Substrate-binding Model of the Chlorophyll Biosynthetic Magnesium Chelatase BchH Subunit*

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Photosynthetic organisms require chlorophyll and bacteriochlorophyll to harness light energy and to transform water and carbon dioxide into carbohydrates and oxygen. The biosynthesis of these pigments is initiated by magnesium chelatase, an enzyme composed of BchI, BchD, and BchH proteins, which catalyzes the insertion of Mg\(^{2+}\) into protoporphyrin IX (Proto) to produce Mg-protoporphyrin IX. BchI and BchD form an ATP-dependent AAA\(^{+}\) complex that transiently interacts with the Proto-binding BchH subunit, at which point Mg\(^{2+}\) is chelated. In this study, controlled proteolysis, electron microscopy of negatively stained specimens, and single-particle three-dimensional reconstruction have been used to probe the structure and substrate-binding mechanism of the BchH subunit to a resolution of 25 Å. The apo structure contains three major lobe-shaped domains connected at a single point with additional densities at the tip of two lobes termed the “thumb” and “finger.” With the independent reconstruction of a substrate-bound BchH complex (BchH-Proto), we observed a distinct conformational change in the thumb and finger subdomains. Prolonged proteolysis of native apo-BchH produced a stable C-terminal fragment of 45 kDa, and Proto was shown to protect the full-length polypeptide from degradation. Fitting of a truncated BchH polypeptide reconstruction identified the N- and C-terminal domains. Our results show that the N- and C-terminal domains play crucial roles in the substrate-binding mechanism.

Chlorophylls and bacteriochlorophylls are integral components of light-harvesting complexes and represent a large family of molecules that harness light energy and sustain the vast majority of life through the process of photosynthesis. Magnesium chelatase (EC 6.6.1.1) performs the first committed step in chlorophyll and (bacterio)chlorophyll biosynthesis with the insertion of Mg\(^{2+}\) into protoporphyrin IX (Proto)\(^2\) (1). *Rhodobacter capsulatus* has become one of the most important model organisms for the study of (bacterio)chlorophyll biosynthesis since the discovery of a 45-kb gene cluster encoding photosynthesis-related genes, including the bacteriochlorophyll biosynthetic genes (2, 3). The *bchH*, *bchD*, and *bchl* genes encode the magnesium chelatase BchH, BchD, and Bchl subunits that interact to form the holoenzyme. In chlorophyll-containing organisms, these subunits are encoded by *chlH*, *chlD*, and *chlI*. On average BchH/ChlH, BchD/ChlD, and Bchl/ChlI have a molecular mass of 140, 70, and 40 kDa, respectively. Beyond intense interest in deciphering the mechanism of magnesium chelatase, the 140-kDa subunit has emerged as a key component in plastid-to-nucleus signal transduction (4–6), chloroplast development (7), regulation of bacteriochlorophyll biosynthesis at the enzymatic level (8), and substrate channeling (9) with an added role in the catalytic mechanism of the subsequent enzyme in the pathway (10). In addition, ChlH was recently identified as an asbscic acid receptor (11).

Toward a better understanding of the enzyme mechanism and auxiliary functions of BchH/ChlH, we have embarked on a complete structural characterization of the magnesium chelatase using the *R. capsulatus* system as a model. Although it has yet to be confidently demonstrated, the three subunits are proposed to form a transient ternary enzyme-Mg\(^{2+}\)-Proto complex (12). Attempts to isolate this complex have not yet been fruitful and emphasize the transient nature of the holoenzyme. Therefore, the alternative approach was to characterize structurally the individual subunits and use knowledge gained to later address the holoenzyme. So far the x-ray crystal structure has been solved to 2.1-Å resolution for the Bchl subunit (13), which is a member of the AAA\(^{+}\) superfamily of proteins that characteristically form ATP-dependent hexameric ring structures (14). Complementary structural studies on Bchl/ChlI have also been performed with negative staining electron microscopy (EM) (15, 16). Homology modeling of BchD, which is composed of an ATPase-inactive AAA\(^{+}\)-like domain and an integrin I domain connected by a negatively charged polypeptide linker (13), has provided insights into the structure of the

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2 The abbreviations used are: Proto, protoporphyrin IX; EM, electron microscopy; DTT, dithiothreitol; BchH-Cdom, BchH C-terminal domain; BchH-Ndom, BchH N-terminal domain; Mg-Proto, magnesium protoporphyrin IX; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
BchD/ChlD subunit. EM and single-particle three-dimensional reconstruction of the BchL-BchD complex show a bipartite ring structure with preferred 3-fold symmetry (17) and attest to an abundance of data that have long predicted these subunits to form a complex (13, 18–25).

