The metabolic pathway known as (bacterio)chlorophyll biosynthesis is initiated by magnesium chelatase (BchI, BchD, BchH). This first step involves insertion of magnesium into protoporphyrin IX (proto), a process requiring ATP hydrolysis. Structural information shows that the Bchl and BchD subunits form a double hexameric enzyme complex, whereas BchH binds proto and can be purified as BchH-proto. We utilized the *Rhodobacter capsulatus* magnesium chelatase subunits using continuous magnesium chelatase assays and treated the BchD subunit as the enzyme with both Bchl and BchH-proto as substrates. Michaelis–Menten kinetics was observed with the BchI subunit, whereas the BchH subunit exhibited sigmoidal kinetics (Hill coefficient of 1.85). The Bchl-BchD complex had intrinsic ATPase activity, and addition of BchH greatly increased ATPase activity. This was concentration-dependent and gave sigmoidal kinetics, indicating there is more than one binding site for the BchH subunit on the BchI-BchD complex. ATPase activity was ~40-fold higher than magnesium chelatase activity and continued despite cessation of magnesium chelation, implying one or more secondary roles for ATP hydrolysis and possibly an as yet unknown switch required to terminate ATPase activity. One of the secondary roles for BchH-stimulated ATP hydrolysis by a Bchl-BchD complex is priming of BchH to facilitate correct binding of proto to BchH in a form capable of participating in magnesium chelation. This porphyrin binding is the rate-limiting step in catalysis. These data suggest that ATP hydrolysis by the Bchl-BchD complex causes a series of conformational changes in BchH to effect substrate binding, magnesium chelation, and product release.

Magnesium chelatase (EC 6.6.1.1) is an AAA+ protein (1, 2) composed of three subunits called BchI/ChlI, BchD/ChlD, and BchH/ChlH (40, 70, and 140 kDa, respectively) and is involved in the first step of (bacterio)chlorophyll biosynthesis (3). For optimal activity, the plant/algal systems also require a fourth protein, Gun4, which binds protoporphyrin and magnesium-protoporphyrin (4–6); however, no Gun4 homolog has been identified in photosynthetic bacteria. It has been recently suggested that BchI, thus far identified only in organisms lacking Gun4, may act as a magnesium-porphyrin chaperone *in vivo* in bacteriochlorophyll biosynthetic systems (7, 8). Magnesium chelatase appears to exist as a complex of the three aforementioned subunits and works as an ATP-dependent molecular machine (3, 9, 10). The current reaction model of magnesium chelation into the red-colored tetrapyrole substrate, protoporphyrin IX (proto), is summarized in a recent review of chlorophyll biosynthesis (11) and has been constructed through a combination of biochemical and structural data. The model was initially built from biochemical studies using plant magnesium chelatases, pea and cucumber (12, 13); and photosynthetic bacterial magnesium chelatases, *Rhodobacter capsulatus* (14), *Rhodobacter sphaeroides* (15, 16), and *Synechocystis* (17). The enzyme from the latter three organisms was purified after heterologous expression of the subunits in *Escherichia coli*. Thus far, the most detailed magnesium chelatase and ATPase kinetic studies were performed with *Synechocystis* enzyme (17–20), whereas the majority of recent structural information is from *R. capsulatus* using crystallization and electron microscopy (EM) analysis of individual subunits (21–25).

Magnesium chelation is best understood by looking at the roles of the individual enzyme subunits of the complex. The Bchl subunit from *R. capsulatus* forms a hexamer upon ATP hydrolysis, and its crystal structure is known (22, 25). Hexameric Bchl particles were confirmed by EM (24), whereas a heptamer was observed with the BchI homolog from *Synechocystis* (26). Only the Bchl subunit has a conserved ATPase domain (3), and ATPase activity of this subunit has been confirmed (27–29), indicating this subunit is responsible for the ATP-catalyzed insertion of magnesium into proto. Studies from *Synechocystis* have shown that ~15 cycles of ATP hydrolysis are required to insert one magnesium into deuteroporphyrin, the more water-soluble analog of proto (19).

The BchD subunit has strong homology to the Bchl subunit at the N terminus known as the AAA domain (22). This subunit also appears to form hexamers observed with EM; however, this oligomerization does not require ATP (21). Biochemical data first demonstrated the likely formation of a Bchl-BchD complex because the lag phase of the magnesium chelatase reaction was overcome by premixing of the Bchl and BchD subunits in the presence of magnesium and ATP (17). Also the BchD subunit requires the Bchl subunit in the refolding process to stay solu-

