Characterization of Three Homologs of the Large Subunit of the Magnesium Chelatase from *Chlorobaculum tepidum* and Interaction with the Magnesium Protoporphyrin IX Methyltransferase*[^5]

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Green bacteria synthesize several types of (bacterio)chlorophylls for the assembly of functional photosynthetic reaction centers and antenna complexes. A distinctive feature of green bacteria compared with other photosynthetic microbes is that their genomes contain multiple homologs of the large subunit (BchH) of the magnesium chelatase which is a three-subunit enzyme complex (BchH, BchD, and BchI) that inserts magnesium into protoporphyrin IX as the first committed step of (bacterio)chlorophyll biosynthesis. There is speculation that the additional BchH homologs may regulate the biosynthesis of bacteriochlorophyll biosynthesis. There is speculation that the different magnesium chelatase complexes from a single species of green bacteria have not yet been compared. In this study, we investigated the activities of all three chelatase complexes from the green sulfur bacterium *Chlorobaculum tepidum* and interactions with the next enzyme in the pathway, magnesium protoporphyrin IX methyltransferase (BchM). Although all three chelatase complexes insert magnesium into protoporphyrin IX, the activities range by a factor of 10^5. Further, there are differences in the interactions between the BchH homologs and BchM; two of the subunits increase the methyltransferase activity by 30–60%, and the third decreases it by 30%. Expression of the chelatase complexes alone and together with BchM in *Escherichia coli* overproducing protoporphyrin IX suggests that the chelatase is the rate-limiting enzyme. We observed that BchM uses protoporphyrin IX without bound metal as a substrate. Our results conflict with expectations generated by previous gene inactivation studies and suggest a complex regulation of chlorophyll biosynthesis in green bacteria.

*Chlorobaculum tepidum* (formerly *Chlorobium tepidum*) is a green sulfur bacterium isolated from anoxicogenic mats found near acidic high sulfide hot springs in New Zealand (1). Because it is naturally transformable and has a fully sequenced genome, *C. tepidum* has become a model system for understanding the physiology of green sulfur bacteria and specifically for understanding chlorophyll (Chl)^2^ and bacteriochlorophyll biosynthesis (2–5).

*C. tepidum* produces three types of Chls/Bcls: bacteriochlorophyll *c*, bacteriochlorophyll *a*, and Chl *a*. Bacteriochlorophyll *c*, the most abundant (B)Chl, is the primary pigment of the chlorosome, a light-harvesting antenna structure, whereas bacteriochlorophyll *a* and Chl *a* are the pigments involved in the primary electron transfer reactions in the photosynthetic reaction center complexes (3, 6, 7). Bacteriochlorophyll *c* is characterized by a C−3^1^ hydroxyl group and the absence of the methoxycarbonyl of ring E. Both characteristics lead to self-assembling properties of bacteriochlorophyll *c* that extend the absorption properties of the supramolecular complex to longer wavelengths (8, 9).

(B)Chl biosynthesis in green bacteria depends on approximately 10 enzymatic steps and begins with the insertion of magnesium into protoporphyrin IX (P^IX^), the intermediate common to both the heme and (B)Chl biosynthetic pathways (10, 11). Metal insertion is followed by methylation of the propionic acid group of ring C of MgP^IX^ to form magnesium protoporphyrin IX monomethyl ester (MgP^IX^ME) (12–15). This reaction is catalyzed by an S-adenosylmethionine (AdoMet)-dependent methyltransferase (BchM). Following methylation, a cyclization reaction generates the ring E characteristic of chlorins. Cyclization in *C. tepidum* is catalyzed by an anaerobic MgP^IX^ME cyclase (BchE), forming protochlorophyllide *a*. Reduction of ring D by a specific oxidoreductase produces chlorophyllide *a*, which recent gene inactivation studies suggest is a likely branch point for the biosynthesis of several types of (B)Chl molecules (3, 5).

Magnesium chelatase, a three-subunit (BchD, BchI, and BchH) enzyme, catalyzes the ATP-dependent insertion of Mg^2+^ to form magnesium protoporphyrin IX (MgP^IX^). The current model for the Mg-chelatase is that a hexameric ring of BchI subunits and a hexameric ring of BchD subunits interact

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[^3]: The abbreviations used are: Chl, chlorophyll; (B)Chl, chlorophyll and/or bacteriochlorophyll; P^IX^, protoporphyrin IX; MgP^IX^, magnesium protoporphyrin IX; MgP^IX^ME, magnesium protoporphyrin IX methyl ester; P^IX^ME, protoporphyrin IX methyl ester; AdoMet, S-adenosylmethionine; Mg-chelatase, magnesium chelatase; HPLC, high performance liquid chromatography; LC, liquid chromatography.

27776 JOURNAL OF BIOLOGICAL CHEMISTRY

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with a single BchH subunit. ATPase activity is located on the AAA-type Bchl subunit, and binding of P^IX and metal insertion occurs on BchH (16–19).

A distinctive feature of the genomes of green bacteria is the presence of multiple homologous BchH subunits; other photosynthetic microbial systems, including purple bacteria, typically have only a single BchH subunit. No biochemical characterization of multiple BchH subunits from the same green bacterium have been described, and the function of the different subunits has not been determined. The *C. tepidum* genome contains three homologs of the Mg-chelatase BchH subunit. Bryant and co-workers (2–4) have suggested that these subunits may have a direct role in the regulation of (B)Chl biosynthesis. In *C. tepidum*, the three homologs of the large subunit have been named BchH (CT1957), BchS (CT1955), and BchT (CT1295).