The BchH/ChlH subunit (but not BchI/ChlI or BchD/ChlD) binds Proto (20, 21, 26) and is thus implicated as the catalytic component of the enzyme. The number of porphyrin-binding sites has been determined to one per BchH/ChlH subunit (20, 27). The R. capsulatus BchH polypeptide consists of 1194 amino acid residues that share global homology only with other BchH/ChlH subunits and the cobalt and nickel chelatase homologs. The length and uniqueness of the primary sequence have impeded structure prediction and homology modeling efforts. Production of crystals suitable for x-ray diffraction has yet to be reported, and the limitations of NMR analysis are grossly exceeded by the size of BchH/ChlH. In this study, controlled proteolysis, EM, and single-particle three-dimensional reconstruction techniques were used to obtain the first insights into the three-dimensional structure of the BchH/ChlH subunit. We visualized substrate-induced conformational changes and mapped the N- and C-terminal domains on the three-dimensional reconstructions. Based on biochemical and structural characterization of truncated BchH polypeptides, we postulate that the N- and C-terminal regions bind Proto cooperatively with the aid of a flexible linker region that facilitates gating between the open and closed states.

**EXPERIMENTAL PROCEDURES**

Production and Purification of BchH Protein—Plasmid pET15bBchH (21) contains the *R. capsulatus bchH* gene cloned into the pET15b vector (Novagen). The *bchH* gene encodes the magnesium chelatase BchH subunit, and when expressed from pET15bBchH, it obtains an N-terminal His tag. Plasmid pET15bBchH was transformed into *Escherichia coli* BL21(DE3) (Novagen), and the His-tagged BchH subunit was produced and purified as described (21) using a 1-ml Ni²⁺-chelating column (GE Healthcare) without addition of 5-aminolevulinic acid during expression to reduce the amount of endogenously bound Proto. Purified protein was desalted on NAP-5 columns (GE Healthcare) into 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, 4 mM dithiothreitol (DTT), and 6% glycerol. All purification and further handling of BchH were performed in darkness or under dim green light.

Magnesium Chelatase Activity Assays—Aliquots of purified BchH were thawed quickly and kept on ice and in darkness for the entire assay procedure. Assays were performed in 100-μl volumes with 1.76 μg of BchH, 0.63 μg of BchI, and 0.79 μg of BchD. The reaction mixture contained 50 mM Tricine, pH 8.0, 15 mM MgCl₂, 1 mM DTT, 10 mM phosphocreatine, phosphocreatine kinase (2 units/reaction), 4 mM ATP, and 1 μM Proto. Assays were incubated for 30 min at 32 °C, and the reaction was terminated with the addition of 10 volumes of Stop mix (acetone, H₂O, 25% ammonia, 80:20:1, v/v/v) followed by centrifugation to remove precipitants. For magnesium protoporphyrin IX (Mg-Proto) detection, the supernatants were analyzed on a FluoroMax-2 spectrophotometer (Spex spectrophotometer system, Jobin Yvon, Longjumeau, France) with excitation set at 412 nm and emission detected between 550 and 650 nm.

Proteolysis and Mass Spectrometry—Proteolysis of BchH was performed under native conditions at an endoprotease Glu-C (Roche Applied Science)/BchH ratio of 1:200. A mixture containing BchH at 0.2 μg/μl, with or without 1 μM Proto, was digested at 32 °C in 50 mM Tricine, pH 8.0, 15 mM MgCl₂, 1 mM CaCl₂, and 1 mM DTT. A volume equivalent to 10 μg of BchH was removed at 5, 10, 15, 30, and 60 min and at 16 h; precipitated with 20 volumes of Stop mix; and centrifuged for 5 min at 16,100 × g. Pellets were resuspended in SDS-PAGE sample buffer and frozen until gel loading. The enzyme/substrate ratio was varied (1:50/1000/2000), and the same final digestion pattern was observed throughout. In-gel digestion with 100 ng/μl endoprotease Glu-C was performed overnight at 37 °C. Matrix-assisted laser desorption ionization mass spectra were acquired with α-cyano-4-hydroxycinnamic acid as matrix on an Applied Biosystems 4700 proteomics analyzer with TOF/TOF™ optics (Applied Biosystems, Darmstadt, Germany) in positive reflector mode as described previously (28, 29).

