6. DISCUSSION

6.1 Overview

The work presented in this thesis (*Papers I-IV*) concentrates on mechanistic studies of the bacteriochlorophyll biosynthetic enzymes; magnesium chelatase and *O*-methyltransferase from *Rba. capsulatus*, and how they may interact. The interaction between magnesium chelatase and BchJ, a protein with no currently assigned biological function in the pathway, was also examined. The key findings from each paper have been compiled into a cohesive discussion chapter. For clarity and continuity, additional experimental data is presented here that is not included in *Papers I-IV*.

6.2 Magnesium chelatase

6.2.1 Is the BchD/ChlD subunit oligomeric?

Similarities between the N-terminus (AAA module) of BchI/ChlI and BchD/ChlD subunits of magnesium chelatase suggests that BchD is oligomeric (Fodje et al., 2001). AAA proteins are typically hexameric or heptameric (Iyer et al., 2004). Since BchI is hexameric (Fodje et al., 2001; Willows et al., 2004), it is hypothesized that BchD could also be hexameric. To test this, a previous strategy that was successful with *chlI* (*xantha-h*) mutants from barley was adopted (Hansson et al., 1999; Hansson et al., 2002). Briefly, *chlI* mutants from barley show a semi-dominant effect on magnesium chelatase activity with heterozygous plants having 25-50 % of wild-type activity (Hansson et al., 1999). *In vitro* assays using *Rba. capsulatus* magnesium chelatase and a 1:1 molar ratio of wild type to mutant BchI results in > 50 % inhibition of magnesium chelatase activity (Hansson et al., 2002). Dominant inhibition is expected with oligomeric proteins where each subunit can contribute to the formation of the multimer, such as BchI (Hansson et al., 2002).

The *in vivo* stability of ChlD depends upon its interaction with ChlI. This is shown with barley *chlI* mutants having significantly decreased amounts of ChlD (Hansson et al., 1999; Lake et al., 2004). It is suggested that ATP hydrolysis is required for ChlI▪ChlD stability and protection from proteolysis since ATPase-deficient *chlI* mutants could not maintain wild type amounts of ChlD *in vivo* (Hansson

et al., 1999; Lake et al., 2004). Mutations in barley *chlD* (*xantha-g*) that have a diminished amount of ChlD *in vivo* did not show a decrease in ChlI and mutations in *chlH* (*xantha-f*) did not reduce the levels of ChlD (Hansson et al., 1999). This indicates that ChlI performs a chaperone-like function for the stabilization of ChlD (Hansson et al., 1999).

Point mutations in barley *chlD* (*xantha-g44*, *xantha-g45*, and *xantha*-g65) *in vivo* were recessive, although *xantha-g45* could still produce significant amounts of chlorophyll (*Paper I*). *Xantha-g44* and *xantha-g65* had a decreased amount of ChlD *in vivo*. The corresponding *xantha-g44*, *xantha-g45*, and *xantha-g65* mutants were generated in *Rba. capsulatus* BchD (T227L, L418F, and G63E respectively) for *in vitro* studies. Mutants were mixed 1:1 with wild type BchD prior to refolding with BchI in magnesium chelatase assays. T227L and G63E mutants had little inhibitory effect on magnesium chelatase activity. These results indicated that *xantha-g44* and *xantha-g65* could not interact with ChlI *in vivo* or *in vitro*, and were prone to degradation *in vivo*. This explains the recessivity of these mutants *in vivo*. One of the mutants, *xantha-g45* still retained wild type levels of protein *in vivo*, so in this case mutant ChlD could still interact with ChlI, and ChlD was therefore protected from degradation *in vivo*. The corresponding mutant in *Rba. capsulatus* (L418F) exhibited a dominant inhibitory effect on magnesium chelatase activity *in vitro*. Therefore this BchD mutant undergoes an oligomeric organization upon refolding, and can form mixed mutant-wild type oligomers similar to BchI (Hansson et al., 2002). Two separate point mutations in the MIDAS motif of BchD (D385A and S389A), a region important for interaction with BchI showed dominant inhibition of magnesium chelatase activity *in vitro*. These mutants could not contribute to magnesium chelatase activity without the inclusion of wild type BchD. This suggested that mixed mutant-wild type BchD oligomers could form but were defective in their ability to interact with BchI.

When equal amounts of mutant and wild type BchD were refolded separately with BchI and later mixed, there was an additive effect upon magnesium chelatase activity. This showed that inhibitory mixed mutant-wild type BchD oligomers were not formed as previously observed. Once the BchI▪BchD complex formed, it was stable and did not undergo rearrangement of subunits. EM of BchD showed a spontaneous oligomerisation of apparently hexameric complex. ATP was not a

prerequisite for oligomerisation of BchD, unlike formation of hexameric BchI (Willows et al., 2004). These results suggested the BchI▪BchD complex exists as a two-level stacked hexameric structure. The double hexameric-shaped BchI▪BchD complex has since been shown by EM (Elmlund et al., 2008). The hexameric shape of each subunit is composed of either three dimers or two trimers (Elmlund et al., 2008).

Collectively the present studies and previous work show that BchD/ChlD has a structural role in the magnesium chelatase reaction as a platform for BchI, forming a catalytic complex. A catalytic cycle was proposed for the formation and turnover of the BchI▪BchD/ChlI▪ChlD protein complex in *Paper I*. The BchD subunit undergoes spontaneous hexamerisation, whereas BchI requires ATP and free magnesium to form this oligomer (Hansson et al., 2002; Willows et al., 2004). If there is sufficient BchI/ChlI, magnesium and ATP present, BchI/ChlI and BchD/ChlD interact and form a stable 6:6 double hexameric BchI▪BchD/ChlI▪ChlD complex. If any of these components are absent or below optimal concentrations, BchD/ChlD is proteolytically degraded *in vivo* (Lake et al., 2004). A decrease in each of these components occurs during prolonged darkness in plants (Usuda, 1988; Papenbrock et al., 1999; Ishijima et al., 2003). The final step in magnesium chelatase catalysis involves the stable BchI▪BchD/ChlI▪ChlD complex interacting with BchH-proto/ChlH-proto. The BchI▪BchD/ChlI▪ChlD catalytic center can presumably perform several rounds of catalysis. In *Paper II* intermediate steps of magnesium chelatase involved in secondary catalysis are described in terms of ATP hydrolysis.

6.2.2 Magnesium chelatase reaction mechanism

To complement the studies directed at the role of BchD/ChlD, it was decided to conduct a thorough kinetic investigation of each magnesium chelatase subunit from *Rba. capsulatus* (*Paper II*). The kinetic properties of each substrate including magnesium, ATP, and proto were determined and each magnesium chelatase subunit was independently analysed in some detail. ATPase assays of the magnesium chelatase complex were included to suggest other roles for ATP hydrolysis apart from chelation of magnesium into proto.