Strains in which individual subunits have been inactivated are all viable but have different phenotypes (3). When BchS is inactivated, bacteriochlorophyll *c* content is greatly reduced, whereas only a slight reduction in bacteriochlorophyll *c* content occurs when BchT is removed. No effect on bacteriochlorophyll content is greatly reduced, whereas only a slight reduction in bacteriochlorophyll *c* content occurs when BchT is not present. This has led to two suggestions for regulation of the amounts of each type of (B)Chl: 1) that the three subunits provide greater control over a positive feedback mechanism that controls biosynthetic flux and 2) that direct interaction between the Mg-chelatase and subsequent enzymes enhances production of specific types of molecules through a mechanism of substrate channeling (3, 4). In the latter scheme, each BchH favors the biosynthesis of specific (B)Chl through interactions with methyltransferase BchM or cyclase BchE.

Substrate channeling is the mechanism of rate enhancement that is gained because an intermediate molecule is passed between enzymes without being released into the bulk solvent (20–23). Several studies have suggested that an interaction between the Mg-chelatase and BchM may be involved in the regulation of (B)Chl biosynthesis (24–27). In vitro studies with isolated BchM and BchH from *Rhodobacter capsulatus* (25) and *Synechocystis* sp. *PCC 6803* (26) showed that methyltransferase activity was stimulated by the presence of BchH. Physical interaction between methyltransferase and Mg-chelatase BchH was shown by yeast two-hybrid experiments with tobacco proteins (27).

In the current paper, we report on the activity of the three BchH homologs and BchM from *C. tepidum*. Mg-chelatase subunits BchT, BchS, BchH, and BchM were overexpressed in *Escherichia coli*, and the recombinant proteins were purified for *in vitro* characterization. In addition, all three Mg-chelatase subunits (BchD, BchH, and BchM) were coexpressed with and without BchM in an engineered *E. coli* strain overproducing P^IX^ (28) to study *in vivo* activities of the proteins. Our results show that the BchH subunits have different Mg-chelatase activities and interact to varying extents with BchM either to increase or to decrease the methyltransferase activity.

**EXPERIMENTAL PROCEDURES**

*Chemicals*—P^IX^ and MgP^IX^ were purchased from Frontier Scientific (Logan, UT) and were resuspended in 0.1% Tween 80 in water. P^IX^ monomethylester (P^IX^-ME) was isolated by preparative high performance liquid chromatography (HPLC) from recombinant *E. coli* cultures overexpressing P^IX^ biosynthetic genes and methyltransferase BchM (see below). Concentrations were determined in 1.5 n HCl using the extinction coefficient of 297 nm^-1 cm^-1 at 408 nm (29). DNA-modifying enzymes were obtained from New England Biolabs (Ipswich, MA). Other chemicals used were obtained from suppliers as described or from Sigma.

**Strains and Growth Conditions**—All plasmid manipulations were performed in *E. coli* JM109 using standard molecular biological techniques (30). *E. coli* JM109 was used for protein expression when using the constitutive promoter on pUCmod (31). For protein expression using the T7 promoter, *E. coli* BL21 cells were used.

**Gene Cloning**—The Mg-chelatase and monomethyl-ester methyltransferase genes from *C. tepidum* were amplified from genomic DNA obtained from the American Type Culture Collection (Manassas, VA; ATCC 49652). Genes cloned in this study were bchH, bchS, and bchT (*C. tepidum* locus tags CT1295, CT1955, and CT1957; GenBank™ accession numbers NP_662183, NP_662832, and NP_662834), bchl (*C. tepidum* CT1297; NP_662185), bchD (CT1296; NP_662184), and BchM (CT1958; NP_662835). The forward primers contained the restriction endonuclease sequence for XbaI, an optimized Shine-Dalgarno and start codon sequence (5‘-AGGAGGAGATCTA-3‘), and 15–18 nucleotides corresponding to the specific gene sequence; the reverse primers contained ~15 nucleotides of the respective gene, a stop codon, and the sequence for the NotI restriction endonuclease. PCR amplification products of methyltransferase and Mg-chelatase subunits were cloned individually into the constitutive expression vector pUCmod (31). All gene sequences were verified by DNA sequencing.

Plasmids containing the three subunits of the Mg-chelatase were constructed for each of the three *C. tepidum bchH* homologs; these plasmids were named pBchDIT, pBchDIS, and pBchDII. Each gene (bchH, bchS, bchT, bchl, and bchD) and its promoter were amplified by PCR from its pUCmod parent plasmids and subcloned into pUCmod to generate a single plasmid with three promoters and three genes. bchl, bchS, and bchT were cloned between the restriction sites XbaI and PaeI, bchl was cloned between the PaeI and EcoRI, and bchD was cloned within the single EcoRI site. bchl and its promoter were amplified from its pUCmod parent construct using primers outside the promoter region and subcloned into pCDF-Duet using the restriction enzyme Ascl to obtain pCDF-BchM^CT1958_.

**Production of Porphyrins in *E. coli***—To produce P^IX^ and the initial intermediates in (B)Chl biosynthesis in *E. coli*, various genes were assembled together on four compatible plasmids derived from pUCmod, pACmod, pBRR1MCS-2 (28), and pCDFDuet™-1 (Novagen, Gibbstown, NJ). Previously, we described overproduction of P^IX^ in aerobically grown *E. coli* transformants harboring plasmid pACmod expressing heme biosynthetic genes hemA, hemB, hemC, and hemD and plasmid pBRR1MCS-2 containing genes hemE and hemF (28).

