

1. INTRODUCTION

1.1 Photosynthesis

At a broad level photosynthesis is either directly or indirectly responsible for the survival of nearly all living organisms, with only chemolithotrophic bacteria not requiring this natural process (Nisbet et al., 1995; Raymond et al., 2002; Xiong and Bauer, 2002; Leslie, 2009). Aerobic photosynthesis in plants, algae, or oxygenic bacteria (cyanobacteria) utilise chlorophyll derivatives such as chlorophyll *a* as the primary pigment involved in absorbing light (Kiang et al., 2007), while anaerobic photosynthetic bacteria may have either bacteriochlorophyll or chlorophyll molecules (Fig. 1, Box 1) (Zeilstra-Ryalls and Kaplan, 2004). There are six derivatives each of chlorophyll or bacteriochlorophyll molecules found in nature including the structures in Fig. 1 (Chew and Bryant, 2007b).

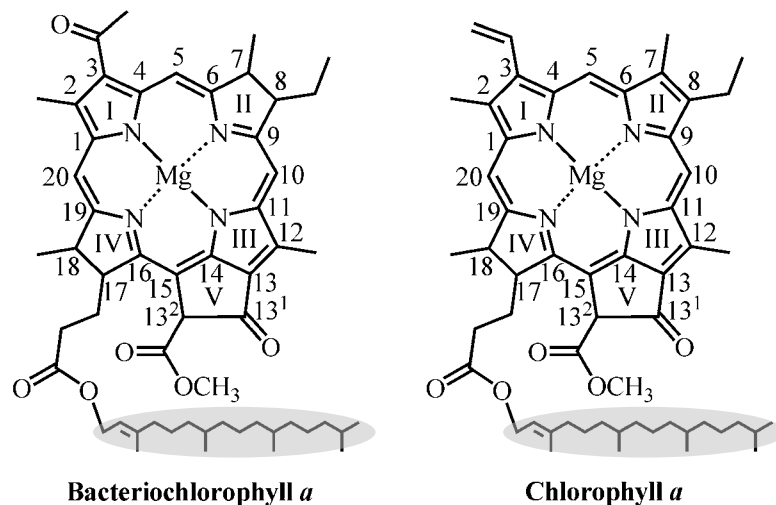


Figure 1. The two predominant light-capturing pigments found in nature. The shaded phytyl chain is typically found in bacteriochlorophyll/chlorophyll synthesizing organisms, but may be replaced with geranylgeranyl or farnesyl chains in some instances (Tamiaki et al., 2007).

Box 1. *Rhodobacter capsulatus* and other anaerobic photosynthetic bacteria

Rhodobacter capsulatus (previously known as *Rhodopseudomonas capsulatus*) (Imhoff et al., 1984) is a freshwater facultative anaerobic photosynthetic α -proteobacterium (Imhoff, 1995; Imhoff, 2001). This genus has the ability to grow as a chemoheterotroph in the presence of oxygen or a phototroph in anaerobic or partially aerobic conditions in the light (van Niel, 1944; Lascelles, 1959; Weaver et al., 1975). Phototrophic growth causes a change in cellular morphology and generates intracytoplasmic membranes (ICM's) (Kaplan, 1981). ICM's in *Rba. capsulatus* have a vesicular-type arrangement, not unlike eukaryotic organelles (Dierstein et al., 1981; Kaufmann et al., 1982; Drews and Golecki, 1995). The cellular invaginations that produce the ICM increase the surface area for photosynthetic structures which are composed of reaction centres and antenna complexes (Zuber and Cogdell, 1995; Bryant and Frigaard, 2006). Some other studied anaerobic photosynthetic bacteria are green sulphur bacteria (e.g. *Chlorobium vibrioforme*, *Chlorobaculum tepidum*), green filamentous bacteria (e.g. *Chloroflexus aurantiacus*), and heliobacteria, with several new species recently identified in this phylum (Asao and Madigan, 2010).

1.2 (Bacterio)chlorophyll biosynthesis

1.2.1 Overview of the pathway

A wealth of information exists on the tetrapyrrole biosynthetic pathway (Fig. 2). Attention will focus on (bacterio)chlorophyll biosynthesis which is regularly updated in reviews (Castelfranco and Beale, 1983; Leeper, 1985, 1987; Beale and Weinstein, 1990; Senge, 1993; Porra, 1997; Beale, 1999; Vavilin and Vermaas, 2002; Willows, 2003; Eckhardt et al., 2004; Beale, 2005; Tanaka and Tanaka, 2007; Chew and Bryant, 2007b; Masuda, 2008; Masuda and Fujita, 2008; Willows and Kriegel, 2009). The steps of the pathway in Fig. 2 are summarised below with gene and enzyme names of bacteriochlorophyll biosynthesis listed in Table 1. Structures in Fig. 2 will be referred to with numbered brackets, [].

The photosynthetic α -proteobacteria (purple non-sulphur bacteria, Box 1) synthesizes δ -aminolevulinic acid (ALA) [1] through the Shemin pathway (Shemin and Wittenberg, 1951; Shemin and Russell, 1953; Kikuchi et al., 1958). This pathway is shared with other non-photosynthetic bacteria, fungi and mammalian systems (Oh-Hama, 1997). ALA synthesis occurs through the condensation of glycine and succinyl-coenzyme A by the enzyme ALA synthase. Most other bacteria, plants and algae have an alternative means to generate ALA *de novo* that is discussed in 1.2.4.

Following ALA synthesis, two ALA molecules are condensed forming porphobilinogen [2]. Four molecules of porphobilinogen then polymerize to form hydroxymethyl bilane [3].

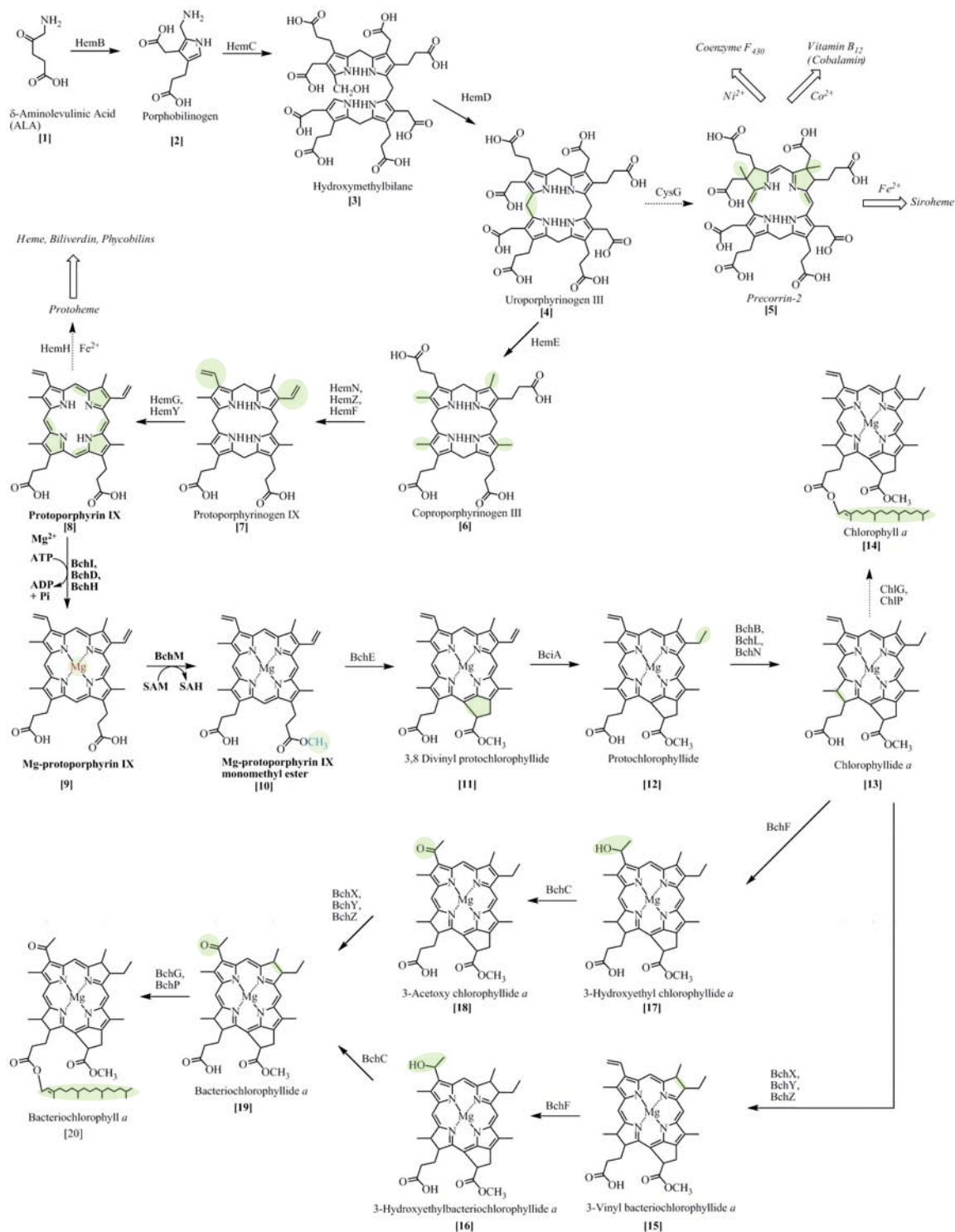


Figure 2. Tetrapyrrole biosynthetic pathway. Thin arrows represent steps in bacteriochlorophyll biosynthesis, while thin dashed arrows represent branchpoints directed to pathways not part of bacteriochlorophyll biosynthesis. Thick open arrows indicate a multistep enzymatic synthesis. Abbreviated enzyme names (Bch*) or BciA above thin arrows refer to bacteriochlorophyll biosynthetic genes, where * refers to a single letter. The gene and enzyme names are listed in Table 1. Other genes/enzymes shown in the pathway are; *hemH*/ferrochelatase, *cysG*/uroporphyrinogen III C-O-methyltransferase, *chlG*/chlorophyll synthase, and *chlP*/geranylgeranyl reductase. Highlighted in bold are the two enzymatic steps from protoporphyrin IX to magnesium protoporphyrin IX monomethyl ester with the required substrates. They are catalysed by BchI, BchD, and BchH; magnesium chelatase, and BchM; S-adenosyl-L-methionine:magnesium protoporphyrin IX O-methyltransferase. ATP is adenosine triphosphate, ADP is adenosine diphosphate, Pi is inorganic phosphate, Mg²⁺ is magnesium, SAM is S-adenosyl-L-methionine, and SAH is S-adenosyl homocysteine.

Table 1. Bacteriochlorophyll biosynthetic gene names from anaerobic photosynthetic bacteria and the enzymes they encode. This information is adapted from Willows and Kriegel (2009).

Gene	Enzyme	EC No.
<i>hemA</i>	ALA synthase	2.3.1.37
<i>hemB</i>	ALA dehydratase	4.2.1.24
<i>hemC</i>	Hydroxymethylbilane synthase	2.5.1.61
<i>hemD</i>	Uroporphyrinogen III synthase	4.2.1.75
<i>hemE</i>	Uroporphyrinogen III decarboxylase	4.1.1.37
<i>hemF, hemN, hemZ</i>	Coproporphyrinogen III oxidase	1.3.3.3
<i>hemG, hemY</i>	Protoporphyrinogen oxidase	1.3.3.4
<i>bchI, bchD, bchH</i>	Magnesium chelatase	6.6.1.1
<i>bchM</i>	S-adenosyl-L-methionine:magnesium protoporphyrin IX O-methyltransferase	2.1.1.11
<i>bchE</i>	Magnesium protoporphyrin IX monomethyl ester oxidative cyclase	1.14.13.81
<i>bciA</i>	8-Vinyl reductase (divinyl reductase, DVR)	1.3.1.75
<i>bchL, bchN, bchB</i>	Dark-operative protochlorophyllide oxidoreductase (DPOR)	1.3.1.33
<i>bchX, bchY, bchZ</i>	Chlorin reductase	
<i>bchF</i>	3-Vinyl bacteriochlorophyllide <i>a</i> hydroxylase	
<i>bchC</i>	3-Hydroxyethyl bacteriochlorophyllide <i>a</i> dehydrogenase	
<i>bchG</i>	Bacteriochlorophyll synthase	2.5.1.62
<i>bchP</i>	Geranylgeranyl reductase	

Hydroxymethyl bilane is reduced and undergoes cyclisation by uroporphyrinogen III synthase to produce the tetrapyrrole uroporphyrinogen III [4]. This is the first branchpoint of tetrapyrrole biosynthesis. Methylation and rearrangement of double bond conjugation in the tetrapyrrole by CysG allows precorrin-2 formation [5]. The multistep synthesis of three different biomolecules can then occur. They are the synthesis of (i) siroheme (an iron-based porphyrin used as a cofactor for nitrite and sulphite reductases) (Raux et al., 2000), (ii) coenzyme F430 (a nickel-based porphyrin) (Ghosh et al., 2001), and (iii) vitamin B₁₂ (also called cobalamin, a cobalt- based porphyrin) (Raux et al., 2000; Heldt et al., 2005). The heme/(bacterio)chlorophyll pathway continues from uroporphyrinogen III [4] with decarboxylation and oxidation reactions [6-7] generating protoporphyrin IX [8]. The second

tetrapyrrole biosynthesis branchpoint occurs here with either iron or magnesium insertion into the protoporphyrin IX nucleus. Iron chelation produces protoheme by ferrochelatase which can be the end of the pathway, such as in mammalian systems. Multistep synthesis from protoheme can generate bilin pigments. This involves re-opening the tetrapyrrole ring and loss of iron (Dammeyer and Frankenberg-Dinkel, 2008). These pigments form covalent bonds with biliproteins and are the primary photoreceptors in plants, fungi, algae, eubacteria, and cyanobacteria (Scheer and Zhao, 2008; Sharrock, 2008).