At first inspection, magnesium and ATP substrates were hyperbolic with respect to magnesium chelatase activity (*Paper II*). However this proved to be incorrect for magnesium due to the failure to consider the magnesium present with protein subunits. Re-investigation of the magnesium substrate showed a sigmoidal relationship (*Paper II* Addendum). This data suggested a regulatory role of the magnesium substrate which agrees with previous results using magnesium chelatase from pea, cucumber, *C. tepidum*, and *Synechocystis* (Richter and Rienits, 1982; Guo et al., 1998; Jensen et al., 1998; Reid and Hunter, 2004; Johnson and Schmidt-Dannert, 2008). This is different to *Rba. sphaeroides* magnesium chelatase which has a hyperbolic response with magnesium (Gibson et al., 1999).

The inclusion of exogenous proto to magnesium chelatase assays generated a greater amount of product than simply using BchH-proto as substrate. This showed that additional rounds of catalysis occurred with magnesium chelatase and a secondary rate of chelation was measured. The secondary rate represented the loss of Mg-proto from BchH, re-loading of BchH with fresh proto and catalysis by the BchI▪BchD complex. The secondary rate was ~26 times slower than the initial rate. Therefore recycling of reacted BchH-Mg-proto for a second round of catalysis was the rate-limiting step of the reaction. BchH does not readily release Mg-proto following catalysis (Sirijovski et al., 2008) which agrees with our data that secondary catalysis is not easily undertaken (*Paper II*).

The addition of BchH-proto/ChlH-proto to the BchI▪BchD/ChlI▪ChlD complex triggers a large increase in ATPase activity in *Synechocystis* and *Rba. capsulatus* (Jensen et al., 1999a) (*Paper II*). ATPase activity of *Rba. capsulatus* BchI▪BchD▪BchH-proto continued after magnesium chelatase activity ceased at reaction equilibrium (*Paper II*). BchH has large conformational changes associated with binding proto (Sirijovski et al., 2008). It is suggested that the continued ATPase activity of the BchI▪BchD complex may be required for structural re-organization of BchH which is needed for undertaking additional rounds of catalysis (*Paper II*). This may explain some of the large amount of ATP hydrolysis required for magnesium chelation in *Synechocystis* where 15 ATP molecules were estimated for insertion of one magnesium atom into proto (Reid and Hunter, 2004). Removal of ATP, or exchange for non-hydrolysable ATP (adenosine 5'-[β,γ-methylene] triphosphate) decreased the

stability of the ChlI▪ChlD complex in *Synechocystis* (Jensen et al., 1999a). Therefore it is also possible that ATPase activity is needed for maintaining the BchI▪BchD/ChlI▪ChlD complex in a conformation ready for catalysis.

The secondary rate of magnesium chelatase was used to estimate a K_m value of 47 ± 9 nM for proto. This represented the K_m^{proto} during additional catalysis by magnesium chelatase and is not a real K_m value which is normally determined from the initial rate. This was the best estimate of the K_m since an effective way to remove proto naturally bound to BchH could not be found (see 1.5.10). However K_m^{proto} is comparable to previous K_m values of proto for chloroplast preparations of cucumber and pea magnesium chelatase using initial rates (25 nM and 13.5 ± 6 nM respectively) (Richter and Rienits, 1982; Guo et al., 1998), and lower than purified magnesium chelatase from *Rba. sphaeroides* (150 ± 50 nM) (Gibson et al., 1999). It is significantly lower than *Synechocystis* (1250 ± 280 nM) (Jensen et al., 1998), and previous stopped assays with *Rba. capsulatus* (1230 nM) (Willows and Beale, 1998). The kinetic experiments mainly utilised continuous assays (*Paper II*) and are expected to be more accurate than stopped assays.

Kinetic experiments with *Synechocystis* magnesium chelatase show that ChlD behaves as an enzyme, while the ChlI and ChlH subunits are the substrates (Jensen et al., 1998). Previous studies with *Rba. capsulatus* magnesium chelatase did not take this into account (Willows and Beale, 1998). In *Paper II* when BchD was used at comparatively lower concentrations, BchI and BchH-proto also responded as substrates. With optimal amounts of BchI and BchH-proto, BchD behaved as an enzyme. The nature of the interaction of BchI and BchH as substrates with BchD was different.

BchI responded hyperbolically at four different BchD concentrations. A global K_m^{BchI} of 20 nM indicated that at this BchI concentration, half of the BchI and BchD subunits form a stable complex. BchI was saturable for magnesium chelatase activity and required an excess of BchI over BchD for optimal magnesium chelatase activity which agrees with previous studies (Jensen et al., 1998; Willows and Beale, 1998; Gibson et al., 1999) (*Paper II*). The proposed stoichiometry of the BchI:BchD/ChlI:ChlD complex is estimated at 2-5:1 depending on the concentration of BchD/ChlD

(Jensen et al., 1998; Willows and Beale, 1998; Gibson et al., 1999). The EM structure of the BchI▪BchD complex is a double hexamer with equal proportions of each subunit (Elmlund et al., 2008). It is now clear that the proposed BchI:BchD ratios are not a true representation of the structure of a stable BchI▪BchD complex. The hyperbolic result with BchI at different concentrations of BchD indicated a 1:1 molar ratio with BchD (*Paper II*) and this correlates with the structural data (Elmlund et al., 2008). It is suggested that excess BchI over BchD required in *in vitro* assays of magnesium chelatase is for the stability of the BchI▪BchD complex. BchD tends to aggregate in the absence of BchI, and so it may be that an excess of BchI is required to prevent this aggregation during refolding of BchD *in vitro*.

BchH was sigmoidal (Hill constant ~2) when measuring magnesium chelatase activity at variable BchI:BchD ratios and concentrations (*Paper II*). ATPase activity of magnesium chelatase with respect to BchH was also sigmoidal. This strongly suggested that there are approximately two BchH subunits that interact with the BchI▪BchD catalytic complex. The $S_{0.5}$ for BchH-proto was ~132 nM which is 6 times greater than the K_m for BchI. Therefore the interaction between BchI and BchD was much stronger than BchH and BchD or BchH and BchI▪BchD. This agrees with a similar kinetic study using *Synechocystis* magnesium chelatase showing a K_m^{ChlI} of 85-107 nM and K_m^{ChlH} of 200-260 nM (Jensen et al., 1998).

6.2.3 Stimulatory/inhibitory effect of detergents upon magnesium chelatase

The addition of increasing amounts of the detergents Tween 80, Tween 20, P-20, or Triton X-100 resulted in more Mg-proto made by magnesium chelatase (Fig. 7A-D, Box 3). The optimal concentration of each of the four detergents occurred near or above their critical micelle concentration (CMC) (Helenius et al., 1979; Neugebauer, 1990). Each of the detergent micelles have similarly large molecular masses. Tween 80 has a molecular weight of ~78,600 Da (de Campo et al., 2004), Tween 20 (P-20) is ~73,680 Da (Garstecki et al., 2005), and Triton X-100 is ~87,920 Da (Neugebauer, 1990). Micelle size was inferred by multiplying their respective aggregation numbers with their molecular weights. Another common feature of each of the stimulatory detergents was their non-ionic form. The

interaction of detergent Tween 80 in the magnesium chelatase reaction was tested further in *Paper IV* and also discussed in 6.4.4. From these results there are two common features of detergents that could be important for stimulatory interaction with magnesium chelatase; 1) large molecular mass micelles, and 2) a non-ionic detergent.