Construction of Truncated BchH Subunits—Two His-tagged truncations of BchH were constructed. BchH-Ndom contains an N-terminal part (amino acid residues 1–733) of the BchH polypeptide, whereas BchH-Cdom contains a C-terminal part (amino acid residues 734–1194). The plasmid pET15bBchH (21) was used as a template for the construction of these truncated polypeptides using the PCR-mediated deletion method (30). Proteins were expressed and purified as described for the full-length polypeptide.

Porphyrin Binding to Protein—Equal amounts (1.85 μM) of the full-length polypeptide and the truncated variants were incubated separately with the various porphyrins (4 μM) in 50 mM Tricine, pH 8.0, 15 mM MgCl₂, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. The incubations were then processed on a 1-ml Ni²⁺-chelating column at room temperature. The loaded column was washed with the incubation buffer plus imidazole until the fluorescence of the fractions no longer exhibited the corresponding porphyrin peak. The protein was then eluted, and all fractions were analyzed for porphyrin content by fluorescence spectroscopy and for protein content by SDS-PAGE.

Electron Microscopy—Carbon-coated 400-mesh copper grids (Ax-labs, Vedbaek, Denmark) were first washed for 1 min with a drop of 50 mM Tricine, pH 8.0, 15 mM MgCl₂, and 1 mM DTT. Excess liquid was removed by brief contact with filter paper. Protein samples were diluted to 0.01 mg/ml with the wash buffer. A drop of the protein solution was applied to the grid for 1 min in darkness, and excess sample was removed. The grid was immediately stained with filtered 2% (w/v) uranyl acetate for 30 s, and excess stain was removed. EM was performed using a 120-kV Philips CM-10 microscope at ×55,000 magnification, giving a sampling size corresponding 4.67 Å/pixel. Particles from different micrographs were automatically selected using Boxer (31). The first zero crossing of the contrast transfer function was determined using the ctfit program, and the individual particles were low-passed filtered accordingly. Using only integer shift to avoid interpolation artifacts, translational alignment was performed on all particles.
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![Image of BchH structure](image)

**FIGURE 1.** Three-dimensional reconstruction of magnesium chelatase BchH. A, EM micrograph showing a field of negatively stained apo-BchH subunits. Scale bar corresponds to 100 nm, and white boxes indicate representative single BchH molecules. B, a gallery of selected projections of the final three-dimensional volume (first column), followed by class average of particles within ±6° from the projection angle (second column) and raw particles that were included in the respective average (third to fifth columns). Box dimensions are 187 × 187 Å. C, the apo-BchH subunit structure. Dotted circles define the major domains (lobes I, II, and III) and the thumb and finger subdomains that are referred to in the text. Scale bar corresponds to 50 Å.

![Image of Eigenimages](image)

**FIGURE 2.** Eigenimages from only translationally aligned data sets of magnesium chelatase BchH (A) and BchH in complex with protoporphyrin IX (B). A 3-fold symmetry is observed in the factors 6 and 8 of the apo-BchH data set (A), which disappears upon protoporphyrin binding to BchH (B).

![Image of Models](image)

**FIGURE 3.** Initial models for the three-dimensional reconstruction of the apo-BchH subunit. Five asymmetrical models were generated from reference-free alignments based on different starting sets of three distinctly different views selected from the 10 most dissimilar and most populated classes. The models were very similar, and all converged to the same solution after three rounds of iterative alignment and model refinement. The boxed model was chosen and used for further rounds of iterative alignment and model refinement.

Using EMAN software (31). Rotationally aligned particles were classified using correspondence analysis and hierarchical ascendant classification as implemented in SPIDER (32). Initial models were generated by angular reconstitution and Fourier interpolation reconstruction as implemented in the EMAN suite of programs (31). The models were refined iteratively by using the projection matching-based image classification algorithm in EMAN.

