Biosynthesis of Bilins from Protoporphyrin and Function of Bilin Lyases

Bilins are biosynthesized from protoporphyrin IX via heme as shown in Fig. 5.7. Fe^{2+} is inserted into protoporphyrin IX by ferrochelatase to make heme. Ferrochelatase is encoded by the *hemH* gene and is a single subunit enzyme with no cofactor requirement apart from the two substrates. The ferrochelatase of *Chlamydomonas* is located in the chloroplast and the heme and is associated with the chloroplast membranes (van Lis et al. 2005). The cellular needs for heme in *Chlamydomonas* are supplied by heme derived from the chloroplast, including heme required in the cytosol and mitochondria (van Lis et al. 2005), suggesting a transport mechanism must exist to supply heme to these compartments.

The initial committed step of bilin biosynthesis is the cleavage of heme by heme oxygenases to afford the first linear tetrapyrrole, biliverdin. The mammalian heme oxygenases are microsomal NADPH-cytochrome P450 dependent enzymes while the heme oxygenases from red algae and cyanobacteria are soluble ferredoxin dependent enzymes. The first ferredoxin dependent heme oxygenase was identified and cloned and expressed in *E.coli* from *Synechocystis* (Willows and Beale 1998). *Synechocystis* has two heme oxygenase genes *ho1* and *ho2*, the *ho1* is required under oxygen sufficient conditions, while *ho2* is required under low oxygen tension conditions and is located next to the oxygen independent coproporphyringen oxidase, *hemN* gene.

Biliverdin produced by heme oxygenase is further reduced by site specific reductases such as PebA, PebB and PcyA, to make, DHBV, 3Z-PEB and 3Z-PCB respectively as shown in Fig. 5.7. Like heme oxygenase these oxidoreductases are ferredoxin-dependent and belong to the interesting family of radical oxidoreductases known as of ferredoxin dependent bilin reductases (FDBRs). In recent years the family of FDBRs has expanded revealing novel activities (Kronfel et al. 2013; Biswas et al. 2011; Schluchter et al. 2010).

Most cyanobacteria make bilins such as 3Z-PCB in order to make PB's but eukaryotic green algae such as *Chlamydomonas* don't have PB's or indeed phytochromes yet they contain *ho* and *pcyA* type genes (Rockwell and Lagarias 2017). Recently it has been shown that these bilins are required in chloroplast nuclear signalling processes and are specifically required for photoacclimation in *Chlamydomonas* and this may also

be important in other algae which lack phytochromes or PB's (Wittkopp et al. 2017; Rockwell and Lagarias 2017; Duanmu et al. 2017; Formighieri et al. 2012).

The DHBV, 3Z-PEB and 3Z-PCB bilins are covalently attached to phycobiliproteins by bilin lyases. During the attachment the bilins can also undergo further isomerisation to bilins such as PUB and PVB shown in Fig. 5.4 (Arciero et al. 1988a; b, c; Fairchild et al. 1992; Swanson et al. 1992; Fairchild and Glazer 1994a, b). The bilin lyases fall into 3 main classes: The E/F-type such as CpcE/CpcF heterodimeric bilin lyase, the T-type including CpcT bilin lyase, and the S/U-type that includes CpcS/CpcU heterodimeric and homodimeric bilin lyases (Zhao et al. 2017).

The CpcE/F bilin lyase was the first bilin lyase characterized (Zhou et al. 1992) and catalyses the attachment of 3Z-PCB to Cys-84 of α-phycocyanin. Other members of this family include PecE/PecF and CpeY/CpeZ (Kronfel et al. 2013; Biswas et al. 2011; Zhao et al. 2000, 2007; Jung et al. 1995; Saunee et al. 2008; Shen et al. 2008; Overkamp et al. 2014). This family of lyases appears to have some members which can isomerize the bilin during attachment to the biliprotein whereas other members are capable of removing the bilin and transferring to a different biliprotein (Schluchter et al. 2010).