LDAO and deoxycholic acid (Box 3) suddenly inhibited magnesium chelatase at break-point concentrations (Fig. 7E-F). The break-point was below the CMC for LDAO and near the CMC for deoxycholic acid. Micelles are not the major inhibitory feature of LDAO, although interestingly the micelle size is small (~17,300 Da) (Herrmann, 1962) in comparison to the stimulatory detergents. LDAO is zwitterionic and the charge contribution may inhibit magnesium chelatase. In contrast to LDAO, micelles appear to play a major part in the inhibition of magnesium chelatase by deoxycholic acid. Deoxycholic acid is anionic and has bulky side groups and either/or a combination of these features could have a detrimental effect on magnesium chelatase. Similar to LDAO, deoxycholic acid has a small micellar size (700 Da) (Helenius et al., 1979) which supports the idea that large-sized micelles may be needed for optimal stimulatory effects upon magnesium chelatase.

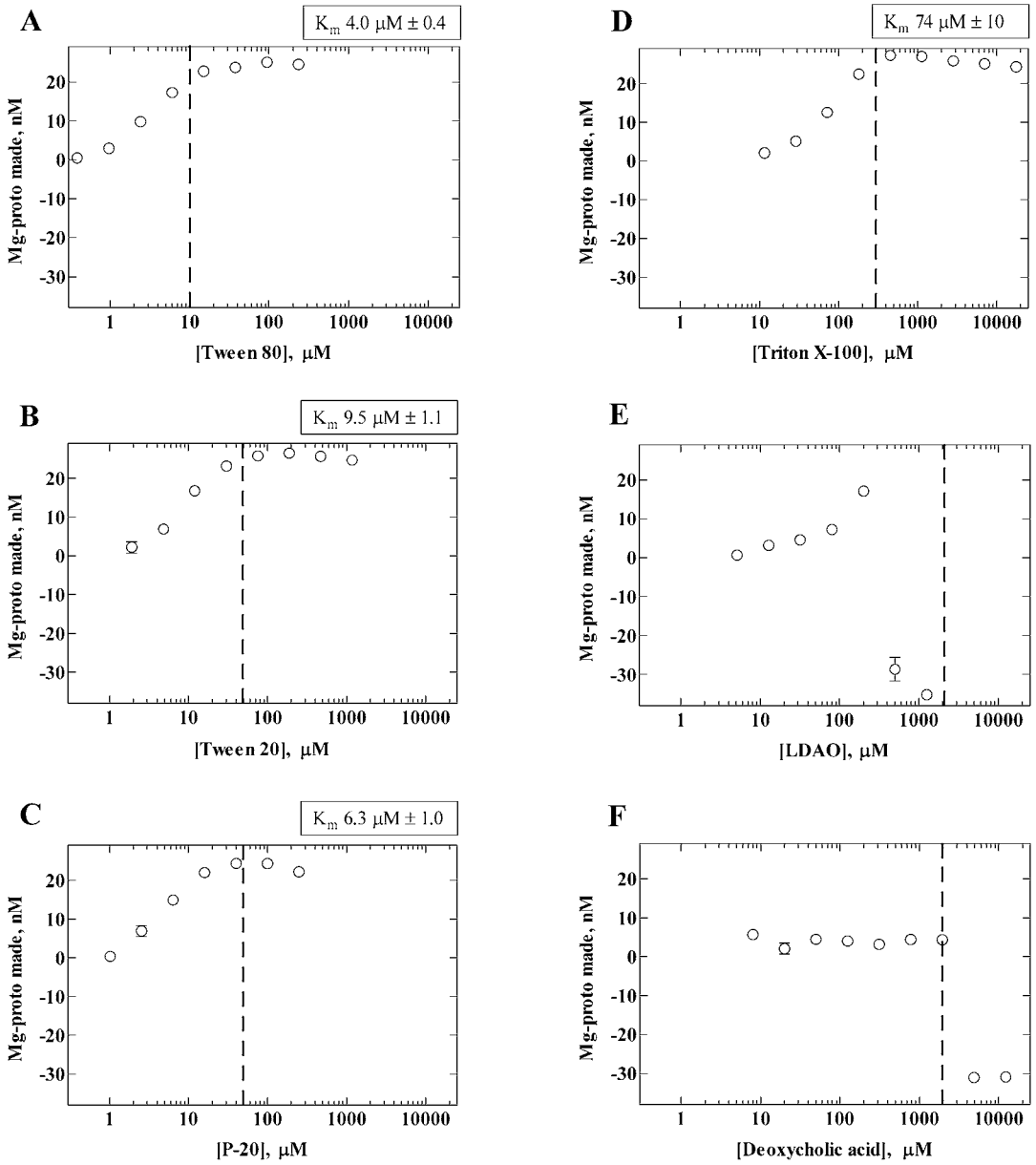
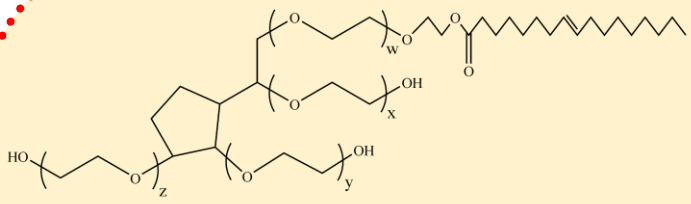
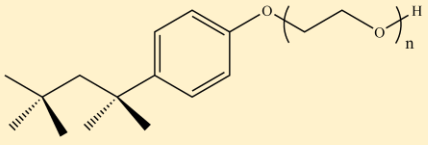


Figure 7. Stimulation or inhibition of magnesium chelatase product formation by detergent. The magnesium chelatase assay was performed with increasing concentrations of six detergents tested (x-axis). The amount of Mg-proto made by magnesium chelatase without any detergent is assigned zero pmol on the y-axis. A-D were analysed using the Michaelis-Menten equation, $V=V_{max}*[S]/(K_m+[S])$, where V is pmol Mg-proto made and [S] is detergent concentration. A negative amount of pmol Mg-proto on the y-axis (E-F) indicated inhibition by the detergent. Assays were performed at 30 °C in 50 mM Tricine-NaOH pH 8.0, 15 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 3.2 mM urea, 44 mM glycerol, 7.8 nM BchD, 15.6 nM BchI, and 60 nM BchH-proto. Dashed vertical lines indicate the CMC of the detergents. CMC values of Tween 80, Tween 20, P-20, and Triton X-100 are determined in water (Neugebauer, 1990), LDAO CMC is determined in water (Herrmann, 1962), and deoxycholic acid at pH 9 (Helenius et al., 1979). Assays were performed according to the method in *Paper II*.