Magnesium insertion into protoporphyrin IX is unique to chlorophyll and bacteriochlorophyll biosynthesis. This step is ATP-dependent and involves a tri-subunit enzyme complex called magnesium chelatase consisting of subunits BchI/ChII, BchD/ChID, and BchH/ChIH with magnesium protoporphyrin IX produced [9]. Magnesium protoporphyrin IX is then specifically methylated at the C13 propionate group by *S*-adenosyl-L-methionine:magnesium protoporphyrin IX *O*-methyltransferase (BchM/ChIM) to form magnesium protoporphyrin IX monomethyl ester [10]. *S*-adenosyl-L-methionine (SAM) is the methyl donor molecule, and is converted into *S*-adenosyl homocysteine (SAH). Formation of a fifth isocyclic ring is catalysed by BchE under anaerobic conditions or AcsF in the presence of oxygen to give 3,8-divinyl protochlorophyllide [11]. Reduction of a vinyl group to form (monovinyl) protochlorophyllide [12] is catalysed by 8-vinyl reductase (DVR). This enzyme is called BciA in the green sulphur bacterium, *Chlorobaculum tepidum*. Ring IV of protochlorophyllide is then reduced to form chlorophyllide by dark-operative protochlorophyllide oxidoreductase (DPOR) [13]. Another branchpoint occurs here directed to either chlorophyll or bacteriochlorophyll biosynthesis. Chlorophyll *a* [14] synthesis is completed with the esterification of the C17 propionate group of chlorophyllide with geranylgeraniol by ChlG (chlorophyll synthase), and reduction to a phytyl chain by ChlP (geranylgeranyl reductase).

Bacteriochlorophyll biosynthesis from chlorophyllide can take two routes. The reduction of C7-8 by chlorophyllide oxidoreductase (BchX, BchY, and BchZ) to form 3-vinyl bacteriochlorophyllide [15], or BchF introduces a hydroxyl group at the 3-vinyl position to form 3-hydroxyethyl chlorophyllide [17]. It is unclear if each route occurs simultaneously or if there is a

preference for one pathway over the other (Willows and Kriegel, 2009). BchC oxidizes the C3 hydroxyl group into a C3 keto group. Esterification of the C17 propionate group of bacteriochlorophyllide *a* with a geranylgeraniol chain is catalysed by BchG. This is followed by reduction to a phytyl chain by BchP to form bacteriochlorophyll *a* [20].

1.2.2 Historical background

The (bacterio)chlorophyll biosynthetic pathway was first pioneered by Granick in the 1940-50's (Granick, 1948a, 1948b, 1950; Bogorad and Granick, 1953; Granick, 1961). These studies utilised mutants of the single-celled green alga *Chlorella vulgaris* to determine accumulated pigments which represent intermediates in the pathway. This included protoporphyrin IX (proto), magnesium protoporphyrin IX (Mg-proto), magnesium protoporphyrin IX monomethyl ester (Mg-proto ester), magnesium vinyl pheoporphyrin a_5 (protochlorophyllide), and other porphyrins with differing numbers of carboxylic groups (Granick, 1948a, 1948b, 1950; Bogorad and Granick, 1953; Granick, 1961). The presence of Mg-proto ester in cells of *Rba. sphaeroides* suggested a common intermediate in algae/photosynthetic bacteria (Jones, 1963). Conversion of Mg-proto to Mg-proto ester with chloroplasts from *Zea mays* indicated a conserved pathway in photosynthetic organisms (Radmer and Bogorad, 1967).

1.2.3 Genetic map of the bacteriochlorophyll biosynthetic pathway

The earliest genetic studies into bacteriochlorophyll biosynthesis and photosynthesis related genes were performed with the model photosynthetic bacteria *Rba. capsulatus* and *Rba. sphaeroides*. Genetic studies were made possible after the landmark discovery by Marris (1974) showed that *Rba. capsulatus* contains a unique gene transfer element. Yen and Marris (1976) demonstrated that this genetic material can be used for physical mapping of photosynthesis-related genes. Marris (1981) employed a known promiscuous plasmid from *Pseudomonas aeruginosa* to mobilize large sections of the *Rba. capsulatus* genome. He discovered one of the plasmids (pRPS404) encompasses most of the photosynthesis related genes involved in carotenoid and bacteriochlorophyll biosynthesis, and the photosynthetic reaction center/light-harvesting proteins (Marris, 1981). Work with this plasmid

involved insertional mutagenesis and complementation together with restriction fragment rescue (Biel and Marrs, 1983; Taylor et al., 1983; Zsebo and Hearst, 1984; Marrs, 2002). This generated a preliminary physical genetic map of several loci involved in photosynthesis which is called the photosynthetic gene cluster (Biel and Marrs, 1983; Taylor et al., 1983; Zsebo and Hearst, 1984). Site-directed mutagenesis of pRPS404 and analysis of the accumulated porphyrins allowed several open reading frames to be allocated to specific stages of the bacteriochlorophyll biosynthetic pathway (Wellington and Beatty, 1989; Young et al., 1989; Yang and Bauer, 1990; Bollivar and Bauer, 1992; Yildiz et al., 1992; Bauer et al., 1993; Burke et al., 1993, 1993; Bollivar et al., 1994b).

The genes within the photosynthetic gene cluster of *Rba. capsulatus* are summarized by Alberti et al. (1995), and are organized in superoperons (Young et al., 1989; Bauer et al., 1991; Wellington et al., 1992; Young et al., 1992). The genes encoding magnesium chelatase subunits BchI and BchD are on the same operon in photosynthetic bacteria, for example *crtAbchIDO* in *Rba. capsulatus* (Xiong et al., 2000; Igarashi et al., 2001; Willett et al., 2007). Depending on the organism *bchH* of magnesium chelatase may exist together with *bchI* and *bchD* or can be located on another operon such as the gene cluster *bchMLHBNF* in *Rba. capsulatus* (Alberti et al., 1995; Petersen et al., 1996; Igarashi et al., 2001).

The bacteriochlorophyll biosynthetic pathway has undergone periodic revision which is still continuing. This is partially due to biochemical characterization of heterologously expressed enzymes revealing some discrepancies from previously inferred mutant studies. For example *bchH* encodes one component of magnesium chelatase and not *O*-methyltransferase (Gibson et al., 1995). *bchM* encodes *O*-methyltransferase, not oxidative cyclase (Gibson and Hunter, 1994; Bollivar et al., 1994a; Bollivar et al., 1994b). *bciA* from the green sulphur bacterium *C. tepidum*, encodes 8-vinyl reductase, not *bchJ* (Suzuki and Bauer, 1995a; Chew and Bryant, 2007a). It was recently proposed that BchJ may act as a porphyrin carrier (Chew and Bryant, 2007a, 2007b) and this is further addressed in *Paper IV*.

1.2.4 Differences between bacteriochlorophyll and chlorophyll biosynthesis

The genetic mapping of the bacteriochlorophyll gene cluster allowed homologous genes from chlorophyll biosynthetic organisms to be identified. Bacteriochlorophyll specific gene names are abbreviated with *bch** where * refers to a single letter (Fig. 2). The genes in chlorophyll biosynthetic organisms are denoted *chl**. Many enzymatic steps from ALA to chlorophyllide are conserved in chlorophyll and bacteriochlorophyll biosynthesis but there are five steps that differ. The differences are at the genetic level resulting in alternative enzymes or novel proteins additionally involved. The five different steps are in ALA synthesis, magnesium chelation, oxidative cyclase, 8-vinyl reductase, and protochlorophyllide oxidoreductase.

In higher plants, algae, and most bacteria ALA is synthesized by the C₅-pathway from the precursor glutamic acid (Beale and Castelfranco, 1974; Beale et al., 1975; Oh-hama et al., 1982; Porra et al., 1983). There are three enzymes involved; glutamyl-tRNA synthetase (EC 6.1.1.17), glutamyl-tRNA reductase (EC 1.2.1.70), and glutamate 1-semialdehyde aminotransferase (EC 5.4.3.8) (Beale, 1999).

Magnesium chelatase (ChlI, ChlD, and ChlH) activity *in vivo* and *in vitro* utilises a fourth protein in oxygenic photosynthetic organisms called Gun4 (genomes uncoupled 4) (Larkin et al., 2003; Davison et al., 2005). There is no known homolog of Gun4 in bacteriochlorophyll biosynthetic organisms such as *Rba. capsulatus*. Several *gun* mutants were originally identified in *Arabidopsis thaliana*, with the common feature of impaired plastid to nucleus signalling (Susek et al., 1993). After treatment with the herbicide norflurazon, an inhibitor of plastid development, nuclear encoded genes (e.g. *lhcb1*) are derepressed in *gun4* mutants (Mochizuki et al., 2001; Larkin et al., 2003). Gun4 from *A. thaliana* and *Synechocystis* are required for optimal magnesium chelatase activity (Larkin et al., 2003; Davison et al., 2005)(see 1.5.11). Most photosynthetic organisms only have one BchI/ChlI subunit. However there are two BchI isoforms in *C. tepidum* (Petersen et al., 1998) and *A. thaliana* (ChlI1 and ChlI2) (Rissler et al., 2002) (see 1.5.7).

Oxidative cyclase is encoded by *acsF* in aerobic photosynthetic bacteria (Pinta et al., 2002), *CHL27* in *A. thaliana* (Tottey et al., 2003), or *chlA₁/chlA₂* isoforms in *Synechocystis* (Minamizaki et al., 2008). The enzymatic reaction uses molecular oxygen. It is suggested that an additional role exists for AcsF since it is present in chlorosomes under anaerobic conditions in *Chloroflexus aurantiacus* (Tang et al., 2009). Anaerobic photosynthetic bacteria have a different gene called *bchE* involved in cyclization (Gough et al., 2000). The enzyme reaction catalysed by BchE from *Rba. sphaeroides* uses water as the oxygen source and requires anaerobic conditions (Porra et al., 1995; Porra et al., 1998; Gough et al., 2000). BchE has an iron-sulphur cluster and uses adenosylcobalamin as a cofactor in a proposed radical SAM reaction (Gough et al., 2000; Sofia et al., 2001; Ouchane et al., 2004).

8-vinyl reductase is encoded by two unrelated genes in (bacterio)chlorophyll biosynthesis, *dvr*, and *cvrA*. Divinyl reductase (DVR) was originally identified in *A. thaliana*, with homologs found in other plants (*Oryza sativa*), algae (*Chlamydomonas reinhardtii*), green sulphur bacterium (*C. tepidum*), and purple non-sulphur bacterium (*Rba. sphaeroides*) (Nagata et al., 2005). The DVR ortholog from *C. tepidum* (BciA) encodes 8-vinyl reductase activity (Chew and Bryant, 2007a). BciA can utilise divinyl protochlorophyllide as a substrate (Chew and Bryant, 2007a), while the major substrate of DVR from *A. thaliana* is divinyl chlorophyllide (Nagata et al., 2007). Therefore it appears that in plants divinyl protochlorophyllide is first converted to divinyl chlorophyllide by protochlorophyllide oxidoreductase (POR), before the 8-vinyl group is reduced by DVR (Fig. 3)(Nagata et al., 2007). Recall that in anaerobic bacteriochlorophyll biosynthesis 8-vinyl reduction occurs first, followed by reduction of ring IV by dark-operative protochlorophyllide reductase (DPOR) to form monovinyl chlorophyllide (Fig. 2).

An unrelated 8-vinyl reductase gene is present in *Synechocystis* called *slr1923* (*cvrA*) (Islam et al., 2008; Ito et al., 2008). Mutants of this gene accumulate divinyl chlorophyll (Islam et al., 2008; Ito et al., 2008). Homologous genes of *cvrA* are found in eukaryotic organisms (*A. thaliana*), purple bacteria (e.g. *Rba. sphaeroides*), green sulphur bacteria, and green filamentous bacteria (Ito et al., 2008). Some photosynthetic organisms do not have *cvrA* or *dvr* homologs so another unidentified 8-vinyl reductase gene may exist (Ito et al., 2008).

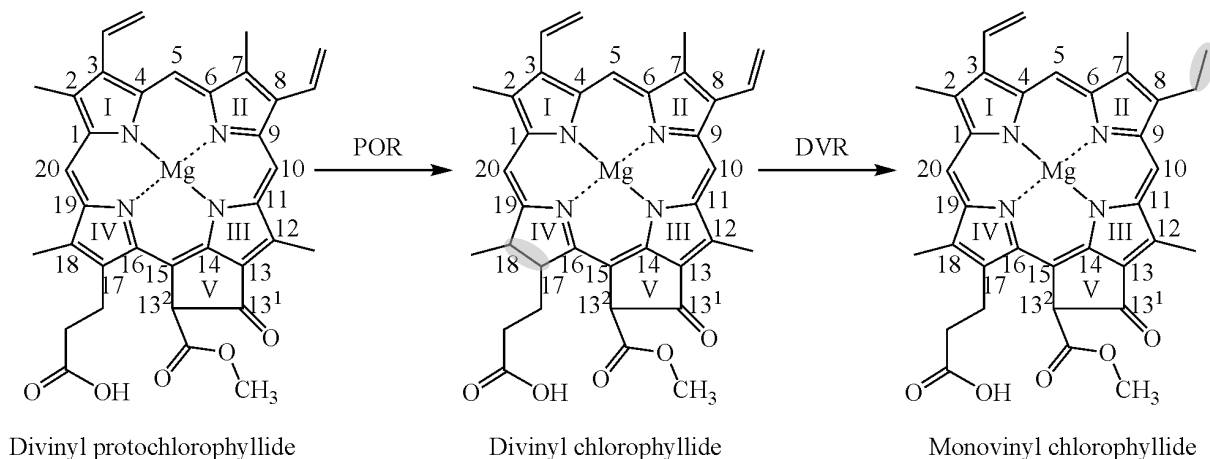


Figure 3. Revised later stages of the pathway for chlorophyll biosynthesis from divinyl protochlorophyllide to monovinyl chlorophyllide. Enzyme-catalysed changes to the structures are shaded in grey. The chlorophyll biosynthetic pathway first reduces ring IV of divinyl protochlorophyllide to form divinyl chlorophyllide predominantly by light-dependent protochlorophyllide oxidoreductase (POR). This is followed by reduction of the 8-vinyl group by divinyl reductase (DVR) to form monovinyl chlorophyllide.

The conversion of protochlorophyllide to chlorophyllide can occur in the light or dark. Angiosperms have only light-dependent protochlorophyllide oxidoreductase (POR) (EC 1.3.1.33), while most other oxygenic photosynthetic organisms have both POR and dark-operative protochlorophyllide oxidoreductase (DPOR) (Masuda and Takamiya, 2004). POR is thought to have evolved from cyanobacteria (Suzuki and Bauer, 1995b). *Synechocystis* has only one gene encoding POR (*por*) (Masuda and Takamiya, 2004). Higher plants have at least two isoforms of POR (Armstrong, 1998; Masuda and Takamiya, 2004). Three genes from *A. thaliana* are differentially expressed during greening in the light; *PORA*, *PORB*, and *PORC* (Oosawa et al., 2000). POR from *Synechocystis* has been thoroughly kinetically studied using purified enzyme. The reaction requires NADPH and involves several intermediate steps in catalysis (Heyes et al., 2003; Heyes and Hunter, 2005; Heyes et al., 2006; Heyes et al., 2008). Anoxygenic photosynthetic bacteria only have DPOR. DPOR is encoded by *bchB/chlB*, *bchL/chlL*, and *bchN/chlN* in bacteriochlorophyll/chlorophyll synthesizing organisms (Burke et al., 1993; Suzuki et al., 1997; Armstrong, 1998; Schoefs, 1999). In *Rba. capsulatus* the enzyme only operates under anaerobic conditions *in vitro* (Fujita and Bauer, 2000; Nomata et al., 2005; Yamamoto et al., 2008).