**Chemicals and Other Methods—**Protein concentration was determined using Bradford reagent (Bio-Rad) according to the manufacturer’s instructions. SDS-PAGE was performed according to Fling and Gregerson (33) with colloidal Coomassie Brilliant Blue staining (34). Proto was purchased from Frontier Scientific Inc. (Logan, UT). Proto was first dissolved in a small volume of 1 M NaOH and resuspended in water. After centrifugation, the concentration was measured in 2.7 M HCl using an ε406 of 262 mmol⁻¹ cm⁻¹ (35).

**RESULTS**

**EM and Single-particle Three-dimensional Reconstruction—**We adsorbed recombinant *R. capsulatus* BchH preparations to carbon-coated grids before negative staining and imaging in the electron microscope. The micrographs revealed discrete entities of a size corresponding to that expected for BchH (Fig. 1A). A few representative views were selected from each micrograph, and additional images were selected semiautomatically using Boxer (31). After two-dimensional alignment and classification, it became evident that the population contained particles in a broad distribution of views (data not shown). We therefore concluded angular reconstitution (36) to be a suitable method for *ab initio* reconstruction. The diameter of the BchH particles was measured to ~100 Å, and no oligomerization was observed. Already at this stage it became clear that the BchH subunit exhibited a compact structure composed of bulbous domains, which in certain orientations possessed a pseudo 3-fold symmetry (Fig. 1B).

**Three-dimensional Reconstruction of the R. capsulatus Apo-BchH Subunit—**After initial single-particle processing (see “Experimental Procedures”), which included translational and rotational alignment, the data set (5176 particles) was compressed to 20 dimensions by correspondence analysis. Reconstitution of the first eight eigenimages (Fig. 2A) revealed a tripartite appearance in factor numbers 6 and 8. Thus, the tripartite appearance did not appear to be a major source of variation in the data set, but confirmed the 3-fold symmetric views described above. Compression of the two-dimensional aligned data set was followed by hierarchical ascendant classification (37) into 77 classes. Of these classes, the 10 most dissimilar and most populated were chosen as a starting set for reference-free alignment. Five initial asymmetrical models were generated from alignments based on different starting sets of three distinctly different views. The models were very similar, and all converged to the same solution after three rounds of iterative alignment and model refinement (Fig. 3). One of these initial models was chosen and used for further rounds of iterative alignment and model refinement. To validate the final reconstructions, clusters of particles were sorted out on the basis of closeness to a set of quasi-evenly distributed Euler angle triplets using the Euclidean norm. For each of the clusters, the reconstructions were projected into the corresponding orientation. The resulting projection averages showed an excellent
agreement with the projections (Fig. 1B). The resolution of the reconstructions was determined to ~25 Å by using a fixed 0.5 Fourier shell correlation threshold value. Plots over the two non-azimuthal Euler angles (Fig. 4) demonstrated that data points were present in the entire Euler angle space, and the resolution was therefore concluded to be isotopic.

There are three major lobes of density extending from a flattened basal root along which lobes II and III extend. The bulk of lobe I stands perpendicular to lobes II and III. All lobes are approximately equal in size with the exception of an additional tilted “thumb”-shaped density at the tip of lobe I. Furthermore, a poorly defined “finger”-shaped density protruding from the tip of lobe III is present (Fig. 1C). The volume is 110 Å across the diagonal axis between lobe III and the thumb of lobe I and 90 Å across lobes II and III with a depth of 35–75 Å. The under-representation of the finger across a range of thresholds probably represents flexibility of this domain, i.e. an average of a range of positions it can occupy. Flexibility of protein domains often leads to poorly defined EM densities and sometimes to complete absence of the domain in a reconstruction (38, 39).