Cultures of JM109 *E. coli* expressing various combinations of genes from the protoporphyrin and bacteriochlorophyll path-
ways were grown for 45 h with shaking in LB supplemented with appropriate antibiotics (50 μg/ml chloramphenicol, 30 μg/ml kanamycin, 100 μg/ml ampicillin, and 50 μg/ml streptomycin) and 1 mM MgCl₂. The cultures were centrifuged, and porphyrins were extracted from the cell pellet by the addition of N,N-dimethyl formamide/acetic acid/water (4:1:1) and incubated in a sonicating water bath at 4 °C for 2 h. The extracts were centrifuged briefly to remove suspended material before further analysis. The amounts of each porphyrin were determined by comparison of integrated peak areas with authentic PIX and MgPIX standards.

High Pressure Liquid Chromatography—Extracts were loaded on a Zorbax 300SB-C18 column (4.6 × 150 mm, 3.5 μm; Agilent Technologies, Palo Alto, CA) using an Agilent 1100 HPLC system equipped with a photodiode array detector. Porphyrins were separated by a 20-min linear gradient of 70–100% mobile phase B at 1 ml/min. The mobile phases were 0.1 M ammonium acetate, pH 5.1 (A), and methanol (B). For quantitation, standard curves were generated using known concentrations of PIX and MgPIX standards, and the peak areas were integrated using the Agilent ChemStation software. The peaks corresponding to PIX, MgPIX, and iron-PIX were assigned by comparison of retention times and UV-visible spectra to known standards. MgPIX ME and PIX monomethyl ester were assigned by expected shifts in retention times and mass spectrometry.

Mass Spectrometry—LC-MS spectra were obtained using a LCQ mass spectrometer (Thermo Finnigan) equipped with an LC system similar to the one described above using an isocratic condition of 80% mobile phase B and electrospray ionization interface. Isocratic conditions were required to obtain reproducible traces on both the HPLC (Agilent) and LC-mass spectrometry systems. After assignment of each of the peaks, the gradient conditions described above were used in subsequent HPLC analysis. The mass range m/z 200–800 was monitored in the positive ion mode using standard tuning parameters. Singly charged ions had m/z values as follows: PIX (563.3), MgPIX (585.2), PPIX monomethyl ester (577.3), and MgPIX ME (599.2). Methanol adducts predominated for MgPIX and MgPIX ME.

Protein Expression and Purification—To simplify protein purification, His₆ tags were added to each of the genes. Initially, the histidine sequence was moved to the C terminus for the histidine-tagged recombinant proteins were used to inoculate low concentrations. The Michaelis constant (Kₘ) and maximum velocity (V_max) were determined from a Lineweaver-Burk plot and linear regression. For the MgCl₂ substrate, Hill plots were constructed to determine the Mg-binding constant (Kₘ).
Mg-chelatase and MgPIX-methyltransferase from C. tepidum

TABLE 1

Porphyrin accumulation in E. coli cells co-expressing PIX and (B)Chl biosynthetic genes

E. coli transformants expressing genes hemA–hemF for PIX overproduction alone or in combination with the three-subunit Mg-chelatase complexes BchDIS and MgPIX methyltransferase BchM were grown for 45 h at 30 °C prior to extraction of porphyrins from cell pellets. The values are mean and S.D. values of three experiments.

| Genes          | MgPPIX | MgPPIXME | PPIX | PPIXME |
|----------------|--------|----------|------|--------|
| hemAF          | ND     | ND       | 2.6  | 0.6    |
| hemAF + bchDIS | 0.03 ± 0.01 | ND     | 4.7 ± 0.8 | ND    |
| hemAF + bchDIS | 0.3 ± 0.1  | ND     | 5.2 ± 0.5 | ND    |
| hemAF + bchDIH| ND     | ND       | 5.1 ± 0.9 | ND    |
| hemAF + bchMT  | ND     | ND       | 2.3 ± 0.2 | 9.8 ± 0.6 |
| hemAF + bchDIH + bchMT | ND     | 0.02 ± 0.01 | 1.5 ± 0.6 | 0.9 ± 0.3 |
| hemAF + bchDI + bchMT | 0.04 ± 0.01 | 0.4 ± 0.1 | 2.7 ± 0.5 | 1.9 ± 0.3 |
| hemAF + bchDI + bchMT | ND     | 3.1 ± 1.0 | 1.9 ± 0.3 |

*ND, not detected.

FIGURE 1. MgPPIX, MgPPIXME, and PPIXME are produced by E. coli overexpressing the initial enzymes of the (B)Chl biosynthetic pathway. Shown are HPLC traces of extracts from JM109 E. coli cells overexpressing the PPIX biosynthetic pathway alone (HemeA–F; includes hemA, hemB, hemC, hemD, hemE, and hemF) and coexpressed with the Mg-chelatase (HemA–F + BchDIS), the MgPPIX methyltransferase (HemA–F + BchM), and both the Mg-chelatase and MgPPIX methyltransferase (HemA–F + BchDIS + BchM). Traces for PPIX and MgPPIX standards are shown for reference, and arrows indicate peaks associated with MgPPIX, MgPPIXME, PPIX, and PPIXME. The minor peak at 13 min in the top three traces was identified as zinc-PPIX by comparison of retention time and absorption spectrum with a standard. Iron-PPIX was not observed in any of the extracts.