The T-type bilin lyases were first identified in *Synechococcus* PCC7002 where CpcT was shown to be involved in PCB attachment to Cys-153 of β-phycocyanin and unlike the other classes it appears to function by itself, probably as a homodimer (Shen et al. 2006; Zhou et al. 2014). The distribution of sequences similar to CpcT among other cyanobacteria suggests that this protein subgroup plays a role in cyanobacterial-type phycoerythrin biosynthesis, probably by attaching 3Z-PEB at the Cys-153 equivalent position of β-phycoerythrin (Schluchter et al. 2010).

The S/U family of lyases includes members such as CpcS, CpcU, CpcV (function unknown) and CpeS and CpeU bilin lyases (Bretaudeau et al. 2013; Six et al. 2007). This family has members which usually form homodimers (CpcS, CpeS) or heterodimers such as CpcS/U but does not appear to perform the transfer or removal of bilins like the other families. However some of these enzymes can recognise many different PBPs and attach bilins at their Cys-82 equivalent positions, and thus have a broader substrate specificity than the other types of lyases (Schluchter et al. 2010; Scheer and Zhao 2008).

Although, bilins have been reported to attach to the phycobiliproteins autocatalytically this is unlikely to occur to any extent *in vivo* as mutants lacking the bilin lyases are unable to assemble functional PBs (Shen et al. 2008).

VII. Biosynthesis of Chlorophylls from Protoporphyrin IX

Magnesium chelatase catalyses the first committed step to chlorophyll synthesis. The first high activity *in vitro* assay was with stromal and membrane extracts from pea chloroplasts (Walker and Weinstein 1991; Walker et al. 1992). This assay system was important as it identified that the enzyme assembly was protein concentration dependent. Subsequently the minimum requirements for magnesium chelatase activity were identified by expressing the three genes *bchH*, *bchD* and *bchI* from the anoxygenic photosynthetic bacteria *Rhodobacter sphaeroides* in *E. coli* and reconstituting the ATP dependent activity (Gibson et al. 1995). The orthologous genes from *Synechocystis*, *chlI, chlD* and *chlH (*Jensen et al. 1996a*)*, and barley, *xantha-H*, *xantha-G* and *xantha-F* (Jensen et al. 1996b) were also identified as magnesium chelatase components corresponding to the *bchI, bchD and bchH* genes of *Rhodobacter* respectively. In organisms that synthesize chlorophyll the genes are generally now called *chlI, chlD* and *chlH*. In plants

and algae these genes are regulated by light with higher expression in the light, particularly of the *chlH* gene, and they are often under circadian clock regulation (Chekounova et al. 2001; Jensen et al. 1996b; Lake and Willows 2003; Stephenson and Terry 2008).

One common feature of the magnesium chelatase from all sources is that it requires an ATP dependent assembly of the ChlI and ChlD subunits to form an activation complex (Willows and Beale 1998; Willows et al. 1996; Walker and Willows 1997; Guo et al. 1998; Petersen et al. 1998; Gibson et al. 1999; Reid and Hunter 2002; Sawicki and Willows 2008; Lake et al. 2004). This ChlI/ChlD complex acts like the "enzyme" to insert Mg^{2+} into protoporphyrin IX bound to the ChlH subunit with this large 132–155 kDa subunit behaving like a substrate in the reaction with a K_m in the 0.1–1 μM range (Willows and Beale 1998; Petersen et al. 1998; Sawicki and Willows 2008; Viney et al. 2007).