1.3 Regulation

1.3.1 Anaerobic/aerobic regulation of bacteriochlorophyll biosynthesis

Anoxygenic/oxygenic regulation occurs mainly through oxygen sensing involving specialized regulatory proteins. The organization of bacteriochlorophyll biosynthetic genes into superoperons allows a global regulation of the pathway. In facultative anoxygenic photosynthesis, phototrophic growth is highly sensitive to oxygen concentration. Bacteriochlorophyll production in *Rba. sphaeroides* ceases upon oxygenic conditions (> 5 % O₂) and strong recovery is observed upon return to anoxia (Cohen-Bazire et al., 1957; Gorchein et al., 1968). Under anaerobic conditions, variations in light give a more modest effect, with dim light showing faster bacteriochlorophyll biosynthesis compared with bright light (Cohen-Bazire et al., 1957). A decrease in oxygen levels from 20 % to 2 % results in a 2-4 fold increase in *bchA*, *bchB*, *bchC*, *bchG*, and *bchH* in *Rba. capsulatus* (Biel and Marrs, 1983). *bchA*, *bchB*, *bchC*, and *bchE* are similarly up-regulated by factors of ~4-6 upon change from aerobic to semi-aerobic conditions in *Rba. sphaeroides* (Hunter and Coomber, 1988).

Aerobic regulation of at least a portion of bacteriochlorophyll biosynthetic genes occurs through an oxygen sensing protein CrtJ in *Rba. capsulatus* or PpsR in *Rba. sphaeroides* (Walker and Willows, 1997; Masuda et al., 2002). Under aerobic conditions, binding of CrtJ to the promoter region of *bchC* causes repression of bacteriochlorophyll transcription (Masuda et al., 2002). The integrity of disulphide bond(s) under aerobic conditions in CrtJ or PpsR allows interaction with DNA (Masuda and Bauer, 2002; Masuda et al., 2002). Under anaerobic conditions, reduction of disulphide bonds causes a weakening of PpsR binding to DNA (Masuda and Bauer, 2002). Anaerobic conditions in the dark allows a PpsR antirepressor, AppA to interact with PpsR (Masuda and Bauer, 2002). This further weakens the interaction of PpsR with DNA thereby allowing RNA polymerase to initiate transcription (Masuda and Bauer, 2002; Bauer, 2004).

Anaerobic regulation of bacteriochlorophyll biosynthesis is directly controlled by a two-component system, RegA and RegB. Under anaerobic conditions, RegA is phosphorylated by RegB allowing RegA to bind to promoter regions which starts transcription of the photosynthetic apparatus

(Sganga and Bauer, 1992; Mosley et al., 1994; Bauer, 2004). This mechanism is conserved amongst other photosynthetic bacteria such as PrrB/PrrA in *Rba. sphaeroides* (Elsen et al., 2004; Elsen et al., 2005). RegA/RegB up-regulates transcription of genes in the bacteriochlorophyll biosynthetic pathway; *bchC* (Abada et al., 2002) and *bchE* (Oh and Kaplan, 2001). RegA/RegB also induces the expression of *bchEJGP*, and *crtAbchIDO* operons in *Rba. capsulatus* (Willett et al., 2007).

1.3.2 Regulation of magnesium chelatase

1.3.2.1 Regulation of BchH

The BchH subunit of magnesium chelatase from *Rba. capsulatus* is oxygen sensitive. There is a decrease in BchH when bacteria are exposed to aerobic conditions in the light or dark (Willows et al., 2003). BchH is inactivated by light in the presence of oxygen, and forms a covalent bond with photooxidised proto (photoporphyrin) *in vitro* (Willows et al., 2003). This causes the formation of large molecular weight inactive aggregates of BchH (Sirijovski et al., 2006). There is a conserved iron-sulphur cluster in BchH from purple non-sulphur bacteria (Sirijovski et al., 2007). Mutations in the iron-sulphur cluster do not significantly affect magnesium chelatase activity so it is unclear how the iron-sulphur cluster functions in BchH (Sirijovski et al., 2007). It is proposed to be involved in responding to changes in oxygen levels (Sirijovski et al., 2007).

1.3.2.2 Light regulation of ChII, ChID, and ChIH

Unlike photosynthetic bacteria, regulation of key steps in chlorophyll biosynthesis in plants has been studied in great detail. The information here will concentrate on the transcriptional and (post)translational regulation of plant and algal magnesium chelatase. Transcriptional regulation of magnesium chelatase in the light occurs through diurnal and circadian cues (Papenbrock et al., 1999; Harmer et al., 2000; Matsumoto et al., 2004; Moulin and Smith, 2005).

ChIH is the core regulatory subunit of magnesium chelatase. This is most obviously seen after prolonged darkness in *A. thaliana* seedlings where ChIH is absent while ChII is present (Stephenson and Terry, 2008). *chlH* is up-regulated upon light induction from a soybean photosynthetic cell culture

(Nakayama et al., 1998), barley seedlings (Yaronskaya et al., 2006), *C. reinhardtii* (Chekounova et al., 2001), tobacco leaves (Papenbrock et al., 1999), and *A. thaliana* mature leaves and seedlings (Matsumoto et al., 2004; Stephenson and Terry, 2008). Studies have shown an increase in ChlH in the light with greening etiolated barley tissue (Jensen et al., 1996a), *C. reinhardtii* (Chekounova et al., 2001), tobacco leaves (Papenbrock et al., 1999), and *A. thaliana* leaf tissue and seedlings (Gibson et al., 1996; Stephenson and Terry, 2008). Therefore the transcriptional up-regulation of *chlH* correlates with an increase in ChlH. *chlI* is up-regulated to a lesser degree than *chlH* in barley seedlings exposed to the light (Jensen et al., 1996b). More recent studies with *A. thaliana* seedlings and mature leaves show that *chlI1/chlI2* and *chlD* are mildly induced by light compared with *chlH* using microarrays or real-time PCR (Matsumoto et al., 2004; Stephenson and Terry, 2008). Consequently the increase or decrease in magnesium chelatase activity under light/dark conditions is mainly attributed to a higher or lower amount of ChlH respectively (Papenbrock et al., 1999).

Under a 12 h light and 12 h dark pattern, *chlH* from tobacco leaves is strongly down-regulated under dark conditions, and is up-regulated at the end of the dark period and beginning of the light period (Papenbrock et al., 1999). Transcriptional regulation of magnesium chelatase from *A. thaliana* leaves or seedlings shows (using microarrays) that *chlH* is under circadian rhythm while *chlI1/chlI2* and *chlD* are diurnally controlled (Matsumoto et al., 2004).

1.3.2.3 Additional regulation of *ChlI*, *ChlD*, and *ChlH*

Studies on the regulation of magnesium chelatase have also included *gun4*. White, red, far-red, and blue light up-regulate *gun4* transcripts during greening of *A. thaliana* seedlings in parallel with *chlH* (Stephenson and Terry, 2008). This suggests an interdependence of ChlH and Gun4 *in vivo* which correlates with previous studies showing the *in vitro* interaction between ChlH and Gun4 with *A. thaliana* and *Synechocystis* (Larkin et al., 2003; Davison et al., 2005; Verdecia et al., 2005; Sobotka et al., 2008).

The light-induction of magnesium chelatase is mainly attributed to far-red/red light photoreceptors (PhyA and PhyB respectively) and a minor effect with blue light cryptochrome

photoreceptor (Cry2) (Stephenson and Terry, 2008). *phyA* mutant *A. thaliana* seedlings have reduced amounts of *chlH* and *gun4* under far-red light after 4 h, while a *phyB* mutant causes a large down-regulation of *chlH* and *gun4* under red light after 8 h (Stephenson and Terry, 2008). *cry2* mutant *A. thaliana* seedlings have a mild down-regulation of *chlH* and *gun4* under blue light after 24 h (Stephenson and Terry, 2008). PhyB is indirectly affected by cytokinin, a plant hormone involved in many aspects of plant growth and development (Kakimoto, 2003). The cytokinin benzyladenine induces the expression of ARR4 (Arabidopsis response regulator 4) (Brandstatter and Kieber, 1998), a protein that interacts with and stabilizes PhyB (Sweere et al., 2001). Benzyladenine also increases cucumber glutamyl-tRNA reductase activity (Masuda et al., 1995). Another cytokinin, kinetin, up-regulates ChlH in greening barley seedlings which corresponds to an increase in magnesium chelatase activity (Yaronskaya et al., 2006).

Early light induced protein (ELIP2), a protein involved in protection against light stress in plants translationally regulates magnesium chelatase. Overexpressed ELIP2 from *A. thaliana* down-regulates ChlH and ChlI, while ChlD is unaffected (Tzvetkova-Chevolleau et al., 2007). This leads to a decrease in magnesium chelatase activity (Tzvetkova-Chevolleau et al., 2007). Glutamyl t-RNA reductase is also down-regulated so it is unclear if there is a direct effect on magnesium chelatase by ELIP2. ELIP2 binds chlorophyll (Adamska, 1997; Hutin et al., 2003) and it is suggested that ELIP2 bound to chlorophyll could be an inhibitor of an unknown step of the chlorophyll biosynthetic pathway (Tzvetkova-Chevolleau et al., 2007).

Regulation of magnesium chelatase from oxygenic species is dependent on the concentration of magnesium. This is observed by sigmoidal substrate-velocity curves with *in vitro* magnesium chelatase assays from *Synechocystis* and pea (Guo et al., 1998; Jensen et al., 1998; Reid and Hunter, 2004). Experiments with magnesium chelatase from the anaerobic bacterium *Rba. sphaeroides* have a hyperbolic response with magnesium which is representative of a non-regulatory effect (Gibson et al., 1999). Recent experiments using magnesium chelatase from *Rba. capsulatus* showed that there was a sigmoidal effect with magnesium (*Paper II Addendum*). In spinach, transition from dark to light causes an increase in magnesium concentration in the stroma from ~0.5 mM to 2 mM (Ishijima et al.,

2003). Regulation of magnesium chelatase by magnesium also involves Gun4 in oxygenic chlorophyll biosynthetic organisms. At ~2 mM magnesium, ChlH from *Synechocystis* interacts with Gun4 and causes an increase in magnesium chelatase activity *in vitro* (Davison et al., 2005). At this magnesium concentration magnesium chelatase alone has little activity *in vitro* (Davison et al., 2005). Therefore Gun4 is critical for the effective function of magnesium chelatase at physiological magnesium concentrations (Davison et al., 2005).

The addition of exogenous proto and Mg-proto increases the amount of ChlH and Gun4 (Adhikari et al., 2009). This suggests a cooperative effect by ChlH and Gun4 to quench these reactive metabolites (Adhikari et al., 2009). The cell produces ChlH in response to stress through Mg-proto accumulation in *A. thaliana chlM* mutants (Pontier et al., 2007). ChlH is up-regulated at the post-transcriptional level, suggesting Mg-proto affords the stabilization of the protein. Without porphyrin bound, BchH from *Rba. capsulatus* is prone to protease degradation *in vitro* (Sirijovski et al., 2008).

1.3.2.4 Regulation of ChII and ChIH by reducing disulphide bonds

Magnesium chelatase is affected by synthetic cysteine-modifying small molecules or thioredoxin. Magnesium chelatase from plant chloroplasts or crude cell lysate from purple non-sulphur bacteria is inhibited by sulphhydryl modifying chemicals such as N-ethyl maleimide (NEM), *p*-chloromercuribenzenesulphonate (PCMBS), or thiomersal (Fuesler et al., 1984a; Fuesler et al., 1984b; Walker et al., 1991; Walker and Weinstein, 1991a; Gorchein, 1997). Purified ChII or ChIH from *Synechocystis* is adversely affected by NEM, and magnesium chelatase activity increases with the reduction of disulphide bonds by the addition of dithiothreitol (DTT) (Jensen et al., 2000). ChIH is partially protected from NEM inhibition through binding proto and MgATP, while ChII is entirely protected through binding magnesium and ATP (Jensen et al., 2000). *A. thaliana* ChII is implicated in thioredoxin regulation through protein-protein interactions using affinity chromatography (Balmer et al., 2003). Both ChII1 and ChII2 isoforms from *A. thaliana* are regulated through reduction of cysteine residues by thioredoxin (Ikegami et al., 2007; Kobayashi et al., 2008). Reduction of ChII by thioredoxin allows each isoform to hydrolyse ATP, a critical feature of this subunit (Kobayashi et al.,

2008). The regulation of ChlII by thioredoxin relies on the presence of light (Ikegami et al., 2007). Key cysteine residues involved in disulphide bond formation are identified at position 354 and 396 in ChlII (Ikegami et al., 2007).

1.3.3 Branchpoint of tetrapyrrole biosynthesis; magnesium or iron insertion

Magnesium chelatase and ferrochelatase share the porphyrin intermediate, proto as a substrate for their respective enzymatic activities. Magnesium chelatase BchH/ChlH subunit and ferrochelatase probably share the same pool of proto substrate since they are located on the chloroplast envelope (Roper and Smith, 1997; Adhikari et al., 2009). Magnesium chelatase from pea chloroplasts has a K_m of approximately 13 nM for proto (Guo et al., 1998), while ferrochelatase has a K_m of 2400 nM (Matringe et al., 1994). Therefore magnesium chelatase is expected to out-compete ferrochelatase for proto substrate as discussed in Walker and Willows (1997).

Inverse magnesium chelatase and ferrochelatase activity is partly attributed to the levels of the three nucleotides; ATP, ADP, and AMP. Addition of ALA and ATP to pea chloroplasts increases Mg-proto ester accumulation and decreases heme levels which corresponds to an increase in magnesium chelatase activity and a decrease in ferrochelatase activity (Walker et al., 1997). Addition of ATP to pea chloroplasts decreases ferrochelatase activity (Cornah et al., 2002; Cornah et al., 2003). Magnesium chelatase from *Synechocystis* is inhibited by ADP (Jensen et al., 1999a) and magnesium chelatase from cucumber chloroplasts is inhibited by AMP (Pardo et al., 1980). The ATP:ADP ratio increases from 1:1 to 2.5-4:1 in maize leaves grown in the light compared to dark (Usuda, 1988). In tobacco leaves magnesium chelatase activity peaks during the onset of light and decreases during the light period, while ferrochelatase steadily increases at the onset of light, and is highest at the later stages of the light period (Papenbrock et al., 1999).