Proteolysis of Native Apo-BchH—As a way to probe the flexibility and domain organization of BchH, we conducted protease digestion under native conditions. The rationale behind this approach is that proteolysis can only occur at exposed flexible sites of the protein chain, which in multidomain proteins can result in the dissection of its constituent domains (40, 41). Endoproteinase Glu-C specifically hydrolyzes peptide bonds at the carboxyl-terminal side of glutamyl and aspartyl residues. No cleavage will occur if a proline residue is on the carboxyl-terminal side (42). In the case of R. capsulatus BchH, which contains 135 theoretical cleavage sites, the longest predicted peptide from a complete digestion is 35 residues, amino acids 232–266. Proteolysis experiments were conducted at pH 8.0, at which the BchH subunit exhibits maximum activity in a magnesium chelatase assay. A time course digestion showed that 120-, 80-, and 50-kDa polypeptides appeared after 5 min and steadily accumulated up to 2 h (data not shown). One major band of ~45 kDa remained after 16 h digestion (Fig. 5, lane 2). We also examined the effect of substrate binding on the proteolytic sensitivity of BchH by preincubating the BchH subunit with Mg²⁺ and Proto. Mg²⁺ did not affect the digestion profile. In contrast, Proto had a stabilizing effect, the Bch subunit remained at its native size after 16 h digestion, and the 45-kDa
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fragment did not appear (Fig. 5, lane 3). Complete digestion of *Bacillus subtilis* ferrochelatase in the presence of Proto showed that addition of porphyrin did not inhibit Glu-C activity (data not shown). These findings suggest that a substrate-induced conformational change of sufficient magnitude occurs in BchH that increases structural rigidity and impedes initial proteolytic nicking of protein sequences that initiate the progressive degradation.

The C-terminal Domain Is Resistant to Proteolysis—The 45-kDa fragment was excised from a denaturing SDS-polyacrylamide gel and digested with Glu-C. Analysis by mass spectrometry revealed peptides from the C terminus of BchH. The most N- and C-terminal peptides identified were \(^7\)TGGV-GRPMTEAERAE \(^7\) and \(^1\)ASDRNYWQPDPATLAALQA-ADE \(^1\) respectively, from a total polypeptide length of 1194 residues. The theoretical molecular mass of a BchH fragment containing residues 734–1183 is 48.7 kDa and agrees well with the observed size of the protease-resistant BchH fragment. Peptides from the \(^1\)LEDRMEGVAAE \(^1\) carboxyl-terminal end were not detected because either they are below the detection threshold of the mass spectrometer or they were cleaved during the native digest. According to the criteria of domain mapping by proteolysis (40), the protein sequence flanking residue 734 is flexible and probably represents a dynamic linker region of the N- and C-terminal domains.

N- and C-terminal Domains Are Required for Optimum Porphyrin Binding—We sought to understand the means by which substrate binding protects against proteolytic attack by first investigating whether the N- and C-terminal domains bind porphyrin. Expression clones of N- and C-terminal regions of the BchH polypeptide were constructed, using the site of the most N-terminal peptide identified in the 45-kDa Glu-C digestion fragment as the division point. BchH-Ndom includes residues 1–733, and BchH-Cdom includes residues 734–1194. Purified proteins were incubated with various porphyrins and then chromatographed on Ni\(^2\+\) chelating columns to assess their ability to retain porphyrin (Fig. 6). The full-length polypeptide could both bind and retain deuteroporphyrin IX, Mg-deuteroporphyrin IX (MgDIX), and N-methylmesoporphyrin IX (N-MMP) through the course of \(\text{Ni}^{2+}\) affinity chromatography. The estimated protein/porphyrin ratios (Fig. 6) agree with a single porphyrin-binding site that has been suggested for *Synechocystis* ChlH and *Rhodobacter sphaeroides* BchH (20, 27). Mg-deuteroporphyrin and N-methylmesoporphyrin, but not deuteroporphyrin, cofractionated with BchH-Ndom to a small extent compared with the full-length polypeptide, but BchH-Cdom did not display porphyrin retention ability. Naturally, it was of interest to determine whether these truncations could perform the magnesium chelatase reaction, but when assayed independently or combined, no activity was detected (data not shown). To investigate whether the His tag on the N terminus of BchH-Cdom obstructed interaction with BchH-Ndom, it was removed by thrombin digestion. However, activity was not reconstituted. Interestingly, when BchH-Ndom was combined in an equimolar ratio in an assay with full-length BchH, a 2-fold stimulatory effect was observed, whereas BchH-Cdom had no stimulatory effects. At present, we cannot explain this observation. In accordance with the binding studies above (Fig. 5), we also found that Mg-Proto was not released from BchH after an *in vitro* assay. Instead, it remained bound after \(\text{Ni}^{2+}\) affinity chromatography (Fig. 7).