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2 The abbreviations used are: proto, protoporphyrin IX; EM, electron microscopy; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; Mg-proto, magnesium-protoporphyrin IX.
ble in *R. capsulatus*, although the expected double hexameric (~660 kDa) complex was never observed (14). Instead, an ~200-kDa complex has been observed in *R. sphaeroides* using size-exclusion chromatography (15). It has been shown that the BchD subunit interacts directly with the Bchl subunit (15, 28, 30–32) and that magnesium chelatase activity requires the Bchl subunit in excess of BchD (14, 15, 17). These results have led to the proposal that the Bchl subunit may act as a chaperone for BchD (31). The lack of ATPase activity of the BchD subunit (27–29) and the absence of the required amino acid in the ATPase domain sites on the BchD subunit (22) prompted the suggestion that it could have a role as a platform for binding the Bchl subunit (21). EM reconstruction of the Bchl-BchD complex has been recently modeled to a double hexamer composed of a trimer of homodimers (33). Thus far, a Bchl-BchD-BchH-proto complex has not been isolated, and it has been presumed to exist transiently because only weak interactions were observed with the BchD and BchH subunits (30).

The recombinantly produced *R. capsulatus* Bchl subunit sequesters proto readily. For optimal magnesium chelatase activity, both the BchH and Bchl subunits appear to be required in slight excess over the BchD subunit (17) presumably to form a stable Bchl-BchD complex that forms in a 1:1 molar ratio (33). Both Bchl and BchH subunits behave kinetically as substrates when the BchD subunit is treated as the enzyme (3). However, the *K*~m~ of the BchH subunit is ~2–3 times that of the Bchl subunit, indicative of a weaker association of BchH with BchD compared with Bchl (17). The structure of the BchH subunit has also been recently investigated by EM, revealing a large conformational change upon binding of the proto substrate (23).

We sought to complete the kinetic characterization of magnesium chelatase activity from *R. capsulatus* using purified subunits and to examine these results based on the current reaction model. These experiments were based primarily upon continuous assays. Our data provide more accurate initial rates than previous results obtained from stopped assays (14). The results provide kinetic profiles of the Bchl, BchD, and BchH subunits and highlight some similarities to and differences from previous reports on *R. sphaeroides* and *Synechocystis* (15, 17). These new findings shed new light in terms of the currently proposed reaction model, regulation of enzyme activity, stability of the complex, and the roles of ATP hydrolysis in the reaction cycle of the complex.

**EXPERIMENTAL PROCEDURES**

Materials—All chemicals were from Sigma, Astral Scientific, and Ajax Finechem unless stated otherwise.

Protein Determination—The protein concentrations of Bchl and His$_\text{sub}$-tagged BchD were determined using the Bradford method (34). The His$_\text{sub}$-tagged BchD concentration was determined using a molar extinction coefficient of ε$_{280}$ = 118,600 M$^{-1}$ cm$^{-1}$ and a molecular mass of 132,000 g/mol. All errors and error bars are shown as means ± S.E. unless stated otherwise.

Bchl:Proto Ratio—The proto concentration bound to the Bchl subunit was determined by adding an aliquot of purified Bchl to an excess of 80% (v/v) acetone and 20% (v/v) 1 M ammonia (acetone/ammonia) and immediately vortexing. The solution was centrifuged at 18,000 × g for 5 min at room temperature, and the supernatant was used to measure the amount of proto present using a PerkinElmer Life Sciences LS 55 luminescence spectrometer and referring to a standard curve at Ex$_{418}$ nm and Em$_{630}$ nm. The BchH used in the experiments had a protein:proto ratio of 1.24–3.26:1.

Porphyrin Solutions—Stock solutions of proto and Mg-proto were prepared as described previously (35).

Protein Expression and Purification—Bchl and His$_\text{sub}$-tagged BchD were purified as described (14, 36) with the modifications that a Superose 6 column was used for the final purification step of Bchl, and 1% (v/v) Triton X-100 was used to wash the inclusion bodies of expressed BchD. His$_\text{sub}$-tagged BchD was purified according to Willows and Beale (14) by Ni$^{2+}$ affinity chromatography with a final wash step of 40 mM imidazole. Bchl was then desalted into 5 mM Tricine-NaOH (pH 8.0) and concentrated (30-kDa cutoff membrane, Millipore) before further purification by anion-exchange chromatography using Source 15Q resin (GE Healthcare). A 10/100 column with a 6-ml bed volume was used at a flow rate of 3 ml/min and a linear gradient from 0 to 75% buffer (5 mM Tricine-NaOH (pH 8.0) with 1 M NaCl) over 20 column volumes with 1.5-ml fractions collected over this range. The red-colored fractions containing purified Bchl eluted between ~51 and 60 ml (~0.32–0.37 M NaCl), and these samples were analyzed by SDS-PAGE before the purest fractions were pooled.