1.3.4 Regulation of S-adenosyl-L-methionine:magnesium protoporphyrin IX O-methyltransferase (BchM/ChlM)

1.3.4.1 Product inhibition

O-methyltransferase is not a major player in the regulation of bacteriochlorophyll biosynthesis. It is mainly regulated in response to metabolites, or post-translationally through protein-protein interactions with BchH/ChlH. O-methyltransferase is product inhibited by SAH or Mg-proto ester (Gibson et al., 1963; Ellsworth et al., 1974). The inhibition by SAH is linked to folate biosynthesis through the C₁ pathway (Hanson and Roje, 2001; Van Wilder et al., 2009; Webb and Smith, 2009). Inhibition of tetrahydrofolate formation in etiolated pea seedlings leads to an increase in the amount of SAH (Van Wilder et al., 2009). The decrease in the SAM:SAH ratio (methylation index) from 7:1 to 1:1 is responsible for reduced O-methyltransferase activity and chlorophyll synthesis by a factor of ca. three (Van Wilder et al., 2009). Regulation is strictly post-translational since gene and protein expression are unaffected (Van Wilder et al., 2009). Porphyrin metabolites Mg-proto ester, protochlorophyllide, chlorophyllide, or chlorophyll *a* each inhibit O-methyltransferase from wheat (Ellsworth et al., 1974; Ellsworth and St Pierre, 1976). Therefore O-methyltransferase is negatively regulated by feedback inhibition of porphyrin metabolites. Product inhibition of O-methyltransferase by Mg-proto ester gives the greatest effect (Ellsworth et al., 1974).

1.3.4.2 Other regulation

Early studies showed that O-methyltransferase activity is increased in the light (Gorchein et al., 1968). The specific activity of O-methyltransferase from *Rba. sphaeroides* increases by ~6-fold upon illumination, and is inhibited in the dark (Gorchein et al., 1968) while *Euglena gracilis* O-methyltransferase increases by ~2-3 fold in the light, and is reduced by a comparable margin in the dark (Ebbon and Tait, 1969). In contrast, O-methyltransferase is not regulated upon light induction in etiolated wheat seedlings after 24 h (Ellsworth and St Pierre, 1976). Unlike magnesium chelatase, O-methyltransferase is not transcriptionally regulated by diurnal or circadian rhythm in *A. thaliana* seedlings or leaves using microarrays (Matsumoto et al., 2004).

O-methyltransferase is post-translationally regulated by magnesium chelatase. An increase in *O*-methyltransferase activity follows shortly after an increase in magnesium chelatase activity in response to light (Papenbrock et al., 1999; Yaronskaya et al., 2006). The increase in magnesium chelatase activity is attributed to *chlH* up-regulation and an increase in ChlH (see 1.3.2.2). The plant hormone cytokinin increases barley ChlM in the light corresponding to an increase in enzymatic activity (Yaronskaya et al., 2006). This parallels an increase in ChlH supporting a co-operative regulation between ChlH and ChlM (Yaronskaya et al., 2006). The mechanism of post-translational regulation of ChlM by ChlH is unknown but an important aspect is that the two proteins can interact to form a complex which involves transfer of Mg-proto (Alawady et al., 2005; Shepherd et al., 2005).

Thiol group(s) are important for optimal *Rba. sphaeroides* and barley *O*-methyltransferase activity. Sulphydryl-targeted molecules (Ag^+ and *p*-chloromercuribenzoate) inhibit *Rba. sphaeroides* *O*-methyltransferase by > 90 % (Gibson et al., 1963). DTT or mercaptoethanol increase barley *O*-methyltransferase activity by 1.5-fold (Shieh et al., 1978). However there is no suggestion of thioredoxin-targeted regulation of *O*-methyltransferase from spinach (Balmer et al., 2003), indicating that reduced disulphide bonds are not a critical feature for activity.

1.4 Cellular localization of protoporphyrinogen oxidase, magnesium chelatase, Gun4, and *O*-methyltransferase

1.4.1 Overview

Cell localization of each protein is mainly studied in plant chloroplasts. Magnesium chelatase ChlH subunit, Gun4, and *O*-methyltransferase are predominantly present at the inner membrane of chloroplasts, whereas there is a stromal ChlI•ChlD complex (Masuda and Fujita, 2008). The current model indicates there is a stromal-based ChlI•ChlD complex that interacts with ChlH and Gun4 at the periphery of the inner envelope (Masuda and Fujita, 2008). Protoporphyrinogen oxidase, the enzyme that synthesizes proto is located on the chloroplast envelope and thylakoids (Matringe et al., 1992; Watanabe et al., 2001). ChlH and Gun4 bind proto and Mg-proto, while ChlM binds Mg-proto, and it

is suggested there is direct movement of porphyrin substrates and products between these proteins in the envelope (Tanaka and Tanaka, 2007). Free porphyrins pose a threat to the viability of the cell with the formation of toxic free radical species (Ricchelli, 1995; Vavilin and Vermaas, 2002). Consequently free porphyrin concentration is expected to be very low *in vivo*.

1.4.2 Magnesium chelatase

A general localization of the magnesium chelatase complex was initially shown in cucumber using inhibitors (Fuesler et al., 1984b). Chloroplasts prepared in buffer with 1 mM Mg^{2+} and pre-treated with a cell membrane-impermeable inhibitor, PCMBS completely inhibit magnesium chelatase, while a known stromal enzyme is mildly affected. Addition of a cell membrane-permeable inhibitor, *p*-chloromercuribenzoic acid (PCMB) inhibits magnesium chelatase to a similar degree. Therefore it was presumed that magnesium chelatase lies on the outer chloroplast envelope (Fuesler et al., 1984b). Contrasting results are found by Walker and Weinstein (1995) with 4 times greater inhibition by PCMB over PCMBS in pea and cucumber chloroplasts at 1 mM Mg^{2+} (Walker and Weinstein, 1995). This suggests that magnesium chelatase is predominantly found in the stroma at low magnesium concentration.

The following studies looked at the chloroplast localization of ChII, ChID, and ChIH using buffer with no magnesium, or magnesium complexed with EDTA. ChII from *A. thaliana* and soybean is found only in the stroma (Nakayama et al., 1995; Gibson et al., 1996), whereas ChID from pea is in the light membrane fraction (Luo et al., 1999). Experiments involving barley plastid lysates and *Rba. sphaeroides* cell lysates show that BchD/ChID is located in the pellet fraction, with BchH/ChIH and BchI/ChII in the soluble fraction after high speed centrifugation (Kannangara et al., 1997). Recent work with pea chloroplasts found ChII and ChID predominantly in the soluble fraction (Adhikari et al., 2009).

At low concentrations of magnesium (~1 mM Mg^{2+}), ChIH is present in the stroma as well as the outer envelope membrane in *A. thaliana* and soybean chloroplasts (Gibson et al., 1996; Nakayama

et al., 1998). At higher magnesium concentrations ($> 5 \text{ mM Mg}^{2+}$) there is a pronounced accumulation of ChlH in the membrane fraction (Gibson et al., 1996; Nakayama et al., 1998).

1.4.3 Gun4 and ChlH

Gun4 is present in all compartments of the chloroplast (stroma, envelope, and thylakoids) in pea and *A. thaliana* with the majority of protein in the stroma (Larkin et al., 2003; Adhikari et al., 2009). In the presence of elevated magnesium, pea ChlH moves from the stroma to the inner envelope membrane or thylakoid membrane where an interaction between ChlH and Gun4 occurs (Adhikari et al., 2009). *A. thaliana* Gun4 and ChlH form a large complex of ~500 kDa in the thylakoid membranes (Larkin et al., 2003). *Synechocystis gun4* mutants have decreased ChlH in whole cells or the membrane fraction which suggests that Gun4 is required for ChlH stability *in vivo* (Wilde et al., 2004; Sobotka et al., 2008).

1.4.4 O-methyltransferase

O-methyltransferase is membrane-associated from bacterial, algal, and plant sources. Early studies measuring O-methyltransferase activity from *Rba. sphaeroides* used crude chromatophores (membranes of lysed cell preparations) (Tait and Gibson, 1961; Hinchigeri et al., 1984). The soluble form of the plant enzyme is generated with addition of sucrose (Radmer and Bogorad, 1967; Ellsworth and Dullaghan, 1972; Ellsworth et al., 1974; Shieh et al., 1978). Despite this, O-methyltransferase is localized in the thylakoid membrane and chloroplast envelope in *A. thaliana* and spinach (Block et al., 2002). O-methyltransferase activity is measured in the envelope membrane from pea (Van Wilder et al., 2009), and approximately 90 % of O-methyltransferase activity is attributed to the inner membrane of etioplasts from barley leaves (Averina et al., 2002).

Solubilisation of *E. gracilis* chloroplast with 0.5 % Tween 80 produces a 2-fold increase in O-methyltransferase activity (Ebbon and Tait, 1969). Therefore it is suggested that the enzyme is lipid-associated. The effect of lipids on O-methyltransferase stability and enzymatic activity from *Rba. capsulatus* was tested in *Paper III*. Interactions between BchH of magnesium chelatase, Gun4, and O-

methyltransferase are expected to occur at the chloroplast inner envelope membrane (Masuda and Fujita, 2008).

1.5 Magnesium chelatase past and present

1.5.1 Overview

Magnesium chelatase catalyses the insertion of magnesium into proto to form Mg-proto (Fig. 4). This occurs in a magnesium and ATP-dependent manner. This tri-subunit enzyme is encoded by the genes *bchI/chlI*, *bchD/chlD*, and *bchH/chlH* (Table 2) with each subunit catering to a different function. BchD/ChlD is a structural platform, BchI/ChlI has ATPase activity required for catalysis, while BchH/ChlH binds proto and Mg-proto. Gun4 is a fourth protein involved in delivering proto substrate to ChlH in oxygenic photosynthetic organisms.

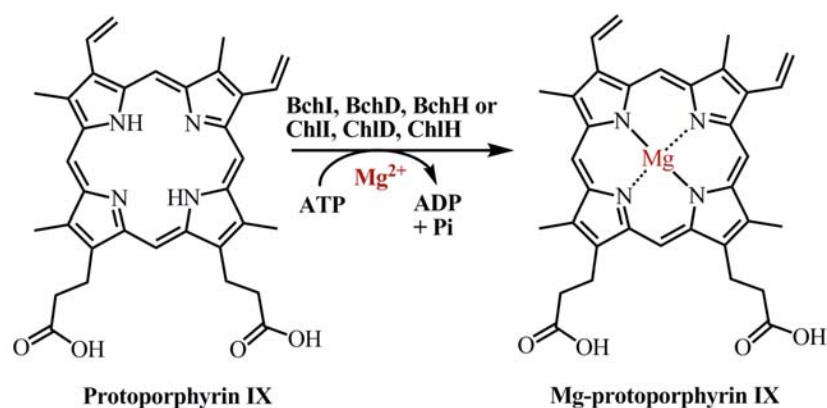


Figure 4. The enzymatic reaction catalysed by magnesium chelatase complex (BchI/ChlI, BchD/ChlD, and BchH/ChlH). The reaction requires magnesium and ATP hydrolysis. The protoporphyrin IX and Mg-protoporphyrin IX binding protein Gun4 which also participates in porphyrin delivery in aerobic photosynthetic organisms is not shown.

1.5.2 Early magnesium chelatase activity

The first evidence for magnesium chelatase activity was observed by Gorchein in 1972 using whole cells of *Rba. sphaeroides* (Gorchein, 1972). Mg-proto ester is produced under anaerobic (nitrogen) conditions in the light following addition of exogenous proto. This gave the

Table 2. Alternate names used for magnesium chelatase subunits. For clarity the main body of text will only use the traditional *bchI/chlI*, *bchD/chlD*, and *bchH/chlH* gene or protein names.

Organism	<i>bchI/chlI</i>	<i>bchD/chlD</i>	<i>bchH/chlH</i>	Reference
	Alternative gene names			
Barley	<i>xantha-h</i>	<i>xantha-g</i>	<i>xantha-f</i>	(Jensen et al., 1996a)
<i>A. thaliana</i>	<i>cs/ch-42</i>		<i>gun5</i>	(Koncz et al., 1990; Gibson et al., 1996) (Mochizuki et al., 2001)
Olive	<i>oli</i>			(Hudson et al., 1993)
<i>O. sativa</i>	<i>chl1</i>	<i>chl9</i>		(Zhang et al., 2006)
<i>E. gracilis</i>	<i>ccsA</i>			(Orsat et al., 1992)
<i>Zea mays</i>	<i>oyl</i>			(Sawers et al., 2006)

first clue for magnesium chelatase and *O*-methyltransferase interactions (described in 1.7). Treatment of whole cells of *Rba. sphaeroides* with various electron transport inhibitors or uncouplers decreases the amount of Mg-proto ester formed (Gorchein, 1973). This strongly suggests an ATP requirement for either magnesium chelatase or *O*-methyltransferase. Developing chloroplasts showed a glutamate requirement for magnesium chelatase activity, although it was unclear why (Castelfranco et al., 1979). Later work found that 10 mM ATP could substitute for glutamate (Pardo et al., 1980). It was concluded that glutamate is supplying the substrate for a contaminating mitochondrial ATP-regenerating system present in the chloroplast preparation (Pardo et al., 1980). In cucumber etioplasts the optimal magnesium and ATP requirement is approximately 10 mM each when added exogenously following removal of excess magnesium with EDTA (Fuesler et al., 1981). ATP hydrolysis is required for magnesium chelatase activity since non-hydrolysable analogs of ATP; adenosine 5'-[β,γ -methylene]triphosphate and adenosine 5'-[β,γ -imido]triphosphate cannot support magnesium chelatase activity (Walker and Weinstein, 1991b).