Three-dimensional Reconstruction of BchH-Proto Reveals a Conformational Change—Given that the N- and C-terminal truncations do not bind deuteroporphyrin IX (Fig. 6), a functional substrate analogue, it is highly likely that residues from both domains are involved in substrate binding. To understand how the apo structure might change upon substrate binding and prevent proteolytic attack, we calculated an independent reconstruction of BchH incubated with Proto. A total of 2208 particles were used to generate a three-dimensional reconstruction of BchH-Proto at \(\sim\) 28-Å resolution (Figs. 4B and 8). The reconstruction clearly revealed a structural change. The fact that the eigenimages of BchH-Proto (Fig. 2B) entirely lack any 3-fold symmetry further supported that a conformational change occurs upon porphyrin binding. To test whether the preparation of apo-BchH included contamination of BchH-Proto and vice versa, the respective data sets were reference-matched against both conformations. Sorting was based on common line correlation to two sets of quasi- evenly distributed reference projections of apo-BchH and BchH-Proto. New volumes were reconstructed from the homogenous subsets of images, but they revealed no significant differences from the original reconstructions.

The most apparent structural change is at the level of thumb and finger domains described in the apo volume (Fig. 1C). Incubation of the same protein preparation with Proto causes these domains to come into direct contact, representing a movement of \(\sim 30\) Å. Despite the major domain movement induced by Proto binding, lobes I, II, and III are clearly recognizable. The

![](https://example.com/image.png)
thumb of lobe I increased its tilted angle, and the finger of lobe III was equally represented in the volume across a range of thresholds in the Proto-bound model, suggesting that flexibility of this domain is related to the occupancy of the porphyrin-binding site.

The N- and C-terminal Domains Fuse upon Substrate Binding—In view of the fact that controlled proteolysis and EM investigations argue that BchH undergoes considerable conformational changes upon substrate binding, it was important to link these studies by identifying the N- and C-terminal domains within the BchH structures. As a step toward this, we first calculated a separate reconstruction of BchH-Ndom using 6982 single particles. The reconstruction was generated using the apo-BchH volume as reference for iterative alignment. The reconstruction converged after eight iterations and is shown aligned with the apo-BchH three-dimensional volume (Fig. 9A). It is clear from this analysis that lobe III and the finger domain are absent in the BchH-Ndom polypeptide. Thus, the thumb domain, lobe I, and lobe II compose the N-terminal domain, whereas lobe III and the finger domain represent the C-terminal domain. Direct translation of these findings to the BchH/Proto reconstruction, where the thumb and finger domains fuse upon substrate binding, supports the hypothesis that BchH is composed of an independently folded C-terminal domain that is separable from the N-terminal domain and that binding of Proto induces interaction between the domains (Fig. 9B).

DISCUSSION
Magnesium chelatase sits at the branch point of the common tetrapyrrole pathway and inserts Mg²⁺ into Proto to produce Mg-Proto, the first unique intermediate of the chlorophyll biosynthetic pathway. It is certain that the BchH/ChlH subunit binds the substrate and, for this reason, is thought to be

![FIGURE 7. Mg-Proto is not released into solution after a magnesium chelatase catalytic cycle. In vitro magnesium chelatase assays were up-scaled 4-fold (400 μl) with one-quarter analyzed by fluorescence spectroscopy directly (A). The peaks at 595 and 630 nm correspond to synthesized Mg-Proto and uncatalyzed Proto substrate, respectively. In A, the solid line represents an assay containing Bchl, BchD, and BchH (IDH), and the dashed line represents an assay with BchH omitted (ID). The remainder of the assays were loaded onto Ni²⁺-chelating columns, and then wash (IDH-WASH) and elution (IDH-ELUTE) fractions were collected. Volumes of 100 μl from 1-ml fractions were precipitated with Stop mix and then analyzed as described for A. The results from the Bchl/BchD/BchH assay are shown in B. The Bchl/BchD assay is shown in C and serves to demonstrate the relative amounts of Proto binding to the column resin and Bchl/BchD, compared with B, which contains BchH, the Proto-binding subunit of magnesium chelatase. In both cases, wash fraction 1 showed the highest level of unbound Proto. Progressive washing steps are illustrated by fractions 2–5 in the upper panels of B and C. The elution of His-tagged BchH (lower panel of B) revealed a spectrum identical to that in A, in that Mg-Proto and Proto were present. The equivalent fraction in the control (lower panel of C) contained the highest level of Proto in that series and probably represents nonspecific binding to Bchl/BchD proteins; BchD contains a His tag but Bchl does not. The fact that Mg-Proto was detected only in the elution fraction from the Bchl/BchD/BchH assay suggests that the reaction product was not released into solution but remained tightly associated with BchH.