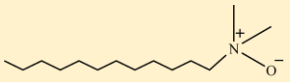
Box 3. Detergents



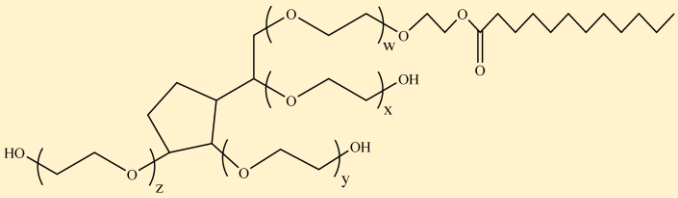
Tween 80 (polysorbate 80)



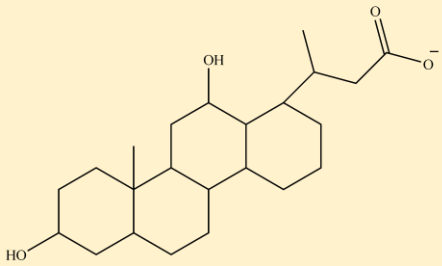
Triton X-100



LDAO (lauryl dimethylamine oxide)



Tween 20 (polysorbate 20)



Deoxycholic acid

Chemical structure of detergents at pH 8. The four repeating units of Tween 80 and Tween 20, w+x+y+z = 20. Tween 20 and P-20 (Biacore Life Sciences) have identical structures except P-20 has a much greater purity than Tween 20. For Triton X-100, n ~ 9.6 (Neugebauer, 1990). Tween and Triton X-100 are non-ionic, LDAO is zwitterionic (pK_a 5.0) (Herrmann, 1962), and deoxycholic acid (pK_a 6.2) (Helenius et al., 1979) is anionic.

6.3 *S*-adenosyl-L-methionine:magnesium protoporphyrin IX *O*-methyltransferase (BchM)

6.3.1 Purification of *O*-methyltransferase

It was necessary to express heterologous His-tagged *O*-methyltransferase from *Rba. capsulatus* in *E. coli* at lower temperatures (15-18 °C) with isopropyl-β-D-thiogalactopyranoside (IPTG) since expression in the range 25-37 °C resulted in very little protein recovery (< 0.1 mg) (*Paper III*). It was beneficial to limit the number of chromatographic steps since the protein is unstable, even at 4 °C. After solubilisation of *O*-methyltransferase from inclusion bodies using detergent P-20 a single chromatographic step using Ni^{2+}-affinity chromatography was enough to purify *O*-methyltransferase to near-homogeneity. It was later found that phospholipids, in particular

phosphatidylglycerol (PG) are crucial for maintaining solubility of *O*-methyltransferase, especially the highly-purified form. The protein from inclusion bodies could be solubilised with other detergents such as Tween 80, Tween 20, or Triton X-100, however it was highly unstable and aggregated within several hours at room temperature. The detergent instability of *O*-methyltransferase from *Rba. sphaeroides* and *E. gracilis* has also been reported (Hinchigeri et al., 1981; Hinchigeri et al., 1984).

6.3.2 Discovering that phospholipids stabilize and stimulate *O*-methyltransferase activity

The first inclinations of the dependence of *O*-methyltransferase upon phospholipids came about as an after-thought following the use of a coupled *O*-methyltransferase assay. This coupled assay employed BchM, Mg-proto, together with an *in situ* system for generating *S*-adenosyl-L-methionine (SAM) (Fig. 8a). *In situ* production of SAM consisted of a crude preparation of SAM synthetase (E.C. 2.5.1.6), L-methionine and ATP. The control *O*-methyltransferase assay simply substituted commercial SAM for the components of the *in situ*-generation of SAM.

The coupled *in situ* method generated the product of the *O*-methyltransferase reaction at a 4-fold faster rate than using commercial SAM (Fig. 8b). It seemed unlikely that this difference in enzymatic activity was due to the reported instability of SAM (Borchardt, 1979; Hoffman, 1986), since a freshly prepared solution of the more stable *p*-toluene sulphonate salt (Fiecchi, 1976) was used. It was of interest to isolate the stimulatory component of *O*-methyltransferase found in the *in situ* SAM synthesizing system.

Crude SAM synthetase with SAM as substrate also increased *O*-methyltransferase activity by ~4-fold (*Paper III*). This ruled out L-methionine and ATP as stimulatory components. When SAM synthetase was replaced by *E. coli* BL21(DE3) Star crude cell lysate, a similar 4-fold stimulatory effect upon *O*-methyltransferase activity was seen. This ruled out the possibility of crude SAM synthetase as the enhancer. Thus a component of *E. coli* cell lysate also contained the stimulatory compound of interest. After boiling *E. coli* crude cell lysate and centrifugation, the supernatant still

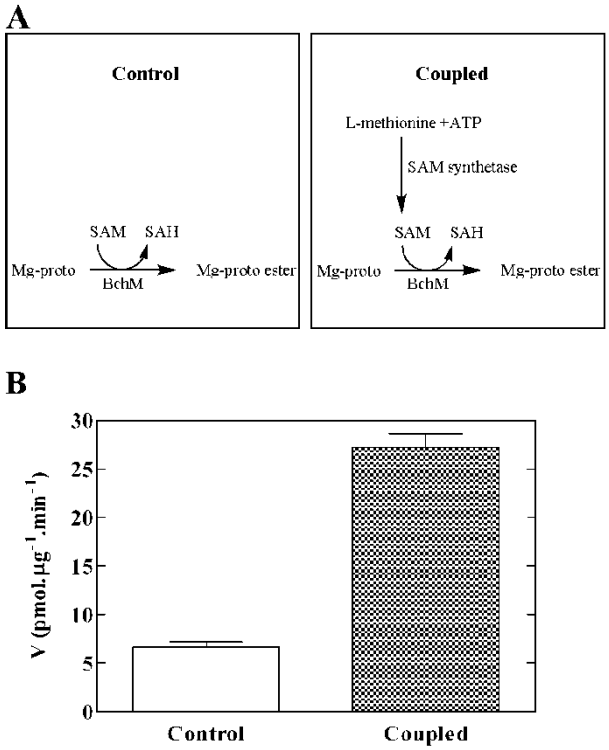
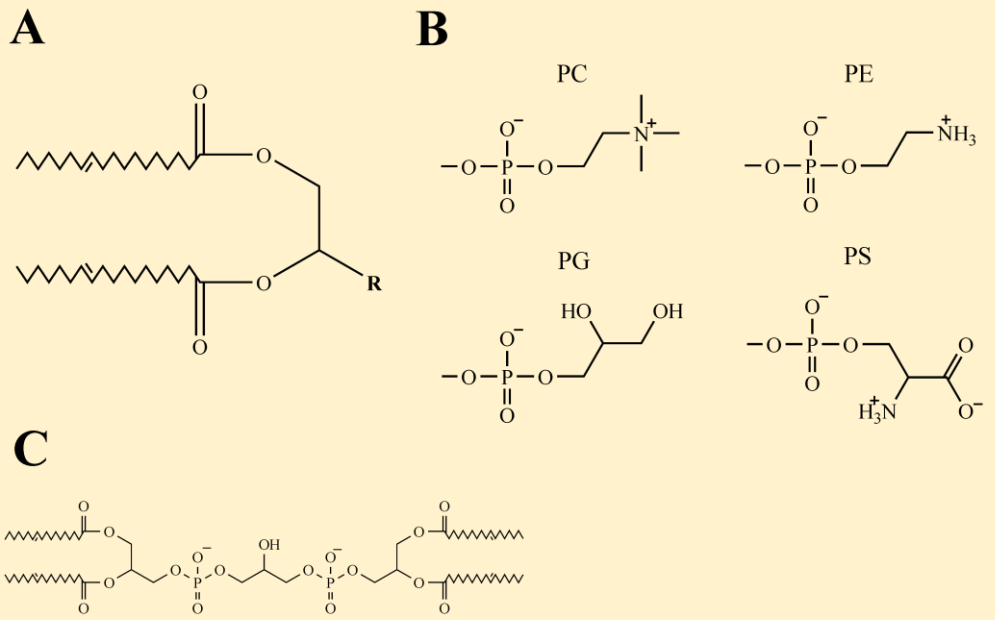