Methyltransferase Assays—Methyltransferase activity was measured using a stopped assay containing 50 mM Tris-HCl, pH 7.5, 0.1% Tween 80, 0.5 mM AdoMet, and either MgPPIX or PPIX. For MgPPIX, the reaction contained 50 nM BchM and was initiated by the addition of AdoMet. Aliquots were removed at 20, 40, 60, and 80 s and mixed with an equal volume of acetone (for higher MgPPIX concentrations, the reaction times were increased to 240 s). For PPIX, the reaction contained 200 nM BchM and was incubated for 16 h. To measure the stopped reactions directly by HPLC, the volume of the reactions increased as the substrate concentration decreased: 20, 50, 100, 250, 750, and 750 μl for substrate concentrations of >30, 30, 10, 3, 1, and 0.3 μM, respectively. The samples were centrifuged, and the porphyrins were separated by HPLC. The absorbance at 420 nm for both MgPPIX and MgPPIXME and 405 nm for both PPIX and PPIXME were integrated for each time point to obtain the velocity of the reaction at each substrate concentration. The Lineweaver-Burk plots of three independent reactions were used to determine the steady-state kinetic parameters.

RESULTS

Mg-chelatase and Methyltransferase Activities in E. coli Overproducing PPIX—We previously showed that overexpression of the heme biosynthetic pathway in E. coli leads to high level production of several porphyrins, including PPIX, the initial substrate for (B)Chl biosynthesis (28). The engineered PPIX pathway was extended with Mg-chelatase complexes (subunits BchD, Bchl, and BchH or BchS or BchT) alone and in combination with methyltransferase BchM to determine the in vivo activities of the C. tepidum proteins.

PPIX-overproducing E. coli expressing Mg-chelatase alone produced a new compound (Fig. 1 and Table 1), which was identified as MgPPIX using retention time and mass spectrometry (Fig. S1). The accumulation of MgPPIX in E. coli, however, depends on whether the BchH, BchS, or BchT subunit is expressed. BchS is the most active subunit (shown in Fig. 1), followed by BchH, and no activity is observed for BchT. These results demonstrate that E. coli is able to express and assemble the multisubunit complex of at least two of the Mg-chelatases without additional factors specific to C. tepidum.

When BchM is co-expressed with the Mg-chelatase, the amounts of PPIX and MgPPIX are reduced, whereas two new products appear. The first peak is MgPPIXME, the expected product of BchM. The second product has an absorption spectrum identical to PPIX, and because it has a longer retention time than PPIX, it appears to be PPIXME. The reduction in MgPPIX levels suggests that the limiting step in this set of reactions is the insertion of Mg2+ into PPIX by the Mg-chelatase. The assignments of both MgPPIXME and PPIXME were confirmed by LC-mass spectrometry (Fig. S1).

The formation of PPIXME may arise in several ways: 1) through a degradative process of MgPPIXME during extraction and analysis in which the metal is lost, or 2) the BchM from C. tepidum is able to transfer a methyl directly to the nonmetal-loaded PPIX. To explore this, we co-expressed only BchM in the PPIX-overproducing E. coli strain. Surprisingly, the total amount of porphyrins produced increased, and most of the porphyrins extracted were PPIXME (Fig. 1 and Table 1). Because no Mg-chelatase was expressed in these cultures, it appears that BchM can use PPIX as a substrate.

Purification of Mg-chelatase Subunits—To investigate the in vitro activities of the three Mg-chelatase complexes from C. tepidum, BchH, BchS, BchT, Bchl, and BchD subunits were overexpressed independently in E. coli for subsequent protein purification. Initial results indicated that the majority of the recombinant protein for BchH, BchS, BchT, and Bchl was expressed in soluble form and could be purified from the cell lysate, whereas BchD was found primarily in inclusion bodies, and the protein was solubilized prior to purification. Hexahistidine affinity tags were added to the N terminus of each of the subunits and were used to purify BchH, BchT, and Bchl by metal affinity chromatography. However, BchS and BchD did not bind to the affinity resin, and the histidine tag was therefore moved to the C terminus for both proteins, which improved binding. The chelatase subunits were estimated to be more than 90% pure, as judged by SDS-PAGE analysis (see Fig. S2). Because the BchH homologs are susceptible to light-induced damage, these subunits...
were purified in low light or dark environments to prevent cross-linking interactions that might inactivate the protein (33).

Samples containing large subunits of the Mg-chelatase eluted from the metal affinity column are significantly colored. Qualitatively, samples containing BchH are red, whereas samples of BchS and BchT have a greenish tint. The absorption spectra for purified BchH, BchS, and BchT are shown in Fig. 2. The prominent Soret transition (400–430 nm) in each spectrum suggests that during protein expression, porphyrins endogenous to _E. coli_ co-purify with each of the BchH paralogs (Fig. 2A). Assuming a single porphyrin-binding site per protein and an extinction coefficient for the Soret transition (29), we estimate that 11, 4, and 5% of BchH, BchS, and BchT contain bound porphyrin, respectively. Fig. 2B shows the distinct differences between the three subunits in the visible region of the absorption spectra. Comparisons with published spectra (34, 35) suggest that BchH binds iron-P\textsuperscript{IX} (iron-P\textsuperscript{IX} has absorption bands at 430, 530, and 563 nm), whereas BchS binds P\textsuperscript{IX} (P\textsuperscript{IX} has absorption bands at 408, 505, 540.5, 578, and 633 nm). The spectrum of BchT suggests that this subunit binds a combination of P\textsuperscript{IX} and iron-P\textsuperscript{IX}.