![FIGURE 8. Three-dimensional reconstruction of the BchH/Proto complex. A, three views of the BchH/Proto reconstruction. Scale bar corresponds to 100 Å. B, three views of the apo-BchH reconstruction aligned with the BchH/Proto views in A. Note the conformational change in the thumb and finger subdomains that are fused in the BchH/Proto reconstruction (red) compared with the apo reconstruction (yellow).]
catalytic component of the enzyme (20, 21, 26). This study has given the first insights into the structure of BchH/ChlH and demonstrated conformational changes upon binding of its porphyrin substrate. We also provide further support that Mg-Proto is not released into solution after catalysis (27). Proteolysis and EM single-particle reconstructions were complementary in showing that BchH is a multidomain protein that is physically divisible into N- and C-terminal fragments. From our fit of the apo and BchH-Ndom structures, we postulate that N-terminal residues 1–733 lie within distinct densities termed the thumb, lobe I, and lobe II. The residual densities in the apo structure, termed lobe III and the finger, are therefore likely to represent residues 734–1194 of the C terminus. The BchH-Ndom and BchH-Cdom truncations do not retain the substrate analogue deuteroporphyrin IX at native levels. Thus, a cooperative binding model is proposed. In such a model, residues from both the N- and C-terminal regions are involved in binding a single Proto molecule that is either accompanied or facilitated by a conformational change where the thumb and finger subdomains fuse. The precise location of the porphyrin-binding site remains to be determined, although it is tempting to speculate that it is clamped between the thumb and finger. Alternatively, Proto could be bound deeper in the cleft closer to the hinge region between the N- and C-terminal domains. In view of the fact that BchH-Ndom retains metal-lated porphyrin, it is probable that the bulk of the porphyrin-binding residues and possibly an axial ligand for magnesium are located within the N terminus. The porphyrin-binding phase probably proceeds in two steps: (i) initial and reversible binding of porphyrin by residues within the N terminus, represented by a structure resembling the apo-BchH model, and (ii) high affinity binding achieved with the involvement of C-terminal residues that lock loosely bound porphyrin in place, represented by the BchH-Proto model. A flexible polypeptide linker at the junction of the N- and C-terminal domains (sequence flanking Gly-734) is predicted to facilitate this cooperativity. Interestingly, the homology between all members of the BchH/ChlH family is lowest exactly at the predicted flexible linker region that we identified by proteolysis. Oxygenic species contain a highly conserved insertion of ~110 residues compared with facultative anaerobes such as Rhodobacter species and Rubrivivax gelatinosus. Apart from chloroplast targeting sequences, this insertion accounts for the majority of mass variations observed between BchH/ChlH subunits, which range in size from 1193 to 1380 residues. Functional characterization of this insertion is eagerly awaited.

The porphyrin-induced conformational changes of different domains in BchH are obvious. The purpose of these changes is less clear, although mechanistic as well as regulatory reasons could be suggested. It has been postulated that insertion of Mg$^{2+}$ into Proto proceeds via a distortion of the porphyrin ring, as N-methylmesoporphyrin and N-methylprotoporphyrin (distorted porphyrins) are inhibitory to magnesium chelatase activity (43). In addition, spectroscopic analysis of the Synechocystis ChlH- and R. sphaeroides BchH-deuteroporphyrin IX complexes showed a red shift in the fluorescence emission maxima, which is indicative of a distorted porphyrin (27). However, based on studies of the better characterized ferrochelatase, it is clear that porphyrin distortion can be obtained without large structural rearrangements of the protein, as in the present case of the magnesium chelatase BchH (44, 45). The fact that BchH-Ndom retains distorted porphyrin, but not planar metal-free porphyrin, during metal ion chromatography suggests that cooperative substrate binding is directly responsible for porphyrin distortion.