GUN4 is a fourth magnesium chelatase accessory subunit which was first identified in *A. thaliana* as being involved in the retrograde signalling system from the chloroplast to the nucleus (Larkin et al. 2003). The ChlH subunit of the *A. thaliana* is also known as GUN5 and is also involved in this signalling process (Mochizuki et al. 2001) suggesting an involvement of the magnesium chelatase in chloroplast to nuclear signalling. GUN4 binds protoporphyrin IX and magnesium protoporphyrin IX and is able to stimulate magnesium chelatase activity (Larkin et al. 2003) through interaction with ChlH (Wilde et al. 2004; Sobotka et al. 2008; Adhikari et al. 2009, 2011; Davison and Hunter 2011). This stimulation of magnesium chelatase activity is required for optimal chlorophyll synthesis (Wilde et al. 2004; Sobotka et al. 2008; Adhikari et al. 2009, 2011; Davison and Hunter 2011). It has been suggested that this porphyrin binding of both ChlH and GUN4 together with light is important in retrograde signalling by producing singlet oxygen (Stephenson and Terry 2008; Brusslan and Peterson 2002; Surpin et al. 2002; Muller et al. 2014; Tarahi Tabrizi et al. 2016).

The interaction of GUN4 with ChlH in plants requires a C-terminal extension of GUN4 that is absent in cyanobacteria (Tarahi Tabrizi et al. 2016; Zhou et al. 2012; Adams et al. 2014; Huang et al. 2014; Richter et al. 2016). In plants the phosphorylation or removal of this C-terminal extension prevents the interaction with ChlH and hence regulates magnesium chelatase activity (Zhou et al. 2012; Huang et al. 2014; Richter et al. 2016) *Chlamydomonas* and other eukaryotic algae also possess a C-terminal extension compared to cyanobacterial GUN4 proteins but it is unclear if this extension serves a similar function to the plant GUN4 in regulating magnesium chelatase activity.

The x-ray and electron-microscopy (EM) structures of magnesium chelatase subunits and complexes have been determined including; GUN4 from cyanobacteria and *Chlamydomonas* (Davison et al. 2005; Verdecia et al. 2005; Chen et al. 2015a; Tarahi Tabrizi et al. 2015); the x-ray and EM structures of BchI and the BchI/BchD complex from *Rhodobacter capsulatus* (Willows et al. 1999; Fodje et al. 2001; Lundqvist et al. 2010, 2013); and the x-ray structure of the cyanobacterial ChlH (Chen et al. 2015b). These crystal structures have informed catalytic models for magnesium chelatase activity which suggest that the ChlI/ChlD subunits form a AAA+ type molecular motor which drives an ATP dependent conformational change in the substrate ChlH-protoporphyrin IX-GUN4 complex to possibly bend the tetrapyrrole and expose and deprotonate the pyrrole nitrogens and allow Mg^{2+} insertion. The GUN4 then is involved in removing the Mg-protoporphyrin IX for trafficking to the next enzyme. One curious finding is that the ChlI subunits appear to be disassembled from the ChlD complex and recycled in each catalytic cycle which may explain a number of the kinetic properties of the enzyme and

the requirement for higher concentrations of ChlI or BchI *in vitro* assays (Lundqvist et al. 2013; Hansson et al. 2002; Zhang et al. 2015; Adams et al. 2016; Adams and Reid 2013; Brindley et al. 2015). To further complicate the regulatory landscape surrounding this enzyme, more than one *chlI* and *chlH* gene is sometimes found in plants and eukaryotic algae. The ChlI2 of *A. thaliana* has been shown to substitute for the ChlI1 protein (Rissler et al. 2002). In contrast, the *Chlamydomonas* ChlI2 has a C-terminal extension and is not able to substitute for the ChlI1 (Brzezowski et al. 2016). However, the *Chlamydomonas* ChlI2 has histidine kinase activity and is involved in activation of the magnesium chelatase by phosphorylation of a histidine on the C-terminal domain of ChlD (Sawicki et al. 2017).