HPLC traces of porphyrins extracted with 7:2 acetone/methanol from samples containing purified BchH, BchS, and BchT are shown in Fig. 3. Comparison of retention times with several standard porphyrins corroborates the assignments based upon the absorption spectra: BchH is purified bound to iron-P\textsuperscript{IX}, BchS is purified bound to P\textsuperscript{IX}, and BchT is purified bound to a mixture of iron-P\textsuperscript{IX}, P\textsuperscript{IX}, and an unidentified porphyrin. For unknown reasons, extraction of the porphyrins bound to BchT was less efficient than for either BchH or BchS.

These data are consistent with previous experiments that associate purified subunits with a brownish color and demonstrate that BchH binds P\textsuperscript{IX} (37). Karger et al. (38) characterized the binding of deuteroporphyrin IX to the BchH subunits from _Synechocystis_ sp. _PCC 6803_ and _Rhodobacter sphaeroides_ and further showed that magnesium deuteroporphyrin IX can form a complex with BchH.

**In Vitro Reconstitution of Mg-chelatase Complexes**—To determine the ratios of the three Mg-chelatase subunits from _C. tepidum_ that give optimal activity, the BchD/BchI ratio was first varied while holding the concentration of BchS constant (30 nM) (BchS was selected as the most active subunit). Under these conditions, maximal activity was observed at an approximate BchD/BchI ratio of 0.4, corresponding to 125 nM BchD and 300 nM BchI in the assay (see Fig. S3A). Holding the concentrations for BchD and BchI constant at this optimum ratio, the activity increased linearly as a function of either the BchS or BchH concentration (Fig. S3B). The Mg-chelatase activity dropped sharply as the BchS concentration was further increased, which could be the result of aggregation. Mg-chelatase activity with BchT was too low (see below) to allow for accurate measurements.

**Kinetic Properties of Mg-chelatase Complexes**—To compare the catalytic activities of the Mg-chelatase complexes, BchD and BchI concentrations were kept in excess at 125 and 300 nM,
The rate dependence on the Mg\(^{2+}\) concentration is sigmoidal, and the data were fit to the Hill equation to determine \(K_{0.5}\). This analysis is based on the total Mg\(^{2+}\) concentration and does not account for the decrease in free Mg\(^{2+}\) because of the presence of ATP. The free Mg\(^{2+}\) concentration is expected to be approximately the total Mg\(^{2+}\) concentration minus the concentration of ATP. For our experiments, this reduces the \(K_{0.5}\) by 4 mM. The Mg\(^{2+}\) binding constants of the chelatase complex were similar for both BchS and BchH.

The slowest rate of Mg\(^{2+}\) chelation was measured with purified BchT in the chelatase complex. In contrast to the in vivo experiments, where no activity was observed, a Mg-chelatase complex containing BchT did have measurable Mg-chelatase activity in vitro. In order to measure the activity, the concentration of the subunit was increased significantly to 6 μM BchT, and the reaction was monitored for 3 h with 0.3 μM PIX and 14 mM MgCl\(_2\) (see Table 2). Values for \(K_{0.5}^{\text{PIX}}\) and \(K_{0.5}^{\text{Mg}}\) were not determined for the Mg-chelatase complex with BchT.

Because BchM was found to convert PIX to PIXME in vivo and in vitro (see below), the activity of Mg-chelatase complexes containing BchH, BchS, or BchT was measured with DIXME as a substrate. Insertion of Mg\(^{2+}\) into DIXME was not detected.

**Purification and Activity of Methyltransferase—** Recombinant BchM was purified from *E. coli* cell lysate using a C-terminal histidine tag and cobalt affinity resin to >90% purity as estimated by SDS-PAGE analysis (see Fig. S2). The majority of the protein existed as a high molecular weight multimer, as determined by gel filtration (see Fig. S4).

The steady-state kinetics for the transfer of a methyl group from AdoMet to MgPIX by BchM were measured using an end point assay. The initial velocity of the reaction was determined by analyzing the amount of substrate and product at several times after initiation of the reaction by the addition of AdoMet. The formation of DIXME was linear with respect to time for all substrate concentrations tested (see Fig. S5). Fig. 5A shows the double-reciprocal plot for the activity of BchM versus MgPIX concentration, which follows Michaelis-Menten kinetics. The kinetic parameters \(k_{\text{m,PIX}}\) and \(V_{\text{max}}\) are 0.6 μM and 320 pmol of MgPIXME min\(^{-1}\) μg of BchM\(^{-1}\), respectively (Table 3).

To characterize the substrate specificity of BchM further, steady-state kinetics of BchM activity were measured using PIX as a substrate (Fig. 5B). With this substrate, the reaction rate was slower by a factor of 10\(^4\) compared with MgPIX. BchM also binds PIX much less strongly than MgPIX (Table 3). Measurements of BchM activity were limited to a concentration range at which PIX is soluble in 0.1% Tween 80 in aqueous buffer. The \(K_{\text{m}}^{\text{PIX}}\), however, lies outside this concentration range (Fig. 5B), and the obtained data were fit using both the double reciprocal plots and a least squares minimization to the Michaelis-Menten equation. The \(K_{\text{m}}^{\text{PIX}}\) was determined to be ~10–50 μM.