Another attractive explanation for the striking substrate-induced conformational changes is the importance of binding Proto with high affinity to direct this intermediate into chlorophyll biosynthesis, as magnesium chelatase is at the branch point between chlorophyll and heme pathways and competes with ferrochelatase for Proto. However, this suggestion has to be considered in light of the cobalt chelatase, which is similar to magnesium chelatase in being an ATP-dependent chelatase consisting of three subunits (46), of which at least the largest and the smallest polypeptides are very similar. The ATP-dependent cobalt chelatase is not at a branch point in the pathway of cobalamin biosynthesis.

A third explanation to the porphyrin-induced conformational change could relate to the enzymatic product Mg-Proto.
The cofractionation of Mg-Proto with BchH from an in vitro activity assay demonstrated that the reaction product is not released into solution after catalysis. This finding is unusual, but it was anticipated because the same BchH preparation could bind and retain Mg-deuteroporphyrin IX along with consistent results obtained with ChlH from the cyanobacterium 

*Synechocystis* and BchH from the proteobacterium *R. sphaeroides*, which have also been shown to bind Mg-deuteroporphyrin IX (27). If Proto is locked between subdomains of BchH/ChlH and if Mg-Proto remains associated after magnesium chelation, then it would seem that the magnesium chelatase employs explicit control over the trafficking of its product. This is plausible because free porphyrins and metalloporphyrins are generally toxic to the cell, which must be a pronounced problem during massive chlorophyll production. The next step in the chlorophyll biosynthetic pathway, catalyzed by Mg-protoporphyrin IX methyltransferase (EC 2.1.1.11), involves the methylation of Mg-Proto to produce Mg-protoporphyrin monomethyl ester (47). 

*Synechocystis* ChlH stimulates methyltransferase activity in vitro and is directly implicated in the catalytic mechanism (10). Our independent reconstructions of apo-BchH and porphyrin-bound BchH probably represent a subset of the repertoire of conformational states BchH/ChlH can adopt. It is probable that rather than free Mg-Proto, a structurally unique BchH/ChlH-Mg-Proto complex is the substrate for the methyltransferase in vivo, thus implicating the need for tight binding of porphyrins and substrate channeling as the reason for conformational changes in BchH/ChlH. Mg-Proto has also been suggested as a signal molecule in chloroplast-to-nucleus signal transduction through the study of Arabidopsis genomes uncoupled (gun) mutants (4–6). It was proposed that the GUN4 protein regulates magnesium chelatase activity by recruiting substrate or removing product from ChlH (5). Thus, the magnesium chelatase ChlH subunit of higher plants must also meet the requirement to avoid uncontrolled release of Mg-Proto that could trigger unwarranted nuclear signaling cascades.

It is obvious that more studies are required before the function of the porphyrin-induced conformational changes is understood. Further dissection of BchH/ChlH into the minimum porphyrin-binding unit, coupled with structural and porphyrin binding investigations, is an obvious avenue in locating the substrate-binding site. We must also consider these findings in respect to the holoenzyme. *R. capsulatus* Bchl and BchD form an ATP-dependent two-tiered hexameric ring structure typical of AAA+ complexes (17). The mechanism by which the asymmetrical BchH/ChlH subunit interacts with an AAA+ dodecamer of 3-fold symmetry is difficult to predict. It is accepted, however, that all three subunits must interact to perform magnesium chelation, although a physical complex is still to be demonstrated. In accordance with our previous statement that the BchH-Proto conformer is recognized by the BchH-BchD complex and that the tightly connected thumb-finger region is structurally unique, we predict that this structure participates in protein-protein recognition and that formation of the holoenzyme drives magnesium chelation into Proto. In continuation of this idea, we postulate that once Mg$^{2+}$ is chelated, the BchH/ChlH subunit adopts a yet unobserved conformation that is able to retain Mg-Proto but no longer maintains structural integrity with the BchI/ChlI-BchD/ChlD complex, at which point the holoenzyme disassociates and BchH/ChlH-Mg-Proto is liberated. This putative structure is predicted to be recognized by the methyltransferase and, in the case of ChlH, GUN4.

We feel confident that this first structure of the catalytic subunit has provided the framework to a better understanding of the primary step in chlorophyll biosynthesis. It is reasonable to assume that all BchH/ChlH subunits and homologs of the cobalt and nickel chelatases involved in the biosynthesis of cobalamins and coenzyme F$_{420}$ respectively, will also have similar underlying structures and substrate-induced conformational changes as those described here.

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