Figure 8. Comparison of control *O*-methyltransferase assay against a coupled *O*-methyltransferase assay involving an *in situ* generation of SAM. A. Schematic of control and coupled *O*-methyltransferase and SAM synthetase assay with *in situ* generated SAM. B. *O*-methyltransferase assay. All assays were performed in triplicate with final concentrations of 50 mM Tris-HCl pH 8.5, 0.3 µM Mg-proto, 2.5 mM DTT, 40 nM BchM, 1.5 mM $MgCl_2$, 3 mM KCl, 0.5 mM ATP at 30 °C for 10 min. The control assay included commercially derived 12 µM SAM. The coupled *in situ* assay required generation of SAM by preincubation of 24 µM L-methionine, 1 mM ATP, 3.3 mM DTT, 3 mM $MgCl_2$, 6 mM KCl, 0.6 µM Mg-proto and 17.7 µg crude SAM synthetase in 50 mM Tris-HCl pH 8.5 at 30 °C for 10 min prior to addition of an equal volume of BchM. Final concentrations of each component in control and coupled assays were identical. *O*-methyltransferase assays were performed according to the method in *Paper III*.

showed a 4-fold stimulatory increase in *O*-methyltransferase activity. Separation by size-exclusion chromatography (Nap-10 column) showed that this heat-stable component had an apparent molecular weight greater than 5,000 Da. This could mean a heat-stable protein or a micellar lipid is the component of interest. The latter was tested by first extracting whole phospholipids from *E. coli* (Osborn and Rothfield, 1966) and this was also stimulatory. This confirmed that phospholipids are the stimulatory component required for optimal *O*-methyltransferase activity. The *E. coli* phospholipid extract was expected to contain ~20-25 % phosphatidylglycerol (PG) and ~70-80 % phosphatidylethanolamine (PE) (Dowhan, 1997) (Box 4).

Box 4. Phospholipids



Chemical structure of some common phospholipids. **A** General phospholipid structure. Both alkyl groups are represented by $(C18:1)_2$ (divaccenic acid , C18:1Δ11) since this is the major fatty acid chain of Rhodobacter (Wood et al., 1965; Kenyon, 1978). R is a variable polar head group **B** Common R groups of the general phospholipid structure (Kenyon, 1978); PC, phosphatidylcholine, PE, phosphatidylethanolamine, and PG, phosphatidylglycerol are found in *Rba. capsulatus*. Phosphatidylserine (PS) is a minor phospholipid present in plants (Vance and Steenbergen, 2005). The charges of the ionic groups are shown at neutral pH (van Dijck et al., 1978; Szoka and Papahadjopoulos, 1980; Cevc et al., 1981; Seddon et al., 1983). **C** Structure of cardiolipin, another common phospholipid present in *Rba. capsulatus* (Kenyon, 1978).

To deduce the phospholipid required for stimulating *O*-methyltransferase activity, a knowledge of the phospholipids from the source enzyme *Rba. capsulatus* is needed. In *Rba. capsulatus* and four other *Rhodobacter* species, the only two common phospholipids are the negatively charged PG and zwitterionic PE (Wood et al., 1965). Phosphatidylcholine, and cardiolipin are some of the other common phospholipids in *Rba. capsulatus* (Wood et al., 1965; Russell and Harwood, 1979). In *Rba. capsulatus* there is a large increase in the proportion of PG at the expense of PE when the growth conditions are changed to photosynthetic (39.3 % to 62.5 % for PG and 33.8 % to 18.7 % for PE) (Russell and Harwood, 1979). Mixed alkyl chain phospholipids PG, PE, and PS were tested for stimulatory *O*-methyltransferase activity. PG had the greatest impact (10-fold), followed by PS (8.6-fold), with PE (1.4-fold) having little effect on *O*-methyltransferase activity. Therefore the *in vitro*

stimulatory effect of *O*-methyltransferase by PG parallels the increase in PG *in vivo* in *Rba. capsulatus* under photosynthetic growth conditions (Russell and Harwood, 1979).

Dioleoyl phosphatidylglycerol (DOPG, $(C_{18:1})_2$) and palmitoyl-oleoyl phosphatidylglycerol (POPG, $C_{16:0}$, $C_{18:1}$) each had a 4-5 fold stimulatory effect on *O*-methyltransferase activity, and it is puzzling that a non-uniform (mixed alkyl chain) phosphatidylglycerol structure worked better than the pure phospholipid structures. The mixed alkyl chain PG that was used from egg yolk lecithin contains approximately 36 % $C_{16:0}$, 33 % $C_{18:1}$, 14 % $C_{18:2}$, and 10 % $C_{18:0}$ (Sigma-Aldrich). A non-uniform micelle structure could be important for interaction with *O*-methyltransferase, or perhaps another untested PG is best, for example dilineoylphosphatidylglycerol ($C_{18:2}$). Further studies with a broad range of pure and mixed PG molecules and using techniques such as electron spin resonance (ESR) is required to determine key features for lipid interactions with *O*-methyltransferase. ESR studies using a variety of spin-labelled lipids has been used extensively with the Na,K-ATPase transporter protein (Esmann and Marsh, 2006). The discovery that PG interacts with *O*-methyltransferase from *Rba. capsulatus* is perhaps not surprising since the enzyme is widely reported as being membrane-associated (Gibson et al., 1963; Hinchigeri et al., 1981; Hinchigeri et al., 1984; Averina et al., 2002; Block et al., 2002).

6.3.3 Structural effects with phospholipids

O-methyltransferase from *Synechocystis* (ChlM) exists as a monomer (Shepherd et al., 2003) BchM from *Rba. capsulatus* was a high molecular weight polymer of unknown size by gel filtration, particularly in the absence of phospholipids (*Paper III-IV*). A high molecular weight form of BchM from *C. tepidum* has also been shown (Johnson and Schmidt-Dannert, 2008). Phospholipids such as PG dispersed BchM from *Rba. capsulatus* into lower molecular weight forms (*Paper III*). This may suggest that more individual BchM molecules are free to participate in the enzymatic reaction. A schematic attempts to show the effect of PG on the structure of BchM in terms of enzymatic activity (Fig. 9).