1.5.3 *In vitro* assays

The first *in vitro* plant assay for magnesium chelatase was developed using cucumber chloroplast membranes although a large proportion of activity is lost upon lysis (Richter and Rienits,

1982). Preliminary kinetic analysis performed with this lysate generates hyperbolic curves for magnesium, ATP, and proto substrates (Richter and Rienits, 1982). Walker and Weinstein (1991b) support a hyperbolic relationship with proto using cucumber chloroplasts. A previous report shows a sigmoidal relationship of proto and ATP with cucumber chloroplasts (Fuesler et al., 1981). Magnesium chelatase activity from pea chloroplasts has since shown a sigmoidal relationship with magnesium and ATP (Guo et al., 1998). The sigmoidal effect with magnesium is also shown with *Synechocystis* and *C. tepidum* magnesium chelatase (Jensen et al., 1998; Reid and Hunter, 2004; Johnson and Schmidt-Dannert, 2008).

A much more efficient *in vitro* assay of magnesium chelatase from lysed pea chloroplasts was developed by Walker and Weinstein (1991c). Activity is present in both soluble (~80 %) and membranous components, with a synergistic effect when mixed together (Walker and Weinstein, 1991c). This indicated that at least two proteins are involved (Walker and Weinstein, 1991c). Loss of activity after disruption of cucumber plastids is overcome by the addition of proto and ATP (Lee et al., 1992). In contrast to pea, only the membrane fraction is required for activity.

A method of separating pea thylakoid membranes containing chlorophyll from envelope membranes and stroma was developed by Walker et al. (1992). This lysate still requires both the soluble and membrane fraction for reasonable magnesium chelatase activity. These authors also developed a continuous magnesium chelatase assay. A “lag-phase” in magnesium chelatase product (Mg-proto) formation was observed over time when the lysate is not pre-incubated with ATP (Walker et al., 1992). Preparation with ATP causes a near-eradication of the “lag-phase” (Walker et al., 1992). This strongly suggests that the magnesium chelatase reaction consists of two stages (Walker et al., 1992).

1.5.4 Reaction mechanism

The discovery of a “lag-phase” in the magnesium chelatase reaction was the starting point for expounding the details of the two-stage reaction mechanism (Walker et al., 1992). A different ATP requirement is observed for the two stages. In the first stage, ATP can be replaced by a slowly

hydrolysable ATP analog (adenosine 5'-[γ -thio]triphosphate), but an obligatory ATP requirement is observed for the second stage (Walker and Weinstein, 1994). Three subunits are required for magnesium chelatase activity, and preincubation of BchI/ChII and BchD/ChID with magnesium and ATP eradicated the "lag-phase" (Willows et al., 1996; Jensen et al., 1998; Willows and Beale, 1998) (see 1.5.9). Therefore the first stage involves pre-formation of a stable complex between BchI/ChII and BchD/ChID (Walker and Willows, 1997). The second stage is the enzymatic magnesium chelatase reaction, which is the insertion of magnesium into proto (Lee et al., 1992; Walker et al., 1992; Walker and Weinstein, 1994; Walker and Willows, 1997).

The optimal stoichiometry of the BchI•BchD/ChII•ChID complex varies depending on the organism, but is concentration dependent. In *Rba. capsulatus* magnesium chelatase, the optimal ratio is 4:1 BchI:BchD at 700 nM BchD, whereas 2.5:1 is sufficient at 1840 nM BchD for optimal magnesium chelatase activity (Willows and Beale, 1998; Willows and Hansson, 2003). Similarly in *Rba. sphaeroides* magnesium chelatase, 5:1 BchI:BchD is optimal at 8.5 nM BchD, whereas 1:1 BchI:BchD is optimal at 27 nM BchD (Gibson et al., 1999; Willows and Hansson, 2003). A 2:1 ratio of ChII:ChID was deemed optimal from *Synechocystis* (Jensen et al., 1998). There is a 1:1 molar ratio of the BchI•BchD structure by EM (Elmlund et al., 2008). Therefore the stoichiometry of these subunits used in *in vitro* assays does not reflect the actual ratio of each subunit in the complex (see 6.2.2). The *in vivo* stoichiometry of the ChII•ChID complex is critical for the proper functioning of magnesium chelatase since both anti-sense and sense *chII* transgenic tobacco plants decrease magnesium chelatase activity (Papenbrock et al., 2000).

There is an obligatory requirement upon ATP hydrolysis for the second stage of the magnesium chelatase reaction, magnesium insertion into proto. Magnesium chelatase activity ceases following removal of ATP during the initial phase of the reaction using a glucose/hexokinase sink (Walker and Weinstein, 1994). Magnesium chelation is sluggish with a slowly hydrolysable form of ATP, adenosine 5'-[γ -thio]triphosphate (Walker and Weinstein, 1994). Magnesium insertion into deuteroporphyrin requires ~15 ATP molecules per formation of a single molecule of Mg-deuteroporphyrin (Reid and Hunter, 2004). ATP hydrolysis is presumed to supply the energy needed

for removal of water molecules electrostatically bound to magnesium prior to insertion of the magnesium atom into the porphyrin nucleus (Beale, 1999). Chemical insertion of magnesium into proto in an aqueous environment is very difficult without a catalyst (Baum et al., 1964). There is evidence suggesting that some ATP hydrolysis is likely to be required during the reorganization of magnesium chelatase BchH subunit and BchI•BchD complex for secondary rounds of catalysis (*Paper II*).

Kinetic experiments show that ChlD at limiting concentrations behaves as an enzyme, with ChII and ChIH acting as saturable substrates in *Synechocystis* (Jensen et al., 1998). The K_m of ChII is 2-3 times lower than that for ChIH (Jensen et al., 1998). Therefore the interaction between ChlD and ChII is stronger than the interaction between ChlD and ChIH (Papenbrock et al., 1997; Gräfe et al., 1999). BchD was also treated as the enzyme in kinetic experiments with BchI and BchH from *Rba. capsulatus* (*Paper II*).

Reactions performed with an excess of *Synechocystis* magnesium chelatase subunits found there was a fast substrate binding step (E-deuteroporphyrin) where E refers to the enzyme (Viney et al., 2007). This is followed by a slow step involving either formation of E-Mg-deuteroporphyrin or the reorganization of magnesium chelatase (E'-deuteroporphyrin) into a form capable of catalysis (Viney et al., 2007). Results suggested a cooperative effect with the first turn-over of substrate generating a more active form of the enzyme (Viney et al., 2007).

1.5.5 Heterologous expression, reconstitution, and purification

The first heterologous expression of magnesium chelatase was performed with BchI, BchD, and BchH subunits from *Rba. sphaeroides* in *E. coli* (Gibson et al., 1995). This was quickly followed for other photosynthetic organisms. This includes ChII, ChIH subunits from soybean (Nakayama et al., 1995; Nakayama et al., 1998), ChlD from pea (Luo et al., 1999), and ChIH from *A. thaliana* (Gibson et al., 1996). All three magnesium chelatase subunits have been expressed from the cyanobacterium *Synechocystis* (Jensen et al., 1996b), tobacco (Kruse et al., 1997; Papenbrock et al., 1997), purple non-sulphur bacterium, *Rba. capsulatus* (Willows and Beale, 1998), green sulphur bacteria *Chlorobium*

vibrioforme (Petersen et al., 1998), *C. tepidum* (Johnson and Schmidt-Dannert, 2008), and the acidic purple bacterium *Acidiphilium rubrum* (Masuda et al., 1999). The first purification of each magnesium chelatase subunit from *Rba. sphaeroides* used non His-tagged proteins (Willows et al., 1996). Multiple chromatographic steps generated highly purified BchI and BchH, while BchD co-purified with chaperonin GROEL (Willows et al., 1996).

From this point forth, the majority of studies use His-tagged protein constructs due to their relative ease of purification by metal affinity chromatography. This was first done using *Synechocystis* ChII, ChID, and ChIH (Jensen et al., 1998), *Rba. capsulatus* and *C. vibrioforme* BchI, BchD, and BchH (Petersen et al., 1998; Willows and Beale, 1998). Purification of magnesium chelatase from *Rba. sphaeroides* and *C. tepidum* has also been performed (Gibson et al., 1999; Johnson and Schmidt-Dannert, 2008). Each protein is His-tagged apart from BchI in *Rba. capsulatus* which needs the native protein to establish magnesium chelatase activity (Petersen et al., 1998; Willows and Beale, 1998). All three proteins from the different organisms are purified to near-homogeneity.

BchD is expressed as inclusion bodies in these studies apart from *Synechocystis* where it is soluble (Jensen et al., 1998). Purification of BchD is performed under denaturing conditions in 6 M urea (Petersen et al., 1998; Willows and Beale, 1998; Gibson et al., 1999; Johnson and Schmidt-Dannert, 2008). Optimal refolding of BchD requires the rapid addition of BchI, together with ATP, magnesium, DTT, and incubation on ice for 1 h (Willows and Beale, 1998). This procedure is commonly employed in *Rba. sphaeroides* and *Rba. capsulatus* (Gibson et al., 1999) (*Papers I and II*). BchD from *C. tepidum* is refolded by dialysis into buffer containing 50 mM Tris-HCl pH 8 with 10 % glycerol (Johnson and Schmidt-Dannert, 2008). Alternatively co-expression of pea ChID with chaperonin DnaK solubilises ChID, with magnesium chelatase activity restored after addition of partially purified ChII and ChIH from pea chloroplast lysate (Luo et al., 1999). Purification of His-tagged ChII, ChID, and ChIH subunits from *Synechocystis* is comparatively straight-forward since all subunits are soluble and pure after metal affinity and ion exchange chromatography (Jensen et al., 1998).

A complete plant magnesium chelatase system has yet to be purified to homogeneity. The only attempt with lysed pea chloroplasts gave partial purification of each magnesium chelatase subunit (Guo et al., 1998). Reconstitution of activity requires the inclusion of each component. *In vitro* magnesium chelatase activity could not be measured in the acidic phototroph *A. rubrum* at pH 9 (Masuda et al., 1999). However bacteriochlorophyll *a* is produced when *bchI*, *bchD*, and *bchH* genes of magnesium chelatase from *A. rubrum* are expressed in a non-photosynthetic *Rba. capsulatus* magnesium chelatase mutant under the *puc* promoter of *Rba. capsulatus* (Masuda et al., 1999). Further study is needed to determine the optimal conditions of this enzyme.

1.5.6 Aerobic cobaltochelate, a homologous enzyme to magnesium chelatase

The cobaltochelate complex (CobS•CobT•CobN) allows insertion of cobalt into hydrogenopyrrolic acid *a,c*-diamide to form cob(II)pyrrolic acid *a,c*-diamide (Debussche et al., 1992; Lundqvist et al., 2009). The aerobic form of cobaltochelate is a class I chelatase, just like magnesium chelatase (Brindley et al., 2003). Both enzymes have three subunits, and require ATP for catalysis (Brindley et al., 2003). The three subunits of cobaltochelate; CobS, CobT, and CobN share significant homology with BchI, BchD, and BchH subunits of magnesium chelatase respectively (Hudson et al., 1993; Willows and Hansson, 2003; Lundqvist et al., 2009). The molecular masses of each homologous pair; CobS/BchI (37/38 kDa), CobT/BchD (71/60 kDa) and CobN/BchH (137/129 kDa) from *Pseudomonas denitrificans* and *Rba. capsulatus* are comparable. Recently the structure of a CobS•CobT complex was solved by electron microscopy (EM), and shows high similarity to the BchI•BchD complex of magnesium chelatase (Elmlund et al., 2008; Lundqvist et al., 2009)(see 1.5.9).

1.5.7 BchI/ChII subunit

The BchI/ChII subunit (~40 kDa) belongs to a metabolically diverse class known as AAA proteins (ATPases associated with a variety of cellular activities) (Neuwald et al., 1999; Vale, 2000; Ogura and Wilkinson, 2001; Iyer et al., 2004). AAA proteins typically form oligomers with large conformational changes upon addition of ATP (Iyer et al., 2004). BchI of *Rba. capsulatus* or *Rba. sphaeroides* forms oligomers in the presence of ATP with an approximate molecular mass of 90-130

kDa by gel filtration, suggesting a dimer or trimer exists (Gibson et al., 1999; Hansson et al., 2002). Oligomerisation of ChII in *Synechocystis* is concentration-dependent. At relatively high protein concentration (8 mg.mL^{-1}) a 208 kDa ChII complex is observed without ATP (Jensen et al., 1998). At lower protein concentrations (0.8 and 2 mg.mL^{-1}), ~ 81 and 108 kDa sized structures are found respectively indicating there is partial oligomerisation (Jensen et al., 1998). The addition of magnesium and ATP at each protein concentration gives a ~ 290 kDa oligomer indicative of a heptameric structure (Jensen et al., 1998).

BchI/ChII is the only magnesium chelatase subunit with ATPase activity (Hansson and Kannangara, 1997; Petersen et al., 1998; Jensen et al., 1999a; Petersen et al., 1999a; Hansson et al., 2002; Lake et al., 2004), a critical feature for catalysis (Hansson et al., 2002) (see 1.5.4 above). Early reports of the BchH subunit having ATPase activity proved untrue after an ATPase contaminant from *E. coli* during heterologous BchH expression from *Rba. capsulatus* was discovered as the source of ATP hydrolysis (Sirijovski et al., 2006). ATPase activity of ChII from *Synechocystis* is dependent upon the concentration of free magnesium, suggesting this subunit may hold the magnesium atom (Reid et al., 2003).

Studies with ATPase-deficient BchI mutants from *Rba. capsulatus* shows the oligomerisation of this subunit only requires ATP binding, not hydrolysis (Hansson et al., 2002). A 1:1 molar ratio of mutant BchI:wild type BchI causes a 65-80 % inhibition of wild type magnesium chelatase activity (Hansson et al., 2002). Strength of inhibition depends upon the capacity of the various mutants to associate and dissociate with wild type BchI. This semi-dominant effect with mutant BchI is expected for oligomeric structures. Therefore each subunit independently contributes to an oligomeric BchI subunit (Hansson et al., 2002). A similar approach using mutant BchD was undertaken to establish if this subunit is also oligomeric (*Paper I*).

Crystallization of BchI from *Rba. capsulatus* reveals a similar structure to the hexameric ATP-dependent protease, HsIU (Willows et al., 1999; Bochtler et al., 2000; Fodje et al., 2001). In the presence of ATP, BchI from *Rba. capsulatus* or ChII from *Synechocystis* form hexamers or heptamers

respectively by EM (Reid et al., 2003; Willows et al., 2004). BchI/ChlI have conserved walker A (P-loop) (G[DH]RGTGKS) and walker B motifs characteristic of ATP binding (Koonin, 1993, 1997; Fodje et al., 2001; Sirijovski et al., 2006), as well as sensor I/II motifs and an arginine sensor/finger, present only in AAA proteins (Neuwald et al., 1999; Vale, 2000; Fodje et al., 2001). It is thought that sensor I and sensor II motifs are important for ATP hydrolysis, while the arginine sensor/finger is involved in ATP-hydrolytic conformational changes (Iyer et al., 2004). The importance of the arginine sensor (Arg²⁸⁹) is demonstrated in *Rba. capsulatus* BchI mutants since substitution by lysine results in a complete loss of ATPase activity (Hansson et al., 2002). Mutation of an adjacent amino acid to the arginine finger (Arg²⁰⁸) from negative to neutral charge (Asp²⁰⁷Asn) also abolishes ATPase activity (Hansson et al., 2002).