The description of *Chlorella* mutants by Granick were pivotal in the characterisation of the next two steps in the pathway, catalysed by the S-adenosyl methionine-Mg-protoporphyrin IX monomethyl ester transferase and the magnesium protoporphyrin oxidative cyclase (Granick and Kett 1948; Granick 1961). The S-adenosyl methionine-Mg-protoporphyrin IX monomethyl ester transferase catalyses the methylation of the C-17 propanoate as shown in Fig. 5.6. The *chlM* gene encodes the single subunit enzyme catalysing this step and the cyanobacterial enzyme has been heterologously expressed in *E.coli* and the enzyme characterised (Shepherd and Hunter 2004; Shepherd et al. 2005; Dorgan et al. 2006; McLean and Hunter 2009). A brown *Chlamydomonas* has been characterised which has a defective *chlM* (Meinecke et al. 2010) and accumulates magnesium protoporphyrin IX. The *chlM* knockout of *Chlamydomonas* has also been performed using CRISPR based gene targeting and the brown phenotype is a useful visual screen to enable selection of mutants (Shin et al. 2016).

The oxidative cyclase has been partially characterised from plant and algal chloroplasts and requires molecular oxygen and NADPH. The enzyme activity can be separated into soluble and membrane components (Wong and Castelfranco 1984; Nasrulhaq-Boyce et al. 1987; Walker et al. 1988, 1991; Vijayan et al. 1992; Whyte et al. 1992; Whyte and Castelfranco 1993; Bollivar and Beale 1995, 1996) and is inhibited by lipid soluble Fe^{2+} chelators like dipyrridyl. Anaerobic photosynthetic bacteria have an oxygen independent cyclase encoded by *bchE* and many cyanobacteria have a *bchE* orthologue called *chlE*, which is not absolutely required for chlorophyll synthesis (Yamanashi et al. 2015). Cyanobacteria also have an aerobic oxygen dependent cyclase encoded by *acsF* or *chlA* (Ouchane et al. 2004; Minamizaki et al. 2008). Homologues of *acsF* were identified in *Chlamydomonas* as *crd1* or *chl27A* and *cth1* or *chl27B,* which are required for chlorophyll synthesis and are differentially regulated under low and high oxygen conditions respectively (Moseley et al. 2000, 2002; Allen et al. 2008). The *Chlamydomonas* CRD1 and CTH1 are di-iron membrane bound proteins and are part of the membrane component required for enzymatic activity of the cyclase. In addition, another part of the membrane component called Ycf54 has been identified (Hollingshead et al. 2012; Bollivar et al. 2014; Chen et al. 2017), while the soluble component of the cyclase activity appears to be ferredoxin reductase (Herbst et al. 2018).

The conversion of divinyl protochlorophyllide to chlorophyllide can be catalysed by two different types of protochlorophyllide reductases. These are known as LPOR and DPOR which stand for light-dependent- and dark- protochlorophyllide reductase respectively. These systems have been very well characterised and have been the subject of a number of extensive reviews (Fujita and Bauer 2003; Gabruk and Mysliwa-Kurdziel 2015; Layer et al. 2017; Rüdiger 2003; Nascimento et al. 2016). The LPOR enzyme requires light as a substrate in the enzymatic reaction and is a single polypeptide encoded

by *por* with many plants and algae having multiple *por* isoforms. The DPOR enzyme is a complex enzyme with three subunits called ChlL, ChlB and ChlN. The sequences of the subunits and structure of the DPOR is similar to nitrogenase (Moser et al. 2013) and like the nitrogenase its activity is oxygen sensitive. Algae and cyanobacteria generally have both types of enzymes so they are able to make chlorophyll in the dark utilizing DPOR or in the light utilising LPOR. In eukaryotes the genes for DPOR are located on the chloroplast genome while the *por* genes are located on the nuclear genome. The DPOR genes in cryptophytes have undergone recent gene loss (Kim et al. 2017) and yellow in the dark mutants of *Chlamydomonas* are the result of nuclear mutations which effect the accumulation of the chloroplast encoded ChlL subunit (Cahoon and Timko 2000).