Magnesium Chelatase Assays—All assays were performed according to Axelson et al. (21) with the following modifications. The final concentrations were 50 mM Tricine-NaOH (pH 8.0), 21 mM glycerol, 15 mM MgCl$_2$, 3.26 mM urea, 2 mM dithiothreitol (DTT), 1 mM ATP (assay buffer), 367 nM Bchl-proto, 263 nM BchH, and 31.7 nM BchD unless stated otherwise in the figure or table legends. Approximately 0.5–μl aliquots of BchD in 6 μM urea buffer (50 mM Tricine-NaOH (pH 8.0), 15 mM MgCl$_2$, and 4 mM DTT) were refolded with rapid addition of 700 μM of variable concentrations of Bchl (at least 0.5:1 Bchl: BchD) on ice giving 63.3 nM BchD and incubated for 2 h. Assays were started by addition of 100 μM of Bchl-BchD and 100 μl of BchH. For continuous assays, fluorescence readings using a gain of 20 were taken immediately with readings every 4–9 s, and the initial rates measured. The standard curve for continuous assays involved reacting Bchl-BchD-BchH-proto with 100 μM Tween 80 and different BchH-proto concentrations until a base-line rate was reached. A linear amount of Bchl-BchD-BchH-Mg-proto was made corresponding to a starting BchH-proto concentration of between 3.8 and 120 nM. The assay with 120 nM was stopped with acetone/ammonia and separated by high pressure liquid chromatography (5–67% acetoni-trile over 15 min) using a previous method (35). The conversion to Bchl-BchD-BchH-Mg-proto was estimated at 87% using various mixtures of proto/Mg-proto concentrations as standards. This was taken into account when the final Mg-proto standard curve was constructed over the various porphyrin concentrations. Stopped assays involved taking a 150-μl sample and adding to 150 or 200 μl of acetone/ammonia. After centrifugation at 18,000 × g for 5 min at room temperature, 250 μl of the supernatant was used for spectrofluorometry (Ex$_{418}$ nm and Em$_{630}$ nm, for proto) with comparison to appropriate standard curves. Unless men-
Magnesium Chelatase Reaction

TABLE 1
Kinetic constants of magnesium chelatase substrates Mg²⁺ and ATP

These values were generated from supplemental Fig. 1. The apparent $K_m$ values for ATP and magnesium were determined with free Mg²⁺ referring to magnesium not forming a complex with ATP. The concentration of free magnesium (Mg²⁺) was determined with WebMaxClite Version 1.15 software at an ionic strength of 0.05 and 30 °C. Both data sets were fitted to the Michaelis-Menten equation.

| Substrate | Apparent $V_{max}$ (nmol Mg-proto/min/nmol BchD) | Apparent $K_m$ (mM) |
|-----------|-----------------------------------------------|-------------------|
| Free Mg²⁺ | 5.1 ± 0.1                                      | 1.3 ± 0.1         |
| ATP       | 4.5 ± 0.1                                      | 0.091 ± 0.009    |

For determining the kinetic parameters, either the Michaelis-Menten equation ($v = V_{max}*[S]/(K_m + [S])$) or the Hill equation ($v = V_{max}*[S]^n/(k + [S]^n)$) was used for nonlinear regression analysis in GraphPad Prism Version 5.01 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Kinetic Properties of Magnesium Chelatase Substrates Mg²⁺ and ATP—Magnesium is required as a cofactor for the magnesium chelatase assay, and an excess was used in all assays performed. The fits of either variable free magnesium (Mg²⁺) with fixed ATP or variable ATP with fixed Mg²⁺ were hyperbolic with the apparent $K_m$ for Mg²⁺ being 14-fold higher than that for ATP (Table 1). The amounts of Bchl, BchD, and BchH subunits used in these assays were close to optimal (see later experiments).

Proto in the Assay—Two separate BchH-proto preparations were used with different amounts of proto bound. The BchH subunit binds proto maximally in a 1:1 molar ratio (16), and the first BchH used contained a 1.26:1 BchH:proto ratio and is termed near-optimal BchH, whereas the second had a 3.26:1 BchH:proto ratio and is termed proto-depleted BchH. Initial rates were 1.47 and 0.78 nmol of Mg-proto/min/nmol of BchD for near-optimal and proto-depleted BchH, respectively, and there was some inhibition as the proto was titrated in both cases (Fig. 1A). However, despite this slight inhibition, the addition of proto to the BchH-proto stock.

FIGURE 1. Effect of exogenous proto on magnesium chelatase activity. Assays were performed with two different BchH stocks, near-optimal (1.26:1) and proto-depleted (3.26:1) BchH-proto. The total proto refers to proto bound to the BchH subunit together with exogenous proto added. Total proto concentrations of 60 and 23 nM refer to proto bound to near-optimal and proto-depleted BchH, respectively, with no exogenous proto added. Assays were performed using 31.6 nM BchI, 15.8 nM BchD, and 75.6 nM BchH.