There are two isoforms of BchI from *C. vibrioforme* (38 and 42 kDa) with both proteins capable of magnesium chelatase activity (Petersen et al., 1998). *A. thaliana* has two ChII subunits (ChII1 and ChII2) (Rissler et al., 2002), and their respective roles in chelation has been debated recently (Apchelimov et al., 2007; Kobayashi et al., 2008). The role of ChII2 in catalysis was questioned since its C-terminus is largely different to ChII1 (Apchelimov et al., 2007). A homozygous *chlI2* mutant produces significantly lower chlorophyll, while a *chlI1/chlI2* double mutant is albino and produces 3 % chlorophyll in the wild type plant (Kobayashi et al., 2008). A mutation in *chlI1* or a *chlI1* knockout has diminished amounts of chlorophyll (~17 %) (Rissler et al., 2002; Huang and Li, 2009). A double *chlI1chlI2* mutant plant recovers wild-type chlorophyll levels following transgenic expression of *chlI2* under the control of *chlI1* promoter (Huang and Li, 2009). The data shows that ChII2 still has the ability to form a functional magnesium chelatase complex and participate in catalysis (Kobayashi et al., 2008; Huang and Li, 2009). The major difference between *chlI1* and *chlI2* occurs *in vivo* with much lower basal expression of *chlI2* (Huang and Li, 2009), which supports earlier observations (Rissler et al., 2002).

1.5.8 BchD/ChlD subunit

BchD/ChlD is the second largest subunit of magnesium chelatase at ~60-70 kDa. There is limited information on the role of this subunit in the magnesium chelatase reaction mechanism. This is partly due to the difficulties in working with this subunit since it is insoluble when isolated (Petersen et al., 1998; Willows and Beale, 1998). The function of BchD/ChlD could only be elucidated through the interaction with the other two subunits. Biochemically BchD/ChlD alone does not appear to be partaking in any clear function. Unlike BchI/ChlI it does not have ATPase activity, and unlike BchH it does not bind proto substrate.

It was established that BchD/ChlD has a structural role in magnesium chelatase, serving as a platform for BchI/ChlI and BchH/ChlH subunits to converge (*Paper I*). The N-terminus of BchD/ChlD is comparable to BchI/ChlI, and is classified as an AAA protein (Fodje et al., 2001). BchD seems to spontaneously form a hexamer in the absence of ATP by EM (*Paper I*). The walker A motif for ATP/GTP binding and hydrolysis is not conserved in BchD/ChlD and is only partially intact depending on the species (Sirijovski et al., 2006). This suggests there is no ATPase activity in this subunit which is supported by previous biochemical studies (Hansson and Kannangara, 1997; Jensen et al., 1999a; Petersen et al., 1999a).

BchD/ChlD has an integrin I domain at the C-terminus (Fodje et al., 2001) which is involved in protein-protein interactions (Takagi, 2007). The BchI/ChlI and BchH/ChlH subunits have integrin I recognition sequences; RGE/D for BchI/ChlI, and LDV in BchH/ChlH (Fodje et al., 2001). Direct interactions are shown between tobacco ChlD and ChlI or ChlH using affinity assays (Gräfe et al., 1999). Within the integrin I domain exists a MIDAS motif (metal ion-dependent adhesion site) which suggests BchD/ChlD binds magnesium ions (Fodje et al., 2001).

ChlD was dismantled into the N-terminus, C-terminus, glutamine/asparagine/proline-rich central region, and flanking sections and rebuilt to form several truncated constructs to elucidate the important regions (Gräfe et al., 1999). The glutamine/asparagine/proline-rich centre with the two flanking regions affords some magnesium chelatase activity (30 %) (Gräfe et al., 1999). Removal of

the N-terminus from wild type ChlD retains wild-type activity, while removal of the C-terminus reduces magnesium chelatase activity by approximately 50-60% (Gräfe et al., 1999). This highlights the importance of the C-terminus over the N-terminus for activity. However when the N-terminus of ChlD is substituted with the N-terminus of ChII, only ~20 % activity remains (Gräfe et al., 1999). Therefore the ChlD N-terminus is quite distinct from the ChII N-terminus despite their homology.

1.5.9 BchI•BchD/ChII•ChlD complex

BchI/ChII and BchD/ChlD interact to form a stable protein complex which is the catalytic centre of magnesium chelatase. This is initially shown through an elimination of the “lag-phase” of the magnesium chelatase reaction after the preincubation of BchI/ChII and BchD/ChlD subunits with ATP and magnesium (Walker et al., 1992; Willows et al., 1996; Jensen et al., 1998; Willows and Beale, 1998)(see 1.5.4 above). A physical interaction between BchD/ChlD and BchI/ChI from *Rba. sphaeroides* and *Synechocystis* in the presence of magnesium and ATP has been demonstrated (Gibson et al., 1999; Jensen et al., 1999a). The ChII:ChlD molar ratio is approximately 1:1 after chromatography and SDS-PAGE (Jensen et al., 1999a). The stability of a *Synechocystis* ChII•ChlD complex is dependent upon some ATP hydrolysis since at least a slowly hydrolysable ATP analog; adenosine 5'-[γ -thio]triphosphate is required for preservation of a 1:1 complex (Jensen et al., 1999a). This is supported by previous observations with *Rba. sphaeroides* BchI•BchD showing a larger increase in ATP to ADP exchange activity compared with BchI alone (Hansson and Kannangara, 1997).

A recent study showed the “lag-phase” of *Synechocystis* magnesium chelatase is eliminated by simply using a high concentration of ChII and ChlD subunits in the absence of ATP (Viney et al., 2007). This is similar to the ATP-independence of the oligomeric formation of ChII at high protein concentration (Jensen et al., 1998) and implies ChII•ChlD stability is dependent upon its concentration, particularly the ChII subunit.

The apparent 1:1 association of BchI/ChII and BchD/ChlD together with the ability of each subunit to form hexamers led to the proposal that the BchI•BchD/ChII•ChlD complex could take the

form of a stacked double-hexamer (Fodje et al., 2001) (*Paper I*). However a BchI•BchD complex is only ~200 kDa by gel filtration from *Rba. sphaeroides* (Gibson et al., 1999). Recent EM structural information of the BchI•BchD complex with ADP has helped in interpreting these results (Elmlund et al., 2008). Each subunit is composed of a trimer of dimers or dimer of trimers which forms a stacked double hexameric complex (Elmlund et al., 2008). The observed 200 kDa BchI•BchD complex by gel filtration (Gibson et al., 1999) could be a stacked BchI•BchD double dimer which would have an theoretical mass of ~200 kDa. It is possible that BchI in the presence of ATP may actually form stable dimers or trimers in solution, with a less stable hexameric form.

The homologous aerobic cobaltochelatase CobS•CobT complex forms a comparable EM structure to the BchI•BchD complex (Lundqvist et al., 2009). There is a two-tiered double hexameric organization of CobS•CobT, with the best fit indicating a trimer of dimers of each subunit (Lundqvist et al., 2009). CobS•CobT is a 450-520 kDa complex by gel filtration which approximately fits with the EM structure (Debussche et al., 1992; Lundqvist et al., 2009). Stability of the EM double hexamer of BchI•BchD may require other components, for example BchM, the next enzyme in the pathway of bacteriochlorophyll biosynthesis.

ChlD is reliant upon chaperone-type proteins for stability *in vivo*. The amount of ChlD diminishes in ATPase-defective *chlI* mutants from barley or *chlI* knockouts in *A. thaliana* (Hansson et al., 1999; Petersen et al., 1999b; Lake et al., 2004; Huang and Li, 2009). ChlD is unaffected in *chlH* mutants from barley or *A. thaliana* (Hansson et al., 1999; Olsson et al., 2004; Huang and Li, 2009) and *chlD* is unchanged in *chlH* mutants from *C. reinhardtii* and barley (Chekounova et al., 2001; Lake et al., 2004). Gene silencing of *chlH* in *Nicotiana benthamiana* only causes a decrease in *chlD* (but does not abolish it) with severe genotypic and phenotypic effects (Hiriart et al., 2002). The decrease in *chlD* could be an after-effect, and suggests that ChlH does not have strict control over ChlD. Instead these results indicate the viability of ChlD *in vivo* is dependent upon ChlI (Lake et al., 2004) (also addressed in *Paper I*). Since BchI/ChlI and BchI/ChlD form a complex *in vitro*, it is suggested BchI/ChlI functions as a chaperone for BchI/ChlD in an ATP-dependent manner (Lake et al., 2004). The role of

BchI/ChII as a chaperone may be compared to HSP70 (DnaK) since DnaK co-expressed with ChID maintains ChID in a soluble, active form in the magnesium chelatase assay (Luo et al., 1999).

1.5.10 BchH/ChIH subunit

The BchH/ChIH subunit (~130-150 kDa) is at the core of the magnesium chelatase enzyme complex because it binds the tetrapyrrole substrate and product (proto and Mg-proto) (Gibson et al., 1995; Willows et al., 1996; Willows and Beale, 1998; Karger et al., 2001; Sirijovski et al., 2008). The strength of proto binding to BchH/ChIH is observed qualitatively by colour through the retention of this red/brown pigment during protein purification. Following heterologous expression of BchH/ChIH in *E. coli*, the protein sequesters proto naturally from the cell (Willows et al., 1996; Jensen et al., 1998; Willows and Beale, 1998). The ability of BchH/ChIH to retain proto through various chromatographic steps differs amongst photosynthetic organisms. For example in *Synechocystis*, proto is depleted from ChIH after purification by ion exchange chromatography (Jensen et al., 1998). In *Rba. sphaeroides*, proto removal from BchH requires a more severe treatment involving detergent and RP-HPLC separation (Hansson and Kannangara, 1997). Incubation of BchH-proto from *Rba. capsulatus* with detergent (Tween 80) and purification with nickel affinity chromatography was not effective in producing undenatured apo-BchH (BchH without proto bound) (Sawicki and Willows, unpublished observation).

The stronger binding of proto or deuteroporphyrin to BchH from *Rba. sphaeroides* compared with ChIH from *Synechocystis* is reflected in their respective K_m values (Willows et al., 1996; Jensen et al., 1998; Gibson et al., 1999; Willows and Hansson, 2003). *Rba. sphaeroides* BchH has a K_m of 0.15 or 0.36 μM for proto from two separate studies (Willows and Beale, 1998; Gibson et al., 1999), whereas K_m^{proto} for *Synechocystis* ChIH is 1.25 μM (Jensen et al., 1998). Unexpectedly *Rba. capsulatus* BchH has a similar K_m^{proto} to *Synechocystis* ChIH (1.23 μM) (Willows and Beale, 1998). This is addressed in *Paper II* and 6.22 with a more comprehensive dissection of the kinetic properties of magnesium chelatase from this organism. Direct comparison between *Rba. sphaeroides* BchH and *Synechocystis* ChIH using tryptophan fluorescence quenching data highlights the stronger binding of

deuteroporphyrin to BchH with a dissociation constant (K_d) of 0.53 μM for BchH against 1.22 μM for ChlH (Karger et al., 2001). Pea magnesium chelatase activity was tested using more water soluble proto analogs, deuteroporphyrin and mesoporphyrin (Walker and Weinstein, 1991b) (Fig. 5). Both deuteroporphyrin and mesoporphyrin have similar specific activities to proto although the apparent K_m for deuteroporphyrin and mesoporphyrin is 4 and 5 μM respectively compared with 1.5 μM for proto (Walker and Weinstein, 1991b).

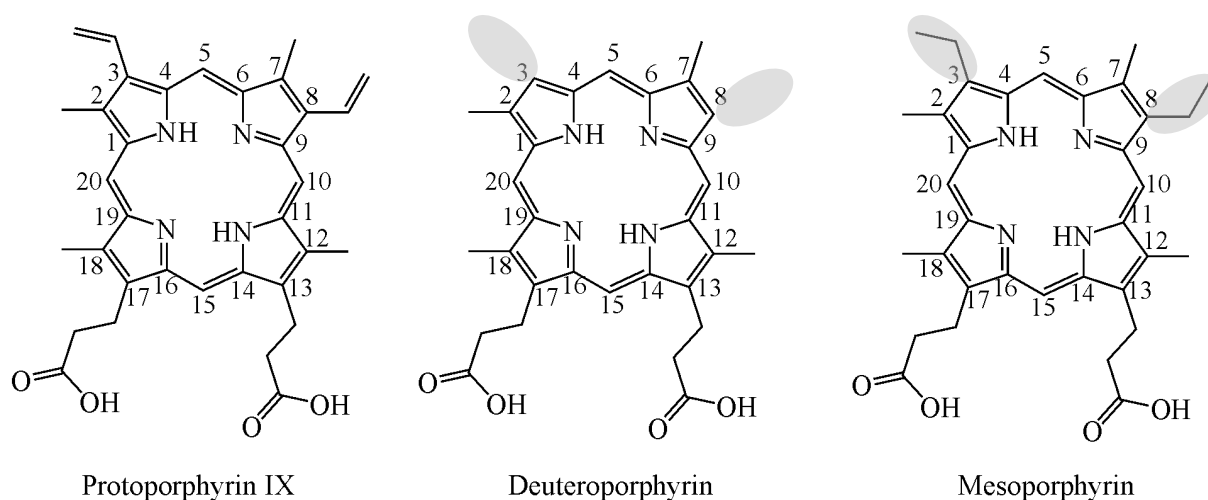


Figure 5. More water-soluble synthetic derivatives of protoporphyrin IX. Changes to protoporphyrin IX structure are shaded at positions C3, and C8; deuteroporphyrin has both vinyl groups removed, while mesoporphyrin has both vinyl groups replaced with ethyl groups.

A unique feature of the green sulphur bacterium *C. tepidum* is the presence of three BchH subunit isoforms, termed BchH, BchS, and BchT (Frigaard et al., 2003; Frigaard and Bryant, 2004). Each isoform exhibits vastly different maximal velocities in magnesium chelatase assays. When tested separately the rate of BchS is two and five orders of magnitude higher than BchH and BchT respectively (Johnson and Schmidt-Dannert, 2008).