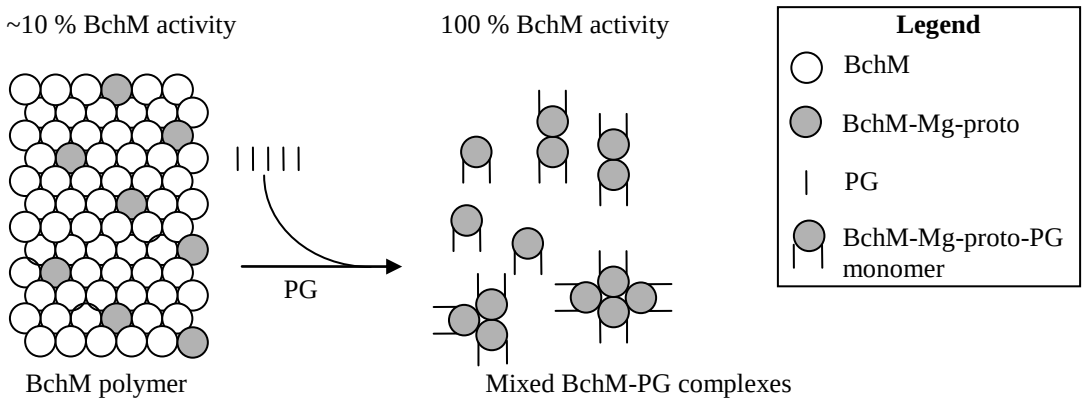


Figure 9. Schematic of proposed disaggregation of BchM into lower molecular weight structural forms by phosphatidylglycerol (PG). In the absence of PG, BchM was a large molecular weight aggregate (the size of the aggregate has not been determined). The addition of PG disaggregated BchM into mainly monomeric, dimeric and other multimeric forms. This schematic does not represent the frequency or identity of the multimers. It is suggested that the BchM polymer cannot bind Mg-proto as efficiently as the dispersed protein. Based on enzymatic assays, ~1/10th of each BchM molecule in the polymer is capable of binding Mg-proto corresponding to 1/10th possible enzymatic activity. Following dispersal of BchM into mixed monomers/multimers it is assumed that there is optimal efficiency of the enzyme (100 %).

6.3.4 Porphyrin substrates of *O*-methyltransferase

Mg-proto is the natural substrate for BchM and has the greatest enzymatic activity, followed by Ca-proto, and Zn-proto (Gibson et al., 1963; Radmer and Bogorad, 1967) (*Paper III*) (Fig. 10). The observed ~10 % *O*-methyltransferase activity with proto from crude membrane preparations of *Rba. sphaeroides* and crude ChlM from *Zea mays* is likely to be a false positive since it is known that magnesium insertion into proto by magnesium chelatase is a difficult and intricate process. Proto from *C. tepidum* has very low *O*-methyltransferase activity (0.15 %) which is a more realistic result for the relative substrate specificity of proto for *O*-methyltransferase (Johnson and Schmidt-Dannert, 2008). Proto is unlikely to be a substrate for *O*-methyltransferase under physiological conditions. Assays with *Rba. capsulatus* could not detect any *O*-methyltransferase activity using proto as a substrate (*Paper III*). Synthetic derivatives of Mg-proto; Mg-deuteroporphyrin, and Mg-mesoporphyrin each have similar sub-optimal *O*-methyltransferase activity (Gibson et al., 1963) (*Paper III*) indicating the importance of the vinyl groups for binding.

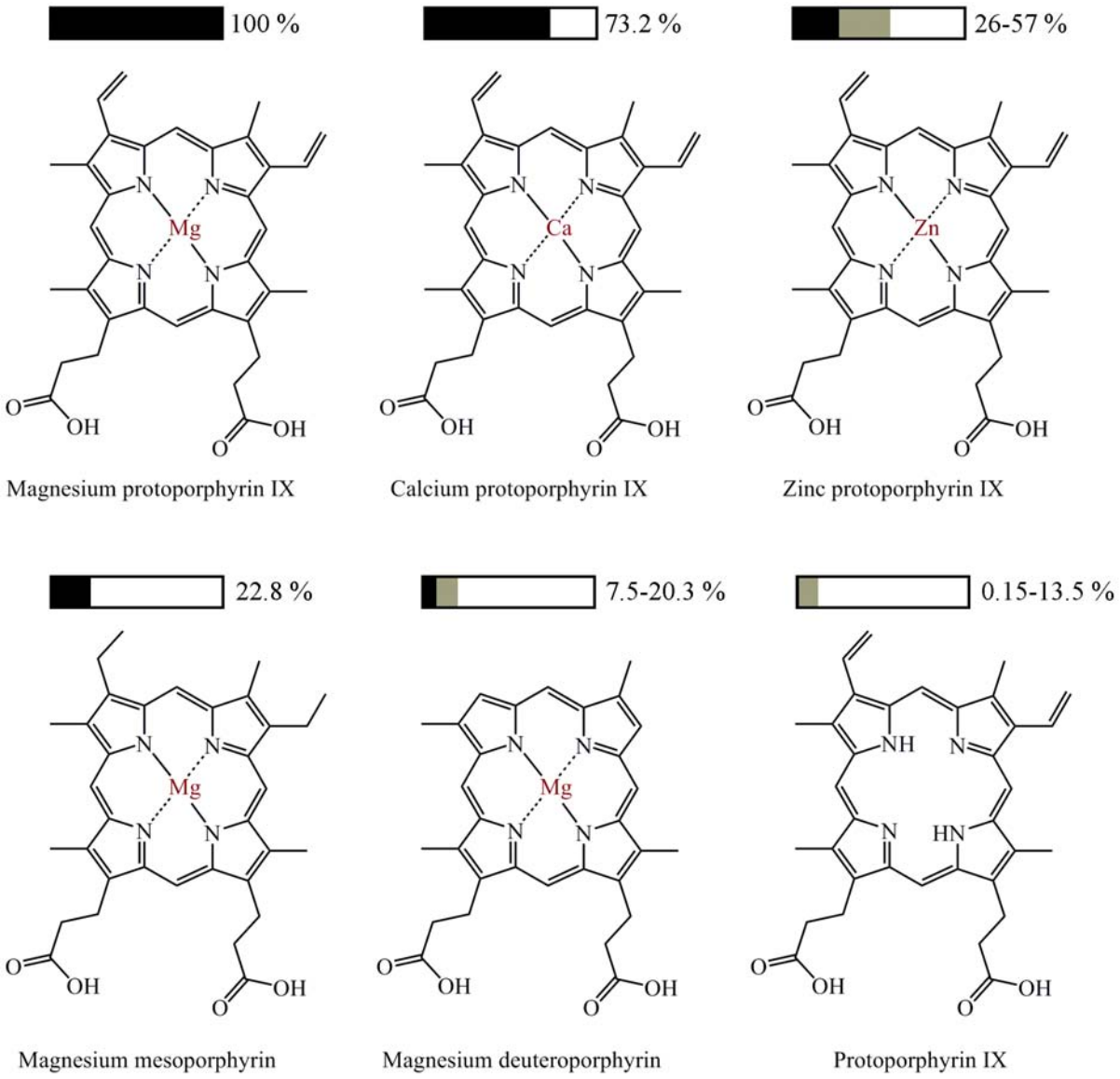


Figure 10. Substrate specificity for *O*-methyltransferase. Filled black bars indicate relative activity compared with Mg-proto. Filled grey bars indicate variation amongst different *O*-methyltransferase sources. Data is taken from crude enzyme preparations from *Rba. sphaeroides* (Ca-proto, Zn-proto, Mg-mesoporphyrin, Mg-deuteroporphyrin, proto) (Gibson et al., 1963), and *Zea mays* (Zn-proto, and proto) (Radmer and Bogorad, 1967), or purified enzyme from *C. tepidum* (proto) (Johnson and Schmidt-Dannert, 2008), and *Rba. capsulatus* (Zn-proto, and Mg-deuteroporphyrin) (*Paper III*).