Error bars indicate the means ± S.D. of 1.26:1 BchH:proto stock; 3.26:1 BchH:proto stock. B, time course following product formation with only three total proto concentrations for each BchH stock shown for clarity: 1.26:1 BchH: proto, 60 nM (○), 110 nM (□), and 560 nM (△); and 3.26:1 BchH: proto, 23 nM (■), 110 nM (▲), and 510 nM (▲). C, secondary rates determined from 3 to 4.95 min (B).

Expression and Purification of BchI, BchD, and BchH—BchI, BchD, and BchH were each expressed in E. coli and purified as described (15). Purification of BchH was simplified by reducing the concentration of DTT from 1 to 0.5 mM, followed by refolding in the absence of DTT and inhibitors, NADPH, and ATP, and eluted with freshly prepared buffer containing 50 mM Tricine-NaOH (pH 8.0), 15 mM MgCl₂, 10 mM DTT, and 2 mM ATP. The BchH-proto stock was prepared as described previously (37, 38), followed by incubation for 5 min with shaking (1200 rpm) at 20 °C, and the absorbance was read at 620 nm. A standard curve with variable concentrations of Na₂HPO₄ was performed at the same time.

For determining the kinetic parameters, either the Michaelis-Menten equation ($v = V_{max}*[S]/(K_m + [S])$) or the Hill equation ($v = V_{max}*[S]^n/(k + [S]^n)$) was used for nonlinear regression analysis in GraphPad Prism Version 5.01 for Windows (GraphPad Software, San Diego, CA).
dional proto was able to participate in the magnesium chelation, as a secondary rate was observed (Fig. 1B). This secondary rate, from 3 to 4.95 min, was used to determine a pseudo-$K_m$ and apparent $V_{\text{max}}$ for proto (Fig. 1C) with both near-optimal and proto-depleted BchH. Both data sets were the same ($p < 0.05$) with a pseudo-$K_m$ of 57 nm and an apparent $V_{\text{max}}$ of 0.057 ± 0.002 nmol of Mg-proto/min/nmol of BchD. As this apparent $V_{\text{max}}$ is the same for both BchH samples but is 14–26-fold lower than the initial rates, the rate-limiting step for the reaction appears to be proto binding to regenerate a functional BchH-proto substrate for the reaction.

BchH as a Substrate—Subsequent experiments utilized BchH-proto as the sole porphyrin substrate without the addition of exogenous proto, and its fate was tested in the magnesium chelatase reaction. Near-optimal and proto-depleted BchH were tested with variable concentrations, and data were analyzed by considering total BchH (BchH containing bound proto as well as BchH with no proto bound) and the remaining BchH subunit both with and without proto bound. A similar trend was seen using the stopped assay with apparent $S_{0.5}$ values of 656 ± 60 and 276 ± 35 nm for the proto-depleted and near-optimal BchH subunits, respectively (Fig. 2B). The difference in apparent $S_{0.5}$ between proto-depleted and near-optimal BchH suggested that the BchH subunit with no proto bound was not participating in the reaction. To test this, it was assumed that only BchH with proto bound (BchH-proto) was contributing to the reaction, and the data were reanalyzed based on the proto bound to BchH (Fig. 2, C and D). This reanalysis showed that only BchH with proto bound was participating in the reaction and that BchH without proto bound did not interfere with the reaction as shown by the identical sigmoidal curves (Fig. 2C). This was also true of the reanalysis of the stopped assays (Fig. 2, compare B and D). Therefore, BchH without proto bound did not participate and did not apparently adversely affect the magnesium chelation reaction. The remaining experiments considered only BchH-proto as a substrate when determining kinetic constants for the magnesium insertion reaction, and those experiments were performed with near-optimal BchH.

The presence of the reacted BchH subunit (BchH-Mg-proto) was then tested for its effect upon a secondary addition of fresh unreacted BchH-proto. As shown in Fig. 3A, the initial magnesium chelatase reaction using 6.1 pmol of BchH-proto was allowed to continue to stationary phase before secondary additions of 3.1, 6.1, or 12.3 pmol of BchH-proto. The initial rates of chelation were 0.52–0.63 nmol of Mg-proto/min/nmol of BchD (Fig. 3B, white bars). A secondary addition of 6.1 pmol of BchH-proto gave essentially the same velocity as the initial rate (0.55 ± 0.06 nmol of Mg-proto/min/nmol of BchD) as shown in Fig. 3B (6.1 black bar). Addition of either 12.3 or 3.1 pmol of BchH-proto resulted in the expected higher and lower rates, respectively, compared with the initial rate. The porphyrins extracted following secondary additions also showed an expected increase in Mg-proto levels (Fig. 3C). These results show there is no inhibition by BchH-Mg-proto in the magnesium chelation reaction, suggesting that BchH-Mg-proto does not compete with BchH-proto in binding to the BchI-BchD complex.