Thus far, the crystal structure of BchH/ChlH has remained elusive. An EM structural representation of the native and proto-depleted *Rba. capsulatus* BchH has been shown (Sirijovski et al., 2008). This study found that BchH undergoes a large conformational change upon binding proto (Sirijovski et al., 2008). Although the particular residues for proto binding in BchH/ChlH have not been determined, it is suggested histidine is a likely candidate (Walker and Willows, 1997). This stems

from the catalytic role of nitrogenous bases needed for the chemical insertion of magnesium into proto (Baum and Plane, 1966). The three histidine residues of BchH/ChlH are shown to be conserved amongst plant (*A. thaliana*, barley, *Antirrhinum majus*) and bacterial species (*Rba. capsulatus*, *Synechocystis*) (Walker and Willows, 1997) and mutant studies may help identify the residues involved.

There is indirect evidence that BchH/ChlH binds magnesium. Preincubation of ChlH with deuteroporphyrin requires the presence of magnesium with or without ATP to reduce the “lag-phase” in the magnesium chelatase assay (Jensen et al., 1998). *Synechocystis* ChlH retains bound deuteroporphyrin more effectively in the presence of magnesium chloride after gel filtration (Karger et al., 2001). The K_d of ChlH binding to deuteroporphyrin increases with the addition of magnesium suggesting allosteric binding of magnesium (Karger et al., 2001). Preincubation of ChlH with proto and MgATP protects ChlH from inhibition by the cysteine-modifying molecule, NEM (Jensen et al., 2000). Preincubation of ChlH with proto alone did not protect against NEM inactivation (Jensen et al., 2000). The homologous BchH/ChlH subunit, CobN from cobaltochelatase binds cobalt using a radioactive isotope, whereas the CobS•CobT complex (homologous to BchI•BchD of magnesium chelatase) does not (Debussche et al., 1992). Radioactive magnesium is unavailable for direct measurement, however these data suggest that BchH/ChlH may bind magnesium.

1.5.11 ChlH and the role of Gun4

Gun4 associates with ChlH and plays a major role in the magnesium chelation step of chlorophyll biosynthesis. The crystal structure of Gun4 from *Thermosynechococcus elongatus* and *Synechocystis* has been solved (Davison et al., 2005; Verdecia et al., 2005). Gun4 and ChlH share the ability to bind porphyrin (proto and Mg-proto) metabolites, however Gun4 has the greater affinity for both ligands (Karger et al., 2001; Larkin et al., 2003; Davison et al., 2005; Verdecia et al., 2005). *In vitro* magnesium chelatase assays show a 1:1 Gun4:ChlH molar ratio is sufficient for near-optimal activity (Davison et al., 2005). The K_m for deuteroporphyrin using Gun4 and magnesium chelatase is approximately 5-fold lower than magnesium chelatase alone (Verdecia et al., 2005). This suggests that

Gun4 preferentially binds deuteroporphyrin and delivers it to ChlH (Verdecia et al., 2005). Gun4 may also play a role in proto delivery to ferrochelatase since *Synechocystis gun4* mutant cells have 20-30 % activity of magnesium chelatase and ferrochelatase compared with wild type (Wilde et al., 2004). Gun4 also binds Mg-proto ester, uroporphyrin III, and coproporphyrin III (Adhikari et al., 2009). Therefore it is possible that Gun4 plays a general porphyrin-carrying role for delivery to other enzymes in chlorophyll and heme biosynthesis (Adhikari et al., 2009).

1.5.12 ChlH is a multi-functional protein

For many years scientists questioned why the BchH/ChlH subunit of magnesium chelatase is so large (130-150 kDa) in comparison to ferrochelatase (~42 kDa). This was puzzling since both proteins have similar tasks in binding proto and taking part in chelation by magnesium and ferrous atoms respectively. Recent discoveries have put BchH/ChlH in line with a relatively new class of proteins with multi-functional roles (Hall et al., 2004; Bhardwaj and Wilkinson, 2005). *A. thaliana* ChlH is involved in plastid to nucleus signalling which is a result of increased transcription of nuclear-encoded *lhcb* with *chlH* mutants (Mochizuki et al., 2001). *Synechocystis* ChlH is an anti-sigma factor (anti-SigE) (Osanai et al., 2009). Sigma factors are part of the RNA polymerase complex and therefore initiate transcription by binding to promoter regions. SigE is implicated in metabolic processes such as glycolysis. ChlH interacts with SigE *in vitro* and represses transcription of RNA polymerase (Osanai et al., 2009). It is reported that ChlH from *A. thaliana* can bind the phytohormone abscisic acid (ABA) (Shen et al., 2006; Wu et al., 2009). However the ability to function as a general plant ABA receptor is inconclusive since there is no evidence for this in barley (Muller and Hansson, 2009).

1.6 S-adenosyl-L-methionine:magnesium protoporphyrin IX O-methyltransferase (BchM/ChlM)

1.6.1 Overview

S-adenosyl-L-methionine:magnesium protoporphyrin IX O-methyltransferase (E.C. 2.1.1.11) (~ 25 kDa) is encoded by a single gene in all photosynthetic organisms (*bchM* or *chlM*). It catalyses

the second step in bacteriochlorophyll biosynthesis (Fig. 6) with the conversion of Mg-proto to Mg-proto ester, and requires SAM (Box 2) as a second substrate (Tait and Gibson, 1961; Gibson et al., 1963). The 13-propionate chain is specifically methylated in preference to the 17-propionate chain *in vivo* (Shiau et al., 1991). Mg-proto is derived from the previous step in bacteriochlorophyll biosynthesis synthesized by magnesium chelatase. SAM is generated through the C₁ pathway (Hanson and Roje, 2001).

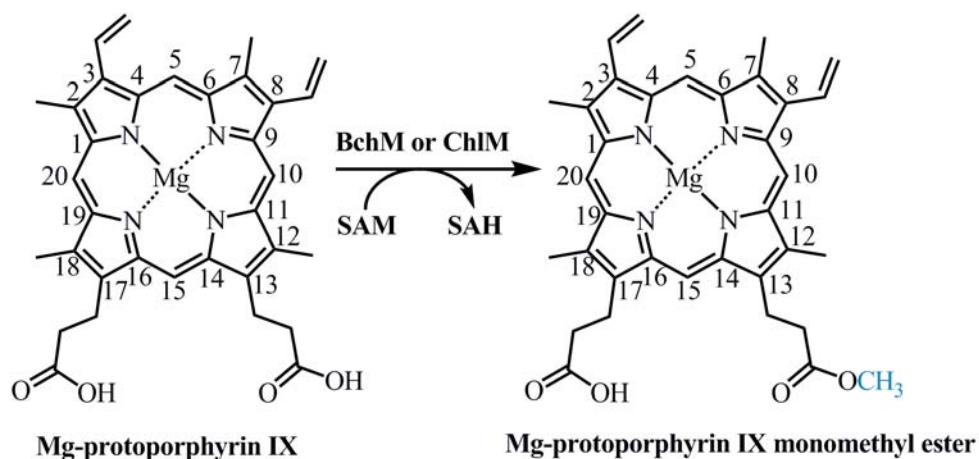


Figure 6. The enzymatic reaction catalysed by *S*-adenosyl-L-methionine:magnesium protoporphyrin IX *O*-methyltransferase (BchM/ChlM). Mg-protoporphyrin IX is methylated by *S*-adenosyl-L-methionine (SAM) to produce Mg-protoporphyrin IX monomethyl ester and *S*-adenosyl homocysteine (SAH).

Box 2. *S*-adenosyl-L-methionine (SAM)

This small molecule metabolite is derived from the substrates L-methionine and ATP by SAM synthetase via the C₁ pathway (Hanson and Roje, 2001). It is the major methylating molecule found in nature (Cantoni, 1975; Chiang et al., 1996). SAM methylates many biomolecules such as DNA, protein, phospholipid, hormone, neurotransmitter, carbohydrate, and porphyrin (Fontecave et al., 2004). SAM is similar to ATP because it is unstable in aqueous solution under physiological conditions (Borchardt, 1979; Hoffman, 1986). Several types of stable functional SAM salts with variable large anions (Morana et al., 2000) such as the *p*-toluene sulphonate salt have been produced (Fiecchi, 1976).

1.6.2 Early *O*-methyltransferase activity

O-methyltransferase was one of the earliest enzymes to be studied biochemically in the (bacterio)chlorophyll biosynthetic pathway. Measurement of *O*-methyltransferase activity involved the

physical separation of porphyrin substrate from product by chromatography. This is because the porphyrin substrate and product have identical absorption/fluorescence spectra.

The first assay measuring *O*-methyltransferase activity was performed in 1961 using chromatophores (membranes of lysed cells) of *Rba. sphaeroides*, Mg-proto and ¹⁴C-labeled SAM (Tait and Gibson, 1961). After acid hydrolysis, TLC of the porphyrins showed a single product that migrated between proto and proto dimethyl ester. This suggested the product made was Mg-proto monomethyl ester (Tait and Gibson, 1961) and this correlated with Mg-proto monomethyl ester reported as an intermediate in *Chlorella* mutants and an intermediate in barley (Granick, 1961). The product of *O*-methyltransferase from *Rba. sphaeroides* was confirmed as Mg-proto monomethyl ester by TLC, IR spectroscopy, and GC (Gibson et al., 1963; Jones, 1963). In all reported cases of *O*-methyltransferase activity, the optimal pH was basic (7.5-7.7) for wheat, barley, *E. gracilis* (Ellsworth and Dullaghan, 1972; Shieh et al., 1978; Hinchigeri et al., 1981). *Rba. sphaeroides* had an optimal pH of 8.5 (Gibson et al., 1963), while cholate-solubilised enzyme from the same organism had an optimal pH of 9.5 (Hinchigeri et al., 1984).

1.6.3 Stopped and continuous assays

O-methyltransferase activity is typically performed for 1-4 h before being stopped by organic solvent for analysis (Gibson et al., 1963; Ellsworth et al., 1974; Ellsworth and St Pierre, 1976; Ellsworth and Murphy, 1978; Hinchigeri et al., 1981; Hinchigeri and Richards, 1982; Hinchigeri et al., 1984; Hinchigeri et al., 1997), or 0.5-30 minutes in more recent assays (Jensen et al., 1999b; Shepherd et al., 2003) (*Paper III*). An automated method for separating porphyrins uses RP-HPLC and absorbance or fluorescence detection (Wong et al., 1985). This technique is utilised for *O*-methyltransferase from *Rba. capsulatus*, *Synechocystis*, and *C. tepidum* (Gibson and Hunter, 1994; Bollivar et al., 1994a; Jensen et al., 1999b; Shepherd et al., 2003; Johnson and Schmidt-Dannert, 2008) (*Paper III*). Mg-proto and Mg-proto ester are separated within ~12-18 min of sample injection. An LC-MS method for *O*-methyltransferase activity monitors SAH formation (Salyan et al., 2006). This is effectively employed for kinetic studies with catechol *O*-methyltransferase (Salyan et al.,

2006). A continuous method for SAM-dependent *O*-methyltransferase is now commercially available from G-Biosciences, St Louis, MO, USA (Dorgan et al., 2006) and involves a coupled assay which enzymatically converts SAH into hypoxanthine which is measurable by UV-light. This has been successfully applied to *Synechocystis* ChlM with similar $K_m^{\text{Mg-proto}}$ values to previous stopped assays using RP-HPLC (McLean and Hunter, 2009).

1.6.4 Purification and stability

Early purification of *O*-methyltransferase did not include SDS-PAGE analysis so the degree of improvement in purity from each source is quoted from their respective specific activities. The first attempt at *O*-methyltransferase purification was from *E. gracilis* and utilised ammonium sulphate and protoamine sulphate precipitation, followed by gel filtration with Sephadex G-200 which resulted in specific activity being increased by 68 times (Ebbon and Tait, 1969). Further purification was not feasible due to the instability of the enzyme. Ammonium sulphate and protoamine sulphate purification of *O*-methyltransferase from barley seedlings has a ~20 fold improvement in purity (Shieh et al., 1978).

A large improvement in the purification method is achieved with affinity chromatography using either SAH or hemin coupled columns. This results in a ~600-2000 fold purification of *O*-methyltransferase from wheat, *Rba. sphaeroides*, and *E. gracilis* (Hinchigeri et al., 1981; Hinchigeri and Richards, 1982; Hinchigeri et al., 1984). Purification of wheat and *E. gracilis* *O*-methyltransferase in this way gives four major protein bands by SDS-PAGE (Hinchigeri et al., 1981).

E. gracilis and *Rba. sphaeroides* *O*-methyltransferase are unstable when attempts are made at purification (Hinchigeri et al., 1981; Hinchigeri et al., 1984). Several detergents are detrimental for solubilising *O*-methyltransferase from *Rba. sphaeroides* with almost all activity lost (Hinchigeri et al., 1984). Only cholate allows solubilisation of *O*-methyltransferase with reasonable remaining enzymatic activity (Hinchigeri et al., 1984). All activity is lost during purification of cholate solubilised *Rba. sphaeroides* enzyme at room temperature while at 4 °C, 84 % activity is lost after 24 h (Hinchigeri et al., 1984). Similarly *E. gracilis* *O*-methyltransferase loses the majority (67 %) of activity during

purification at 4 °C (Hinchigeri et al., 1981). Ascorbate stabilizes *E. gracilis* *O*-methyltransferase during purification (Hinchigeri et al., 1981).

Currently heterologous expression systems in *E. coli* use His-tagged *O*-methyltransferase and allow single-step purification. This has been successful for *O*-methyltransferase from *Synechocystis* (Shepherd et al., 2003), and most recently, *C. tepidum* (Johnson and Schmidt-Dannert, 2008). This procedure was used for *Rba. capsulatus* *O*-methyltransferase, however an initial partial purification step with detergent was included (*Paper III*).