With a few exceptions, such as *Prochloron*, chlorophylls in the photosystems of most chlorophyll containing organisms have an 8-ethyl group. This 8-ethyl group is formed by reduction of the divinyl-chlorophyllide *a* to monovinyl chlorophyllide *a* by an divinyl reductase. The 8-vinyl-reduction is catalysed by an NADPH dependent or ferredoxin dependent divinyl reductases. The enzyme is called DVR in eukaryotes such as *Chlamydomonas* and is NADPH dependent. Cyanobacteria have two types of enzyme with the BciA, homologous to DVR, being NADPH dependent while the second type known as BciB being ferredoxin dependent (Nagata et al. 2005; Liu and Bryant 2011; Wang et al. 2013; Canniffe et al. 2014; Chen et al. 2016). The BciA and BciB are unrelated to each other but both proteins have been found in cyanobacteria such as *A. marina*, where the genes had been misannotated as *nmrA* and *frhB* respectively, but both were shown to have divinyl reductase activity (Chen et al. 2016).

The final steps of chlorophyll *a* synthesis are catalysed by geranylgeranyl-pyrophosphate reductase and chlorophyll synthase, abbreviated ChlP and ChlG respectively (Addlesee et al. 1996; Oster et al. 1997). The ChlP catalyses an NADPH reduction of geranylgeranyl-pyrophosphate to phytyl-pyrophosphate and in plants this reductase is also required for tocopherol biosynthesis (Grasses et al. 2001). The plants with reduced levels of ChlP have chlorophyll *a* with geranylgeraniol esterified instead of phytol indicating chlorophyll synthase can use either geranylgeranyl-pyrophosphate or phytyl-pyrophosphate as a substrate (Grasses et al. 2001). The ChlG of plants, cyanobacteria and algae form a complex with the protein insertase, YidC/Alb3 and an assembly factor Ycf39, suggesting that chlorophyll *a* formed from ChlG is delivered directly to the photosystem proteins as they are inserted into the membrane (Proctor et al. 2018).

VIII. Synthesis of Chlorophyll *b*, *d* and *f*

The formyl oxygens present in all of these modified chlorophylls are derived from molecular oxygen (Schliep et al. 2010; Garg et al. 2017; Porra et al. 1993, 1994; Porra and Scheer 2001). The gene encoding chlorophyll *a* oxidase (CAO) was first identified using *Chlamydomonas* mutants (Tanaka et al. 1998) and the *Arabidopsis* gene was expressed in *E. coli* and the product shown to have CAO activity *in vitro* but it has not been completely characterised (Oster et al. 2000). The activity of the CAO from various organisms has been confirmed by mutation and/or overexpression with resulting changes in the chlorophyll *a* to chlorophyll *b* ratios (Tanaka et al. 2001). More recently the CAO from *Micromonas* was shown to consist of two homologous subunits CAO1 and CAO2 with both subunits required for chlorophyll *b* formation in an *Arabidopsis* chlorophyll *b*-less mutant (Kunugi et al. 2013).

The chlorophyll *f* synthase is a light dependent enzyme with a structure like the PSII reaction centre (Ho et al. 2016) but the

enzyme system which makes chlorophyll *d* is unknown. The enzymes that incorporate a formyl group into chlorophyll *a* require further investigation, especially in regard to their enzymatic mechanisms.

IX. Concluding Remarks

Future work on bilin and chlorophyll synthesis in algae will be to fill the obvious gaps in our knowledge of the structure and mechanism of various enzymes involved in their synthesis. In addition one of the more interesting aspects to bilin and chlorophyll synthesis in algae is the observation that chlorophyll *b* containing algae do not contain PB's and vice versa. The recent discovery that bilins are still synthesized by chlorophyte algae and appear to have a regulatory role suggests the pathways for bilin and chlorophyll synthesis are intertwined after they branch, at least in a regulatory sense. Future work on how this regulation occurs would advance our understanding of how pigment synthesis is regulated and coordinated with the pigment binding proteins.

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