Assembly of the BchI-BchD Complex—The assembly of the BchI-BchD complex was investigated by varying the concentrations of the BchI and BchD subunits in the magnesium chelatase assay. This is shown in Fig. 4A with a variety of curves at different BchD concentrations fit to the Michaelis-Menten equation to generate apparent $V_{\text{max}}$ values. A linear relationship was found as the BchD concentration was increased up to 31.7 nm (Fig. 4B), therefore, BchD was treated as the "enzyme"
in all analyses, and activities are given as per nmol of BchD subunit in all other experiments. Two substrate kinetic analyses assuming BchI and BchH-proto as the two substrates were conducted at fixed BchD concentrations (Fig. 5). A sigmoidal relationship was clearly observed with BchH as the substrate (Fig. 5A), indicating cooperativity at all BchI concentrations. The secondary plot of the apparent V\textsubscript{max} against BchI concentration (Fig. 5B) was clearly hyperbolic with a K\textsubscript{m} for BchI of 20.2 ± 3.2 nM and V\textsubscript{max} of 5.1 ± 0.3 nmol of Mg-proto/min/nmol of BchD.

ATPase Activity of the BchI-BchD Complex—The BchI subunit contains low intrinsic ATPase activity. The ATPase activity of BchI-BchD was 1.66-fold higher compared with that of BchI, and both exhibited a linear response over the concentrations tested (Fig. 6). Negligible ATPase activity was detected from BchH-proto (supplemental Fig. 2) (39). Addition of BchH-proto to produce a complete magnesium chelatase (BchI-BchD-BchH-proto) complex caused an ∼33-fold increase in the rate of ATPase activity of the BchI-BchD complex (Table C).

FIGURE 3. Effect of secondary addition of BchH-proto on rates of magnesium insertion into proto. All magnesium chelatase initial assays contained 63.2 nM BchI and 30.6 nM BchH-proto (6.1 pmol). Secondary additions (5 μl) involved either assay buffer (as indicated under “Experimental Procedures”) or BchH-proto with half (3.1 pmol), equal (6.1 pmol), or double (12.3 pmol) the amount of BchH-proto used in the initial assay. Velocities are given as V (nmol of Mg-proto/min/nmol of BchD) ± S.D. A, time course monitoring of Mg-proto made (pmol over time). Left, initial assay; right, secondary additions at 9 min. ○, no secondary addition; □, assay buffer; △, 3.1 pmol of BchH-proto; ■, 6.1 pmol of BchH-proto; ●, 12.3 pmol of BchH-proto. B, comparison of initial rates with no secondary addition (—) and secondary additions of the BchH-proto subunit. Error bars represent the means ± S.D. White bars, initial velocities of the initial assay; black bars, secondary addition of either BchH-proto or assay buffer. Secondary additions of BchH-proto or assay buffer were stopped using acetone/ammonia at 12.3 min. C, extracted porphyrin amounts using acetone/ammonia after 9 min (—) or after the secondary assay buffer or BchH-proto additions at 12.3 min. White bars, Mg-proto levels extracted; black bars, proto levels extracted.

FIGURE 4. Kinetic properties of the BchD subunit in magnesium chelatase assays. Variable concentrations of BchI (13.2–329 nM) were used at fixed BchD concentrations (1.58 nM (E), 3.16 nM ( ), 6.33 nM ( ), 12.7 nM ( ), 25.3 nM ( ), and 31.7 nM ( )) and the BchI:BchD ratios were at least 0.5:1. A, the Michaelis-Menten equation was fitted to the data to generate apparent V\textsubscript{max} values for the given BchD concentrations. Error bars represent the means ± S.D. B, apparent V\textsubscript{max} values generated in A are plotted against the fixed BchD concentrations, and linear regression was applied and fitted through zero.
The differences in apparent $V_{\text{max}}$ for magnesium chelatase activity using the stopped and continuous assays, respectively. However, the rate of magnesium insertion was only linear for $30\text{ min}$ and continued after magnesium insertion had ceased (supplemental Fig. 2, A–C). Two different types of magnesium chelatase assays (continuous and stopped) were used with variable BchI-proto concentrations (15–481 nM). Hill equations were applied to each experiment, giving apparent $V_{\text{max}}$ values (nmol/min/nmol of BchD or BchI), with the resulting apparent $S_{0.5}$ values referring to BchH-proto.