1.6.5 Identification of BchM/ChlM as *O*-methyltransferase

BchH was originally identified as putatively encoding *O*-methyltransferase (Bollivar and Bauer, 1992). This is mainly because *bchH* mutants completely abolish *O*-methyltransferase activity (Richards et al., 1991), whereas *bchI* and *bchD* mutants have wild-type *O*-methyltransferase activity (Gorchein et al., 1993). The presumption that BchH encodes *O*-methyltransferase seemed unlikely after distinct homology was found between BchH and CobN, a gene already shown to be part of the cobaltochelate complex (Debussche et al., 1992; Hudson et al., 1993)(see 1.5.6 above). BchH is now known as one third of the magnesium chelatase complex (Gibson et al., 1995).

bchM was previously suggested as being a two-part component together with *bchE* in the cyclase reaction due to the accumulation of Mg-proto ester in both of these mutants (Yang and Bauer, 1990). The accumulated porphyrin is now suggested as a degradation product of Mg-proto (Bollivar, 2003). Biochemical assays of heterologously expressed *bchM* from *Rba. capsulatus* or *Rba. sphaeroides* in *E. coli* confirms that BchM has *O*-methyltransferase activity (Gibson and Hunter, 1994; Bollivar et al., 1994a). The homologous *chlM* gene from *Synechocystis* is identified through hybridization of *Rba. capsulatus* *bchM* mutant with the *Synechocystis* DNA cosmid library (Smith et al., 1996). A sequence similarity of 29 % exists between the two genes. The expression of *Synechocystis* *chlM* plasmid in a *bchM* mutant strain of *Rba. capsulatus* under the control of the *Rba. capsulatus* anaerobic *puc* promoter gives wild-type bacteriochlorophyll (Smith et al., 1996). This indicates that both enzymes function in a similar manner.

1.6.6 Reaction mechanism

Product inhibition of *O*-methyltransferase by SAH (Gibson et al., 1963) and Mg-proto ester (Ellsworth et al., 1974) has allowed the determination of the reaction mechanism (Ellsworth et al., 1974; Hinchigeri and Richards, 1982; Hinchigeri et al., 1984; Shepherd et al., 2003) (*Paper III*). The reaction mechanism of *O*-methyltransferase has been deduced from photosynthetic bacteria (*Rba. sphaeroides*) (Hinchigeri et al., 1984), plant (wheat and barley) (Ellsworth et al., 1974; Shieh et al., 1978; Yee et al., 1989), and alga (*E. gracilis*) (Ebbon and Tait, 1969; Hinchigeri et al., 1981; Hinchigeri and Richards, 1982) (Table 3). All kinetic data prior to 1990 used crude or partially purified enzyme. Highly purified enzyme was utilised in recent kinetic analysis from *Synechocystis* (Shepherd et al., 2003), *C. tepidum* (Johnson and Schmidt-Dannert, 2008), and *Rba. capsulatus* (*Paper III*). The reaction mechanism varies depending upon the organism with wheat *O*-methyltransferase showing a ping-pong reaction (Ellsworth et al., 1974; Hinchigeri et al., 1981; Yee et al., 1989), whereas *E. gracilis*, *Rba. sphaeroides*, and *Synechocystis* each have sequential mechanisms (Hinchigeri and Richards, 1982; Hinchigeri et al., 1984; Shepherd et al., 2003).

Kinetic characterization of wheat *O*-methyltransferase produces double-reciprocal Lineweaver-Burke plots for both SAM and Mg-proto substrates (Ellsworth and St Pierre, 1976). This generates a series of parallel lines when plotting the inverse of velocity versus inverse of substrate. This is characteristic of a ping-pong mechanism, where the binding and release of one substrate and product occurs before the binding of the other substrate (Cleland, 1967). The most likely mechanism for *O*-methyltransferase is the binding of SAM and methylation of the enzyme prior to binding of Mg-proto. The binding of wheat *O*-methyltransferase to hemin-coupled sepharose requires the preincubation of the enzyme with SAM (Hinchigeri et al., 1981). Exchange reactions with reacted wheat *O*-methyltransferase converts labelled SAH into labelled SAM in a reverse reaction (Yee et al., 1989). This confirms that the enzyme is methylated during the reaction (Yee et al., 1989).

Kinetic analysis of *O*-methyltransferase from *E. gracilis* suggests a random sequential reaction mechanism (Hinchigeri and Richards, 1982). The K_m values for Mg-proto and SAM are 10-34 and 20-

230 μM (Table 3) (Ebbon and Tait, 1969). The variations in the K_m of either substrate are dependent upon the preparation of cell extract. Tween 80 solubilised chromatophores purified by gel filtration have the lowest K_m values (10 μM for SAM and 20 μM for Mg-proto) (Ebbon and Tait, 1969). The random substrate binding reaction mechanism is supported through affinity chromatography with porphyrin and SAH (Hinchigeri et al., 1981). *O*-methyltransferase binds to hemin-sepharose or Mg-proto-sepharose in the presence or absence of SAM (Hinchigeri et al., 1981). This enzyme also binds to SAH-coupled sepharose without the addition of Mg-proto (Hinchigeri et al., 1981). *O*-methyltransferase binding to hemin or SAH represents binding to Mg-proto and SAM respectively because hemin and SAH are competitive inhibitors against Mg-proto and SAM respectively (Hinchigeri and Richards, 1982). A portion of *O*-methyltransferase not preincubated with Mg-proto elutes in the void volume after application to SAH-coupled sepharose (Hinchigeri and Richards, 1982). This is explained by a weaker affinity of SAM compared with Mg-proto to *O*-methyltransferase through kinetic studies (higher K_m of SAM) (Hinchigeri and Richards, 1982) (Table 3). Affinity chromatography and kinetic data indicate that *E. gracilis* *O*-methyltransferase can either bind to SAM or Mg-proto first.

Kinetic analysis of *Rba. sphaeroides* *O*-methyltransferase shows an ordered sequential reaction mechanism with Mg-proto binding to the enzyme first (Hinchigeri et al., 1984). Highly purified *O*-methyltransferase from *Synechocystis* and *Rba. capsulatus* each has random sequential mechanism (Shepherd et al., 2003) (*Paper III*). Interestingly the latest results with *C. tepidum* *O*-methyltransferase shows the K_m for Mg-proto is at least an order of magnitude lower than previous findings (Johnson and Schmidt-Dannert, 2008) and this is also the case in *Rba. capsulatus* (*Paper III*) (see Table 3).

Table 3. Kinetic properties of substrates and reaction mechanism of *O*-methyltransferase

Source of <i>O</i> -methyltransferase (Organism)	K_m^{SAM} (μ M)	$K_m^{Mg-proto}$ (μ M)	Mechanism	Reference
<i>Rba. sphaeroides</i>	55	40	N.D.	(Gibson et al., 1963)
	106	N.D. (near zero)	Sequential, (Mg-proto binds first)	(Hinchigeri et al., 1984)
Wheat	44	22	Ping-pong (SAM binds first)	(Ellsworth et al., 1974; Yee et al., 1989)
<i>E. gracilis</i>	20-230	10-34	Random sequential	(Ebbon and Tait, 1969; Hinchigeri and Richards, 1982)
Barley	39	48	N.D.	(Shieh et al., 1978)
<i>Synechocystis</i> [^]	38 \pm 17	<100*	Random sequential	(Shepherd et al., 2003)
<i>C. tepidum</i> [^]	N.D.	0.6 \pm 0.1 [†]	N.D.	(Johnson and Schmidt-Dannert, 2008)
<i>Rba. capsulatus</i> [^]	45 \pm 7	0.11 \pm 0.02	Random sequential	<i>Paper III</i>

* Synthetic Mg-deuteroporphyrin is used as the porphyrin substrate in this case. [†] A K_m of 53 \pm 27 μ M for proto is also found in these experiments. [^] These enzymes are purified to homogeneity. N.D. = not determined.

There were concerns that earlier experiments involving minute or hour scale assays did not accurately show the initial rate of *O*-methyltransferase. Millisecond time-scale experiments using *O*-methyltransferase from *Synechocystis* were performed using stopped-flow assays (Shepherd and Hunter, 2004). There is a slow isomerisation of the enzyme, which is followed by fast substrate binding and catalysis. There is a “lag-phase” that exists for ~150 ms, and during this stage, there is the rapid formation of an unknown intermediate molecule. After the “lag-phase”, the intermediate degrades while Mg-deuteroporphyrin monomethyl ester is produced. The intermediate is more hydrophobic than the product and shares the same spectrofluorometric properties as the product. Although unidentified, it is possible this intermediate could be Mg-deuteroporphyrin dimethyl ester (Shepherd and Hunter, 2004).

1.7 Evidence for magnesium chelatase and *O*-methyltransferase associations

1.7.1 Early studies

Early *O*-methyltransferase assays gave hints at the closeness of the enzyme with magnesium chelation. Proto is converted to Mg-proto ester using chromatophores or whole cells of *Rba. sphaeroides* (Tait and Gibson, 1961; Gibson et al., 1963; Gorchein, 1972), or the chloroplast pellet from *Zea mays* (Radmer and Bogorad, 1967). Specific activity of *O*-methyltransferase with proto substrate is ~ten times slower than Mg-proto as a substrate (Gibson et al., 1963; Radmer and Bogorad, 1967). The low conversion rate of proto is presumably attributable to sub-optimal conditions for magnesium chelatase, it being discovered later that the enzyme has ATP and magnesium requirements (Pardo et al., 1980).

A landmark study in 1972 using whole cells of *Rba. sphaeroides* showed conclusively that magnesium chelatase and *O*-methyltransferase are coupled (Gorchein, 1972). Addition of the *O*-methyltransferase specific inhibitor DL-ethionine causes the accumulation of proto (Gorchein, 1972). Together with previous data it is concluded that magnesium chelatase and *O*-methyltransferase rely upon one another for *in vivo* activity (Gorchein, 1972). This idea was supported much later with each of three mutants of the BchH subunit of magnesium chelatase from *Rba. sphaeroides* halting *O*-methyltransferase activity (Gorchein et al., 1993). These authors also showed that BchI and BchD mutants poison magnesium chelatase activity yet *O*-methyltransferase activity is unaffected, thus alluding to the potential prospects of a specific *O*-methyltransferase-BchH/ChlH interaction (Gorchein et al., 1993).

1.7.2 BchH-BchM/ChlH-ChlM interactions

Studies now focus on the BchH/ChlH-BchM/ChlM interaction and the effect upon *O*-methyltransferase activity. This was first tested using crude preparations of each protein from *Rba. capsulatus* heterologously expressed in *E. coli* (Hinchigeri et al., 1997). The addition of crude, soluble BchH to a crude membrane fraction of BchM increases *O*-methyltransferase activity up to 6.8 times

(Hinchigeri et al., 1997). This result is inconclusive since the effect of lipids cannot be discounted (Hinchigeri et al., 1997). Exogenous proto added to extracts of co-expressed magnesium chelatase and *O*-methyltransferase results in Mg-proto ester formation (Jensen et al., 1999b). This led to the idea that BchH delivers Mg-proto to BchM. Preliminary *in vitro* experiments with purified magnesium chelatase and *O*-methyltransferase suggests a 1:1 molar ratio of ChlH to ChlM is optimal, however a detailed investigation was not undertaken (Jensen et al., 1999b). A more thorough kinetic investigation of the effect of *O*-methyltransferase from *Rba. capsulatus* upon magnesium chelatase, with reference to BchH, has now been undertaken (*Paper IV*).

ChlH from tobacco or *Synechocystis* increases *O*-methyltransferase activity (Alawady et al., 2005; Shepherd et al., 2005). Other magnesium chelatase subunits from tobacco have little effect on *O*-methyltransferase activity with ChlI producing a slight increase, while ChlD has no effect (Alawady et al., 2005). The K_m for Mg-deuteroporphyrin with *Synechocystis* ChlM is not significantly altered upon addition of ChlH (Shepherd et al., 2005). Therefore ChlH-Mg-deuteroporphyrin is not a better substrate for ChlM compared with raw Mg-deuteroporphyrin. The effect of *Synechocystis* ChlH upon ChlM activity is seen at a millisecond time-scale (Shepherd et al., 2005). Firstly, the “lag-phase” of the *O*-methyltransferase reaction is reduced from 100-150 ms to 50 ms with the addition of ChlH (Shepherd et al., 2005). In assays with *O*-methyltransferase alone this “lag-phase” is proposed to involve reorganization of ChlM into an active form thus suggesting ChlH may be directly involved in a conformational change of ChlM (Shepherd and Hunter, 2004).

Assays with ChlM-ChlH show the intermediate of the ChlM reaction is rapidly depleted from 0-160 ms, while ChlM alone has a hyperbolic increase in the intermediate during the same time period (Shepherd et al., 2005). If the reaction intermediate is Mg-deuteroporphyrin dimethyl ester, ChlH seems to improve the specificity of ChlM to produce the correct product Mg-deuteroporphyrin monomethyl ester. If double methylation occurs, Mg-proto dimethyl ester is demethylated by an esterase in cucumber chloroplasts to form Mg-proto, which is then converted to Mg-proto monomethyl ester, and eventually protochlorophyllide (Walker et al., 1988).

Tobacco ChlH and ChlM are transcriptionally regulated in parallel using transgenic *chlM* plants (Alawady and Grimm, 2005). *chlH* is transcriptionally up-regulated in *chlM* sense plants with a parallel increase in the enzymatic activity of magnesium chelatase and *O*-methyltransferase. Anti-sense *chlM* plants have a coupled decrease in transcription of *chlH* and *chlM*, and enzyme activities of magnesium chelatase and *O*-methyltransferase (Alawady and Grimm, 2005). The biochemical changes are due to ChlH-ChlM interactions since a physical interaction between tobacco *O*-methyltransferase and magnesium chelatase is specific to the ChlH subunit of magnesium chelatase using a yeast 2-hybrid system (Alawady et al., 2005).

1.8 BchJ

1.8.1 The role of BchJ in bacteriochlorophyll biosynthesis

Until recently it was thought that *bchJ* encodes the enzyme that catalyses the 8-vinyl reductase step of the bacteriochlorophyll biosynthetic pathway. This is because *Rba. sphaeroides* cells with mutant *bchJ* have an increased accumulation of divinyl protochlorophyllide (Suzuki and Bauer, 1995a). Some monovinyl protochlorophyllide is still present therefore the results are inconclusive (Suzuki and Bauer, 1995a). It has since been shown that BchJ does not exhibit 8-vinyl reductase activity (Chew and Bryant, 2007a). Thus currently the role of BchJ is undesignated however it has been proposed as a substrate-channelling protein similar to Gun4 in chlorophyll synthesizing organisms (Chew and Bryant, 2007a). There is a suggestion that BchJ may be able to bind porphyrins with bioinformatics predicting a small molecule binding domain (COG1719, 4VR domain) in *bchJ* (Anantharaman et al., 2001). The ability of BchJ from *Rba. capsulatus* to bind the porphyrins, proto and Mg-proto were tested and kinetic assays undertaken to assess interactions between magnesium chelatase and BchM or BchJ (*Paper IV*).