**Effects of BchH, BchS, and BchT on BchM—** Previous studies have suggested an interaction between BchH and BchM (24–27). To investigate whether BchH, BchS, or BchT from *C. tepidum* affects BchM activity and to determine whether they influence BchM activity differently, as would be expected if flux through different (B)Chl pathways in *C. tepidum* is controlled by the Mg-chelatase, BchM activity was measured in the presence of a 20-fold excess of each of the three subunits. The nor-

respectively, whereas BchS and BchH concentrations were 50 and 300 nM, respectively. The Michaelis-Menten constant for DIX (\(K_{\text{m,PIX}}\)), the binding constant for Mg\(^{2+}\) (\(K_{\text{m,Mg}}\)), and the maximum reaction velocities (\(V_{\text{max}}\)) for Mg-chelatase complexes containing BchS and BchH were measured by either varying the PIX concentration in the presence of saturating levels of MgCl\(_2\) or vice versa (Fig. 4 and Table 2).

Following Michaelis-Menten kinetics, the rate of chelation increases hyperbolically as a function of PIX concentration up to 0.4 μM for both subunits. The rate decreases for concentrations above 0.4 μM PIX, probably due to PIX becoming insoluble in aqueous buffer (39). Linear regressions to double-reciprocal plots were used to determine \(k_{\text{m,PIX}}\) and \(V_{\text{max}}\). Although the \(k_{\text{m,PIX}}\) values for both subunits were similar, the catalytic activity of the chelatase complex with BchS was 2 orders of magnitude greater than when BchH was part of the complex.

**TABLE 2**

| Enzyme   | \(K_{\text{m,PIX}}\) (μM) | \(K_{\text{m,Mg}}\) (μM) | \(V_{\text{max}}\) (pmol MgPIX min\(^{-1}\) μg BchH\(^{-1}\)) | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(k_{\text{cat}}/K_{\text{m,PIX}}\) (s\(^{-1}\) μM\(^{-1}\)) |
|----------|----------------------------|--------------------------|-------------------------------------------------------------|-----------------------------|---------------------------------|
| BchH     | 0.33 ± 0.02                | 1.7 ± 0.4                | 0.6 ± 0.1                                                   | 0.2 ± 0.02                  | 3.3 ± 0.7                        |
| BchS     | 0.33 ± 0.02                | 1.7 ± 0.4                | 0.6 ± 0.1                                                   | 0.2 ± 0.02                  | 3.3 ± 0.7                        |

* This measurement is total Mg\(^{2+}\) concentration in the presence of 4 mM ATP. The free Mg\(^{2+}\) concentration available to the enzyme is expected to be approximately 4 mM lower.

**FIGURE 4.** Plots used to calculate \(K_{\text{m,PIX}}\), \(K_{\text{m,Mg}}\), and \(V_{\text{max}}\) for Mg-chelatase. The concentrations were 50 nM (BchS) and 300 nM (BchH); Bch and BchD concentrations were fixed at 300 and 125 nM, respectively. Lineweaver-Burk plots are shown for both BchS (A) and BchH (B). The PIX concentration was varied from 0.07 to 0.3 μM in the presence of 14 mM MgCl\(_2\). Hill plots used to determine \(K_{\text{m,PIX}}\) are shown for both BchS (C) and BchH (D). The PIX concentration was 0.3 μM, whereas the MgCl\(_2\) concentration was varied from 4 to 18 mM. The error bars represent the S.D. of three independent measurements.
Mg-chelatase and Mg$^{2+}$-methyltransferase from C. tepidum

which is in contrast to purple bacteria and cyanobacteria that have only single homologs of this gene. The purpose for three H-subunit paralogs remains unclear, and, previous to the current study, multiple Mg-chelatase complexes from a single species of green bacteria have not been characterized biochemically. The three (B)Chl biosynthetic pathways may be regulated, in part, by the activities of the BchH homologs, since Mg-chelatase sits at a branch point between the heme and (B)Chl pathways. Previous results have shown a direct interaction between Mg-chelatase and BchM and that the catalytic rate of BchM changes in the presence of BchH (26, 40).

Three paralogs of the large Mg-chelatase subunit (BchH, BchS, and BchT) are present in the C. tepidum genome, at least two of which are active, because inactivation of individual genes does not eliminate (B)Chl biosynthesis (3). Expression of the Mg-chelatase complexes (BchDIH, BchDIS, and BchDIT) from C. tepidum in E. coli overproducing P$^{2+}$ resulted in the accumulation of Mg$^{2+}$ for two of the three complexes (BchDIH and BchDIS). The most active Mg-chelatase complex contained BchS, resulting in ~5% of the extracted porphyrins metallated with Mg$^{2+}$. This is consistent with genetic studies of C. tepidum, which demonstrated that inactivation of BchS had the most dramatic phenotype, causing a large reduction in bacteriochlorophyll c content (3, 5). The results obtained in E. coli for the latter two complexes containing BchT and BchH contrast with expectations from the corresponding gene inactivation studies (3, 5). Although BchT is inactive in E. coli, the inactivation of BchT in C. tepidum had the second largest effect on bacteriochlorophyll c accumulation. The opposite occurred for BchH; E. coli expressing BchH produced small amounts of Mg$^{2+}$, and inactivation of BchH in C. tepidum had no effect. This difference may arise through regulatory elements not present in the E. coli host.