**Summary of kinetic constants for the magnesium chelatase protein substrate BchI and BchH-proto**

These values were generated from Figs. 5B and 7B, with $V_{\text{max}}$ given as nmol of Mg-proto/min/nmol of BchD and $K_{\text{cat}}$ given as min$^{-1}$.

| Substrate | $V_{\text{max}}$ or $K_{\text{cat}}$ | $K_m$ | Hill coefficient | $S_{0.5}$ | $K_{\text{cat}}/(K_m$ or $S_{0.5}$) |
|-----------|----------------------------------|-------|------------------|-----------|----------------------------------|
| BchI      | 5.1 ± 0.3                         | 20.2 ± 3.2 | 2.00 ± 0.06     | 132 ± 20  | 252                              |
| BchH-proto| 5.4 ± 0.1                         | 1.85 ± 0.06 | 132 ± 20  | 41        |

**TABLE 2**

Comparison of the ATPase and magnesium chelatase activities of the BchI:BchD:BchH-proto complex

All values were determined from supplemental Fig. 2 (A–C) with the same final concentrations for each assay with limiting Bchl to allow comparison of ATPase and magnesium chelatase activities. Two different types of magnesium chelatase assays (continuous and stopped) were used with variable BchI-proto concentrations (15–481 nM). Hill equations were applied to each experiment, giving apparent $V_{\text{max}}$ values (nmol/min/nmol of BchD or BchI), with the resulting apparent $S_{0.5}$ values referring to BchH-proto.

| Substrate                  | $S_{0.5}$ | $V_{\text{max}}$ | Hill coefficient |
|---------------------------|-----------|------------------|------------------|
| ATPase                    | 119 ± 48  | 128 ± 6          | 2.27 ± 0.27      |
| Magnesium chelatase (continuous) | 61 ± 12  | 2.71 ± 0.05      | 2.00 ± 0.11      |
| Magnesium chelatase (stopped) | 118 ± 45 | 3.10 ± 0.10      | 1.29 ± 0.15      |

2). The ATPase activity of BchI:BchD:BchH-proto was expressed per nmol of Bchl subunit because this is the only magnesium chelatase subunit with ATPase activity; however, a 1:1 Bchl:BchD ratio was used in these experiments, and hence, ATPase activity is directly comparable with magnesium chelatase activity. The apparent $V_{\text{max}}$ of ATPase activity was 41- and 47-fold higher compared with the corresponding apparent $V_{\text{max}}$ for magnesium chelatase activity using the stopped and continuous assays, respectively. However, the rate of magnesium insertion was only linear for $30\text{ s}$, whereas the rate of ATP hydrolysis was linear for $>30\text{ min}$ and continued after magnesium insertion had ceased (supplemental Fig. 2, D and E). The differences in apparent $V_{\text{max}}$ in Table 2 compared with Table 3 are due to suboptimal Bchl concentrations used to generate Table 2 data to allow for ATPase measurements with a minimum background of ATPase activity from the Bchl subunit.

**Cooperativity of the BchH-Proto Subunit—**Two substrate kinetic analyses with BchI and BchH-proto treated as the substrates were performed (Fig. 7A). A sigmoidal plot was obtained when the apparent $V_{\text{max}}$ values for BchI were plotted against BchH-proto concentrations (Fig. 7B) and gave a Hill coefficient of 1.85 and an $S_{0.5}$ of 132 ± 20 nM. This sigmoidal curve was obtained when measuring both magnesium chelatation activity and ATPase activity and resulted in similar Hill coefficients: 2.0 and 2.3 (Table 2). However, the apparent $S_{0.5}$ value was 2-fold higher for the ATPase activity compared with the magnesium chelatase activity in a continuous assay (Table 2). The apparent $S_{0.5}$ and Hill constants obtained with stopped assays were significantly different from those obtained with continuous assays, with stopped assays approaching hyperbolic curves. This was also observed in previous experiments (Fig. 2). The reason for

**FIGURE 5.** Kinetic properties of the Bchl subunit in magnesium chelatase assays. Variable concentrations of BchH-proto (11.5–489 nM) were used at fixed combinations of Bchl (3.16–202 nM) and BchD (3.16–25.3 nM). Glycerol concentrations were from 15.5 to 21 mM. Data were fitted to the data to generate apparent $V_{\text{max}}$ values for the given Bchl concentrations. Error bars represent the means ± S.D. Only 7 of 16 of the fixed data sets are shown for clarity: 3.16 nm BchD and 3.16 nm Bchl (□), 3.16 nm BchD and 12.6 nm Bchl ( ), 6.3 nm BchD and 25.3 nm Bchl (△), 12.6 nm BchD and 12.6 nm Bchl ( ), 12.6 nm BchD and 101 nm Bchl (■), 25.3 nm BchD and 25.3 nm Bchl (▲), and 25.3 nm BchD and 202 nm Bchl ( ). Apparent $V_{\text{max}}$ values generated in A are plotted against the fixed concentrations of Bchl with the Michaelis-Menten equation fitted to the data. The four variable BchD concentrations are plotted on the same graph: 3.16 nm ( ), 6.3 nm ( ), 12.6 nm (△), and 25.3 nm ( ).
the differences between the stopped and continuous assays appears to be due to loss of magnesium from Mg-proto when stopping the assay. This loss of magnesium from Mg-proto was reduced or absent when the assay was stopped after magnesium chelation had apparently ceased, suggesting that the chelation reaction proceeds through an intermediate labile form of Mg-proto.