6.3.5 *O*-methyltransferase reaction mechanism

Kinetic analysis of *Rba. capsulatus O*-methyltransferase revealed the K_m of the two substrates of the enzyme; SAM, and Mg-proto (*Paper III*). The K_m for SAM was 45 μM, and is comparable to previous studies shown in Table 3. The K_m for Mg-proto was 0.11 μM which is similar to a recent study with purified *C. tepidum* enzyme (0.6 μM) (Table 3) (Johnson and Schmidt-Dannert, 2008).

Previous work with crude plant and algal *O*-methyltransferase showed a much higher $K_m^{Mg\text{-}proto}$ of ~10-48 μM (Ebbon and Tait, 1969; Ellsworth et al., 1974; Shieh et al., 1978; Hinchigeri and Richards, 1982).

Kinetic analysis of each substrate with *O*-methyltransferase from *Rba. capsulatus* fitted to the Michaelis-Menten equation produced a pattern that was characteristic of a sequential reaction mechanism (Cleland, 1967) (*Paper III*). Product inhibition with SAH was non-competitive with respect to SAM and Mg-proto. The same pattern of product inhibition was observed with Mg-proto ester. Later experiments involving coupled magnesium chelatase and *O*-methyltransferase assays showed that addition of BchM produces more Mg-proto by magnesium chelatase in the absence of SAM (*Paper IV*) which implied that BchM can bind Mg-proto prior to SAM. Together these results indicated the reaction mechanism was random with respect to substrate binding and removal of product. This is called a random sequential or random Bi Bi reaction mechanism (Cleland, 1963), and this reaction type is the same as *Synechocystis* and *E. gracilis* ChlM (Hinchigeri and Richards, 1982; Shepherd et al., 2003), but differs from wheat ChlM (ping-pong) (Ellsworth et al., 1974; Yee et al., 1989) or *Rba. sphaeroides* (ordered sequential mechanism) (Hinchigeri et al., 1984)(Table 3).

6.4 Interactions between magnesium chelatase and *O*-methyltransferase, BchJ, or detergent Tween 80

6.4.1 Effect of magnesium chelatase on *O*-methyltransferase activity

Magnesium chelatase and *O*-methyltransferase assays are coupled (Gorchein, 1972; Hinchigeri et al., 1997; Alawady et al., 2005; Shepherd et al., 2005). The interaction is between BchM/ChlM and the BchH/ChlH subunit of magnesium chelatase and is thought to entail the transfer of Mg-proto. Kinetic studies highlighting the interactions between magnesium chelatase and *O*-methyltransferase are directed at monitoring *O*-methyltransferase activity in response to the addition of BchH/ChlH (Hinchigeri et al., 1997; Alawady et al., 2005; Shepherd et al., 2005). BchH-proto or other combinations of magnesium chelatase subunits had no distinct stimulatory effect upon *O*-

methyltransferase activity from *Rba. capsulatus* (*Paper III*). It should be noted that in these experiments Mg-proto was used as the substrate and BchH had proto bound when used. The difficulty in obtaining isolated BchH-Mg-proto was a weakness of the experiments. If BchH-Mg-proto was used instead of Mg-proto and BchH-proto, a different result may have been found. The addition of a fully functional BchI▪BchD▪BchH complex (which has BchH-proto and BchH-Mg-proto) to *O*-methyltransferase had no clear-cut stimulatory effect on *O*-methyltransferase activity. This is probably because exogenous Mg-proto used in the assay is at a saturating concentration. With these limitations in the assays, it was decided to concentrate on the effect of *O*-methyltransferase upon magnesium chelatase activity with no addition of exogenous porphyrin (*Paper IV*). The effect of BchJ on magnesium chelatase was also examined since it has no defined role in bacteriochlorophyll biosynthesis (Chew and Bryant, 2007a, 2007b).

6.4.2 Aggregation of BchM and BchJ with magnesium

Magnesium chelatase requires millimolar concentrations of magnesium for optimal activity (*Paper II*) so the effect of magnesium upon BchM and BchJ solubility was tested (*Paper IV*). Greater than approximately 2 mM magnesium caused aggregation of BchM and to a lesser degree BchJ (*Paper IV*). As the magnesium concentration increased up to 12.5 mM, the solubility of BchM and BchJ decreased. The addition of increasing concentrations of NaCl or KCl up to 200 mM prevented aggregation of BchM and BchJ by magnesium (results not shown). However as the higher salt concentrations inhibited magnesium chelatase activity this was not pursued any further. Assays involving interactions between magnesium chelatase and BchM or BchJ used 12.5 mM $MgCl_2$. Therefore interactions involved aggregates of BchM and partial aggregates of BchJ. Preliminary assays showed that BchM, BchJ, and Tween 80 stimulated magnesium chelatase product formation in a similar way. This shows the aggregation of BchM or BchJ did not affect the interaction with magnesium chelatase.

6.4.3 Binding of proto and Mg-proto to BchM or BchJ

Mg-proto naturally binds to *O*-methyltransferase as a substrate and BchJ was also tested for binding exogenous proto and Mg-proto by absorption spectroscopy. The addition of proto or Mg-proto to BchM or BchJ caused a shift in the soret region of the absorbance spectrum. Spectra were shifted by approximately 10 nm for Mg-proto which is indicative of binding and a change in the surrounding environment of Mg-proto (Shelnutt et al., 1998). In contrast a negative control protein, aldolase had no shift in the soret spectrum after addition of Mg-proto.

Secondary structure variations in BchM and BchJ alone were analysed by CD. This was compared with equal molar amounts of BchM or BchJ with Mg-proto. The CD spectrum of BchJ changed in the far-UV region but not the soret region after binding Mg-proto. The proportion of alpha helices increased, while beta strands were decreased by the same margin. In contrast the CD spectrum of BchM is relatively unchanged in the far-UV region (within experimental error) upon binding Mg-proto, but there is a change in the CD soret. Therefore it appeared that BchJ secondary structure undergoes a conformational change after binding Mg-proto, while BchM secondary structure had a fixed conformation with a presumed distortion of Mg-proto which is a common occurrence in protein binding of porphyrins (Shelnutt et al., 1998).