The in vitro rates obtained for the three Mg-chelatase complexes of C. tepidum containing different BchH paralogs correlate well with the amounts of Mg$^{2+}$ obtained in the coexpression experiments in E. coli. The reaction velocities for insertion of Mg$^{2+}$ into P$^{2+}$ measured using purified protein ranged over 5 orders of magnitude for the three Mg-chelatase complexes. The fastest Mg-chelatase complex contained BchS and had an activity ~3-fold higher than the enzyme from Synechocystis sp. PCC 6803 (18), whereas the complex containing BchH had an activity similar to the Mg-chelatase from R. sphaeroides (17), and the complex containing BchT was the slowest Mg-chelatase enzyme. The substrate affinities of the Mg-chelatase complexes

| Enzyme        | V (%) |
|---------------|------|
| BchM          | 100  ± 4 |
| BchM + BchH   | 68 ± 2 |
| BchM + BchS   | 157 ± 2 |
| BchM + BchT   | 132 ± 2 |

* Velocity is reported as a percentage of the reaction with only BchM present.

**TABLE 3**

Steady-state kinetic parameters of BchM from C. tepidum

Reaction conditions and determination of $V_{\text{max}}$ for BchM were as described under “Experimental Procedures” and in the legend to Fig. 5. $K_{\text{cat}}$ and $V_{\text{max}}$ were determined from the Lineweaver-Burk plot, and the error was determined from the S.D. of three independent experiments.

TABLE 4

BchM activity in the presence of BchH, BchS, and BchT

Reaction conditions were as described under “Experimental Procedures” with 20 $\mu$M Mg$^{2+}$ and the addition of different Mg-chelatase BchH homologs. BchH, BchS, and BchT were present in 20-fold excess; the reaction contained 0.05 $\mu$M BchM and 1 $\mu$M BchH, BchS, or BchT. The error was determined from the S.D. values of three independent experiments.

![FIGURE 5. Lineweaver-Burk plots plot used to determine $V_{\text{max}}$ and $K_{\text{cat}}$ for BchM. A, Mg$^{2+}$ concentrations were varied 0.3–30 $\mu$M in the presence of 50 nm BchM. B, P$^{2+}$ concentrations were varied 1–30 $\mu$M in the presence of 200 nm BchM. Reaction conditions were as described under “Experimental Procedures.” The error bars represent the S.E. of three independent measurements.](image-url)
are comparable with those described previously \( (K_m^{\text{PIX}} = 0.2–1.3 \mu M \text{ and } K_{Mg}^{\text{Bu}} = 3–5 \text{ mM}) \) (17, 18, 37, 41).

The observations both *in vivo* and *in vitro* that show that the slowest Mg-chelatase complex is the one that contains BchT are surprising, both because they are in contrast with the inactivation studies in *C. tepidum* and because BchT is the only bchH paralog clustered with the other Mg-chelatase subunits, bchD and bchl, on the *C. tepidum* chromosome. Moreover, BchT is 97% identical at the amino acid level to BchH from *Chlorobium vibrioforme*, which has been shown to be part of an active Mg-chelatase complex *in vitro*, although detailed kinetics have not been reported (41). A major difference between these two BchH homologs is that the protein from *C. vibrioforme* has seven amino acids at the N terminus that are missing in BchT. Inspection of the genomic sequence for the annotated bchT in *bchT* shows seven amino acids at the N terminus that are missing in BchT. The sequence is annotated correctly, and bchT does not encode these seven amino acids. The current data are insufficient to determine whether this difference, other amino acid changes, or a requirement for additional factors leads to reduced activity in *E. coli* and *in vitro*.

A simple sequence analysis also contradicts our initial expectation that BchH paralogs forming chelatase complexes with similar reaction rates have the most similar amino acid sequences. Alignment of the amino acid sequences reveals that BchT and BchS, forming the slowest and fastest chelatase complexes, share the most sequence identity. BchT is 65% and 34% identical to BchS and BchH, respectively, whereas BchS and BchH are 33% identical. Further analysis of any of the three *C. tepidum* BchH paralogs with BchH subunits from either *Synechocystis* sp. PCC 6803 or *R. sphaeroides* shows an identity of roughly 35%. More detailed sequence analysis and site-directed mutations may reveal the nature of PIX binding and residues important in catalysis.

*In vitro* characterization of the subsequent enzyme in the (B)Chl pathway of *C. tepidum*, Mg^IX^-methyltransferase BchM, showed that this enzyme is faster than the analogous enzymes from *Synechocystis* sp. PCC 6803 and *R. capsulatus* by a factor of 3.5 and 5, respectively (40, 42). The \( K_m^{\text{PIX}} \) for *C. tepidum* and *R. capsulatus* are comparable, and the \( K_M^{\text{Bu}} \) for *Synechocystis* sp. PCC 6803 is slightly higher (43). BchM from *C. tepidum* shares with the *R. capsulatus* enzyme the tendency to form high molecular weight aggregates, whereas ChlM from *Synechocystis* exists as a monomer in solution (40).