In summary, the kinetic properties of the Bchl and BchH-proto protein subunits for the BchD enzyme are quite different (Table 3). The Bchl subunit has a $K_m$ of 20 nM and does not display cooperativity, whereas the BchH-proto subunit has an $S_{0.5}$ of 132 nM and shows cooperativity. Thus, the Bchl subunit forms a tight association with BchD to form a Bchl:BchD complex, with BchH forming a transient substrate-like association with the Bchl:BchD complex. This is consistent with previous data from *Synechocystis*, for which the $K_m$ values for the Bchl and BchH subunits were 85–107 and 200–260 nM, respectively.

DISCUSSION

The BchH subunit of magnesium chelatase from *R. capsulatus* bound the porphyrin substrate, proto, very tightly such that it was difficult to remove without loss of enzyme activity. This differs from the *Synechocystis* homolog ChlH, whereupon the proto is lost during anion-exchange chromatography (17). Therefore, in our experiments, the BchH-proto subunit effectively acted as the porphyrin substrate for the magnesium chelatase reaction, and activity could be measured without addition of proto. In our experiments, proto remained tightly bound to the BchH subunit even after extensive washing with detergent during purification. Therefore, we could not determine a real $K_m$ value for proto using normal steady-state kinetics of the initial rate. However, we found that addition of exogenous proto resulted in a continued increase in Mg-proto made compared with no additional proto. This allowed us to calculate a pseudo-$K_m$ for proto using the continued increase in rates with addition of variable proto (Fig. 1C) at a fixed BchH concentration. The effect of proto addition was tested on two BchH preparations, near-optimal and proto-depleted BchH. The pseudo-$K_m$ for proto of 47 nM with either near-optimal or proto-depleted BchH is significantly lower than previous results obtained with *R. sphaeroides* (150 nM) (15) and *Synechocystis* and *R. capsulatus* (1250 and 1230 nM, respectively) (14, 17). This pseudo-$K_m$ for proto approaches the $K_m$ values reported for chloroplast preparations from pea leaves and cucumber: 13.5 and 25 nM, respectively (12, 13).

These data suggest a role for Bchl:BchD in facilitating binding of porphyrin as shown in Scheme 1 (step 1). Mg-proto usually remains bound to the BchH subunit following chelation (23), but we observed an additional amount of Mg-proto made with exogenous proto. This suggests that Mg-proto normally stays bound to BchH unless exogenous proto is present, with which it can be exchanged. Although proto does appear to bind to proto-depleted BchH when mixed and preincubated (14), it is not in a state that can immediately participate in magnesium chelation (see Fig. 1 and previous results). This implies that BchH* shown in Scheme 1 is a transient porphyrin-binding intermediate form of BchH. The binding of proto to BchH* appears to be the rate-limiting step in magnesium chelation because the secondary rate of catalysis has a 14- and 26-fold lower rate than the initial rates of the proto-depleted and near-optimal BchH samples tested (Fig. 1, A and C). This loading of...
extract the porphyrin.

This suggests that there is an intermediate bound form of Mg-
proto during extraction rather than to any fluorescent artifact.

The stopped assay gave lower initial rates and a different
kinetic profile compared with the continuous assay (Fig. 3,
(15)). This appears to be due to loss of magnesium from the
proto during extraction rather than to any fluorescent artifact.

The Mg-proto released does not have a large inhibitory
impact on magnesium chelation, with only 16% inhibition
at 71 nm Mg-proto. The rate of binding of proto to BchH may be
higher in vivo if it is facilitated by a bacterial equivalent to the
plant protein Gun4, which acts like a proto chaperone (4–6),
and a candidate for this has been suggested to be BchJ (7, 8).

Interestingly, the ATPase activity of BchI-BchD-Mg-proto con-
tinues at a similar rate after the magnesium chelation has
stopped, suggesting that the trigger for ATP hydrolysis is sim-
ply binding of Mg-proto to BchH. From a regulatory
point of view, a second molecule/protein may be required
in vivo to halt the BchI-BchD ATPase activity cycle once mag-
nesium has been inserted, for example, by binding to BchD and
stopping its interaction with BchI-BchD. One possibility for this
is BchM/ChlM, the next enzyme in the biosynthetic pathway,
because interactions between BchM/ChlM and BchH have
been reported (41–44).