6.4.4 Effect of *O*-methyltransferase, BchJ, and Tween 80 on magnesium chelatase activity

A time-course of magnesium chelatase product formation showed that BchM had a significantly dominant effect over BchJ and Tween 80. This suggested that BchM was the primary interacting partner of magnesium chelatase with potentially another role for BchJ. At the optimal concentration of BchM, BchJ, or Tween 80 additives magnesium chelatase converted up to 100 % of proto to Mg-proto. This is compared with 71 % using magnesium chelatase alone. Therefore each additive altered the equilibrium position of magnesium chelatase to favour product formation. BchH from *Rba. capsulatus* normally retains Mg-proto following catalysis (Sirijovski et al., 2008) and so it is suggested the addition of either BchM, BchJ, or Tween 80 causes the removal of Mg-proto from BchH. *O*-methyltransferase and BchJ had a concentration-dependent interaction with BchH of

magnesium chelatase. The concentration-dependent effect of BchM or BchJ relied upon changes in BchH concentrations. At each BchH-proto concentration the K_m of BchM or BchJ was approximately half. This indicated there is a 1:1 interaction between BchM and BchJ with BchH-Mg-proto. In contrast at each BchH-proto concentration, the K_m of Tween 80 was essentially the same so the effect of Tween 80 was independent of BchH concentration. The K_m of Tween 80 was 3.6 μM which is below its CMC of 10 μM (Neugebauer, 1990). Optimal effect of Tween 80 was nearing the CMC so the detergent apparently depended upon micelle formation for interaction with magnesium chelatase (6.2.3 above). The cylindrical micelle structure of Tween 80 micelles has been shown by small-angle X-ray scattering (SAXS) (Aizawa, 2009). The structure changes to a discus-shaped micelle in a more hydrophobic environment (1,4-dioxane) and this could be important for removal of hydrophobic Mg-proto from BchH, as well as the large micelle size described in 6.2.3.

SDS-PAGE of magnesium chelatase with BchM/BchJ supported the kinetic observations of BchM/BchJ-BchH interactions since BchH associated with either BchM or BchJ through aggregation of each protein (*Paper IV*). This may indicate a membranous interaction between these proteins *in vivo* which would fit the current model of porphyrin translocation among enzymes in plants presumably occurring at the inner envelope of the chloroplast (Masuda and Fujita, 2008). Fluorescence of Mg-proto from soluble and insoluble fractions of the assay at completion indicated that BchM released Mg-proto into the soluble fraction while BchJ retained Mg-proto (*Paper IV*). A mixed assay with magnesium chelatase, BchM, and BchJ showed that BchM had the dominant effect in terms of Mg-proto binding and release at equilibrium. The dominant effect of BchM supported time course experiments. Since the kinetic patterns of the interaction of BchM/BchJ with magnesium chelatase were comparable, it is suggested that BchJ may be able to deliver Mg-proto to BchM. Thus BchJ potentially has a porphyrin-binding role in bacteriochlorophyll biosynthesis as originally proposed by Chew and Bryant (2007a, 2007b).

7. CONCLUSIONS AND FUTURE WORK

The stages of the magnesium chelatase reaction mechanism that require ATP hydrolysis are not well-defined. The work in *Paper II* proposes intermediate catalytic steps of the magnesium chelatase reaction mechanism that may require ATP hydrolysis. For example, the removal of Mg-proto from BchH and re-loading with proto was suggested to involve interaction with the BchI▪BchD complex and ATPase activity. This is not definitive and needs further study. Novel protein-protein interaction studies may be required to resolve this since isolation of a BchI▪BchD▪BchH complex has not been successful.

Kinetic evidence suggests that the BchH subunit of magnesium chelatase from *Rba. capsulatus* is likely to have two or three binding sites on the BchI▪BchD complex (*Paper II*). It shall be interesting if this is a common feature amongst the other magnesium chelatase enzymes from other photosynthetic bacteria, algae, and plants. Currently the model system for plant magnesium chelatase centres on *Synechocystis* since it synthesizes chlorophyll. The major difference in chlorophyll and bacteriochlorophyll biosynthetic organisms is the presence of Gun4 in chlorophyll biosynthesis. It should be tested if Gun4 with proto bound can deliver proto to ChlH for magnesium chelatase activity. Green sulphur and purple non-sulphur bacterial genomes produce BchJ which is not present in chlorophyll-synthesizing organisms. BchJ is suggested to have a similar role to Gun4 (Chew and Bryant, 2007a), and can stimulate magnesium chelatase in *Rba. capsulatus* (*Paper IV*). Studies using BchJ from other photosynthetic bacteria will help elucidate its function in magnesium chelatase or other roles in bacteriochlorophyll biosynthesis, for example as a porphyrin delivery protein.

There are some uncommon features of magnesium chelatase from *C. tepidum* and *A. thaliana*. In each of these organisms there are two BchI/ChlI isoforms (Petersen et al., 1998; Rissler et al., 2002), while there are three isoforms of BchH in *C. tepidum* (Frigaard et al., 2003; Frigaard and Bryant, 2004). We do not yet understand the role of these isoforms in terms of the reaction mechanism. We now have the means to express and purify plant magnesium chelatase, and this may give us a broader understanding of the magnesium chelatase reaction mechanism. There are certainly

going to be differences between bacterial and plant systems, but also between green sulphur, and purple non-sulphur bacteria.

Structural information has greatly helped in our understanding of the magnesium chelatase reaction mechanism. The crystal structure of BchI (Fodje et al., 2001) and Gun4 (Verdecia et al., 2005) is now known. There is an EM structure of BchH with and without proto (Sirijovski et al., 2008) and it would be of significant help if there was supporting X-ray crystal structural information of ChlH, or perhaps co-crystallization of ChlH/Gun4. Apart from assessing the interaction of ChlH with ChlI▪ChlD, this may also provide information to clarify if ChlH is an ABA-binding protein (Muller and Hansson, 2009). Further to this, the interaction of BchH/ChlH with *O*-methyltransferase has been shown using enzyme kinetics (Shepherd et al., 2005) (*Paper IV*). It would be advantageous to have an X-ray crystal structure of *O*-methyltransferase to model BchH-BchM interactions. This will be difficult with *O*-methyltransferase from *C. tepidum* and *Rba. capsulatus* since it has no defined multimeric structure (Johnson and Schmidt-Dannert, 2008) (*Paper III*). There is a greater chance with cyanobacterial *O*-methyltransferase since it is monomeric (Shepherd et al., 2003). Interactions between other (bacterio)chlorophyll biosynthetic enzymes should be examined since the cascade of porphyrin delivery from one enzyme to the next is likely to be a common theme in the pathway.

Phospholipids stimulate *O*-methyltransferase activity in *Rba. capsulatus* (*Paper III*), and this lipid-effect should be tested with *O*-methyltransferase from plant, algae, and other photosynthetic bacteria. This is likely to be a common theme amongst *O*-methyltransferase since the enzyme is typically membranous (Tait and Gibson, 1961; Hinchigeri et al., 1984; Block et al., 2002). Enzyme kinetics of purified plant *O*-methyltransferase has not yet been conducted. Studies should include the porphyrin binding protein Gun4 since it can bind Mg-proto substrate for *O*-methyltransferase in *A. thaliana* (Adhikari et al., 2009).

Tentative evidence of interactions between BchM and BchJ has been provided (*Paper IV*). Further work is needed to substantiate this finding such as employing affinity chromatography through the immobilisation of one protein on a solid matrix, and assessing any interaction of the second

protein. The potential interaction between BchM and BchJ may also be studied *in vivo*, which is expected to be membrane-associated.

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