The substrate specificity for BchM from *C. tepidum*, however, is different from Mg^IX^-methyltransferases characterized from *Synechocystis* sp. PCC 6803 and *R. capsulatus*. Sawicki et al. (40) found that the presence of both the vinyl group and a complexed metal in the porphyrin structure is required for activity of the methyltransferase from *R. capsulatus*. In our *in vivo* experiments in *E. coli* and kinetic assays, the BchM from *C. tepidum* was able to transfer a methyl to \( ^{\text{PIX}} \) without bound metal. *In vitro*, the reaction occurred more slowly relative to reactions containing Mg^PIX^, and the \( K_m^{\text{PIX}} \) for Mg^PIX^ increased by ~100-fold compared with the \( K_m^{\text{Bu}} \) for Mg^Bu^IX^-ME. These results are similar to previous experiments on BchM from *R. sphaeroides* (13). None of the Mg-chelatase complexes from *C. tepidum* were able to insert Mg\(^{2+}\) into \( ^{\text{PIX}} \)-ME, and the formation of \( ^{\text{PIX}} \)-ME appears to be a dead end product in (B)Chl biosynthesis.

In *E. coli* expressing Mg-chelatase and BchM from *C. tepidum*, Mg^PIX^ is transformed to a much greater extent by BchM than by the Mg-chelatase, and, consequently, the \( ^{\text{PIX}} \)-pool available for Mg\(^{2+}\) chelation is decreased.

We do not know whether this activity of BchM is of physiological significance in *C. tepidum*, although there is no evidence in the literature for the presence of \( ^{\text{PIX}} \)-ME in cultures of *C. tepidum*. There are several possibilities that may explain the absence of \( ^{\text{PIX}} \)-ME in *C. tepidum*: 1) the steady-state concentration of \( ^{\text{PIX}} \) in *C. tepidum* may be maintained at a low level, which may limit the formation of \( ^{\text{PIX}} \)-ME; 2) the Mg-chelatase activity in *C. tepidum* may be much higher than the BchM activity, increasing the concentration of Mg^PIX^ relative to \( ^{\text{PIX}} \) and limiting the formation of \( ^{\text{PIX}} \)-ME; and 3) substrate channeling between the Mg-chelatase and BchM may occur in order to increase the formation of Mg^PIX^ME relative to \( ^{\text{PIX}} \)-ME.

Interactions observed between BchM and the large Mg-chelatase BchH subunit suggest a direct mechanism for regulation of (B)Chl biosynthesis. The *Synechocystis* ChlM activity increases by a factor of 10 in steady-state kinetic measurements in the presence of a 15-fold excess of ChlH (26). The opposite occurs for the *R. capsulatus* BchM in which the rate is decreased slightly in the presence of BchH (40). This raises the possibility that BchH may have different roles in (B)Chl regulation that depend on the specific organism.

The three BchH paralogs of *C. tepidum* affect the activity of BchM, and although there is less than a 2-fold change *in vitro*, the effect of each of the subunits is distinct. Two of the paralogs (BchT and BchS) cause an increase in the activity of BchM, and the third (BchH) causes a slight decrease in activity (Table 4).

The effects of the three BchH paralogs on the BchM rate correlate with the apparent binding affinities for Mg^PIX^. BchH binds metallated porphyrin more strongly than either of the other BchH paralogs (see Fig. 2) and reduces BchM activity. The stronger binding of Mg^PIX^ may decrease the release of Mg^PIX^ from the Mg-chelatase and limit the amount of substrate available to BchM and slow the reaction. On the other hand, BchS appears to have a stronger interaction with Mg^PIX^ than with metallated Mg^PIX^ and may release Mg^PIX^ more readily for subsequent action by BchM.

The mechanism for the change in Mg^PIX^-methyltransferase activity induced by BchH, BchS, and BchT is not understood. It is possible that the BchH homologs 1) accelerate the formation and decay of a catalytic intermediate and/or 2) orient Mg^PIX^ to BchM, which accelerates activity via substrate channeling. In evidence of the first possibility, transient kinetics on the formation and decay of a catalytic intermediate have been measured for the *Synechocystis* ChlM (26, 43). In the presence of ChlH, a significant increase in the formation and decay of the catalytic intermediate for ChlM was observed, although the rate-limiting step remains the decay of the intermediate.

Substrate channeling has been characterized in several systems using competition experiments with an inactive enzyme (44, 45). These experiments depend on a mutation that disrupts the catalytic activity of the enzyme but not its interaction with its channeling partner. So far, no structure of BchH is available for the design of a suitable mutant enzyme for substrate chan-
Mg-chelatase and Mgp^{IX}-methyltransferase from C. tepidum

neling experiments. Initial structural characterization of BchH by electron microscopy suggests that the N and C termini of BchH undergo a conformational change and clamp together to bind porphyrins (36). Further sequence analysis and structural characterization of the three BchH subunits may therefore herald new opportunities for studying substrate channeling interactions in the (B)Chl biosynthetic pathway.

In conclusion, we report on the activities of Mg-chelatase complexes and the Mgp^{IX} methyltransferase of C. tepidum. Our results point toward a more complex regulation of (B)Chl biosynthesis in C. tepidum than in organisms containing only a single homolog of the Mg-chelatase large subunit. The conflicts between the gene inactivation studies and our in vitro and in vivo Mg-chelatase activities together with the unusual activity of BchM with P^{IX} highlight the variety of interactions that can occur during (B)Chl biosynthesis. Moreover, although BchT appears to be almost inactive in vitro, it does enhance the activity of BchM and has a significant effect on bacteriochlorophyll c production in C. tepidum. Although BchM had no affect on the activity of any of the three Mg-chelatase complexes, other enzymes directly or indirectly involved in (B)Chl biosynthesis may interact with the complexes (e.g. to increase the activity of the BchT-containing complex). Additional studies with subsequent enzymes of the (B)Chl pathway in C. tepidum and structural studies of the Mg-chelatase complex will be necessary to unravel the control mechanisms of this pathway.

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