Assembly of the BchI-BchD Complex—A previous report sug-
gested that the BchD subunit acts as a platform for binding of
the BchI and BchH subunits to form the complete functional
magnesium chelatase complex (21). This type of arrange-
ment suggests that the BchD subunit is acting as an enzyme with
the BchI and BchH subunits behaving as substrates, and this was
also seen in a previous study with Synechocystis (17). In our
study, the behavior of BchD was tested by keeping it at a fixed
concentration while varying the BchI concentration. A linear
response was obtained when plotting apparent $V_{max}$ versus
BchD concentration, suggesting that the BchD subunit in the
1.58–31.7 nm range can be treated as the enzyme in the analy-
ses. The $K_m$ of the BchI subunit was found to be 20 ± 3.2 nm,
which means that only 50% of the BchD forms functional
BchI-BchD complexes at this BchI concentration (Fig. 5B).
Approximately 100–200 nm BchI is required to form a fully
functional BchI-BchD complex, and BchD needs to be in excess
over BchD. These results are consistent with previous studies
showing that an excess of BchI subunit is required for optimal
magnesium chelatase activity (14, 15, 17) and highlight the
dynamic nature of the BchI-BchD complex in vitro.

Cooperativity and Regulation of Pigment Synthesis—Magnes-
ium chelatase commits proto to chlorophyll/bacteriochloro-

ATP is unclear; however, considering that there is strong
kinetic data (shown here) and structural evidence (23) for mul-
tiple forms of BchH, it seems likely that ATP hydrolysis by
BchI-BchD functions to modify the conformation of BchH
through a sequential series of conformational changes. The
model for catalysis shown in Scheme 2 has a minimum of five
conformational forms for BchH that are consistent with the
data presented here. These five forms in a catalytic cycle would be
1) BchH, a form that does not specifically bind proto;
2) BchH*, a form capable of binding proto;
3) BchH-proto;
4) BchH-Mg-proto*, a labile intermediate form of BchH;
and 5) BchH-Mg-proto. The BchH with no proto bound (form 1)
cannot bind proto readily and needs to change conformations by
interacting with the BchI-BchD complex, which drives this
process by hydrolyzing ATP and gives BchH* (form 2). The
BchH* can now bind proto to form BchH-proto (form 3), the
substrate for magnesium chelation by BchI-BchD. Chelation
produces an unstable BchH-Mg-proto* intermediate (form 4),
which can lose magnesium in basic conditions with acetone.
This is then converted to a stable BchH-Mg-proto product
(form 5). In the presence of exogenous proto and BchI-BchD,
Mg-proto is released from BchH, and the cycle can continue
from form 2. It should be qualified that the necessity for ATP
hydrolysis at each transition is speculative.

The stopped assay gave lower initial rates and a different
kinetic profile compared with the continuous assay (Fig. 3, A
and C). This appears to be due to loss of magnesium from the
proto during extraction rather than to any fluorescent artifact.
This suggests that there is an intermediate bound form of Mg-
proto that is unstable when the proteins are denatured to
extract the porphyrin.

The BchI subunit and BchI-BchD complex show relatively
low ATPase activity that is stimulated by 33-fold upon addition
of the BchH subunit to the latter (Table 2). A 16–20-fold
increase in ATPase activity was observed with
Synechocystis (17). In our
study, the behavior of BchD was tested by keeping it at a fixed
concentration while varying the BchI concentration. A linear
response was obtained when plotting apparent $V_{max}$ versus
BchD concentration, suggesting that the BchD subunit in the
1.58–31.7 nm range can be treated as the enzyme in the analy-
ses. The $K_m$ of the BchI subunit was found to be 20 ± 3.2 nm,
which means that only 50% of the BchD forms functional
BchI-BchD complexes at this BchI concentration (Fig. 5B).
Approximately 100–200 nm BchI is required to form a fully
functional BchI-BchD complex, and BchD needs to be in excess
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dynamic nature of the BchI-BchD complex in vitro.

Cooperativity and Regulation of Pigment Synthesis—Magnes-
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Mg2+ and Mg2+•ATP binding to the BchI and BchH-proto subunits facilitates Mg2+•ATP hydrolysis, as indicated by the concentration-dependent decrease in Mg2+•ATP fluorescence. The Mg•ATP fluorescence decay rate is concentration-dependent, with a Hill coefficient of 1.85 ± 0.23 (Table 2 and 3). This is consistent with the regulation of BchH stability in vivo by light and oxygen levels (40).

Conclusion—Our results show that the BchI and BchH-proto subunits can be treated as protein substrates for the BchD subunit in a kinetic analysis. The BchH-proto subunit shows cooperativity with both magnesium chelatase and ATPase activities and continuous magnesium chelatase assays (Hill coefficient of 2 suggests that either two or three BchH-proto subunits bind per BchI:BchD complex. Thus, the concentration of the BchH subunit is an important factor in regulation of the R. capsulatus enzyme, and this is consistent with the regulation of BchH stability in vivo by light and oxygen levels (40).

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