

Magnesium-protoporphyrin chelatase of *Rhodobacter sphaeroides*: Reconstitution of activity by combining the products of the *bchH*, *-I*, and *-D* genes expressed in *Escherichia coli*

(protoporphyrin IX/tetrapyrrole/chlorophyll/bacteriochlorophyll/photosynthesis)

LUCIEN C. D. GIBSON*, ROBERT D. WILLOWS†, C. GAMINI KANNANGARA†, DITER VON WETTSTEIN†, AND C. NEIL HUNTER*

*Krebs Institute for Biomolecular Research and Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, S10 2TN, United Kingdom; and †Carlsberg Laboratory, Department of Physiology, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

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ABSTRACT Magnesium-protoporphyrin chelatase lies at the branch point of the heme and (bacterio)chlorophyll biosynthetic pathways. In this work, the photosynthetic bacterium *Rhodobacter sphaeroides* has been used as a model system for the study of this reaction. The *bchH* and the *bchI* and *-D* genes from *R. sphaeroides* were expressed in *Escherichia coli*. When cell-free extracts from strains expressing *BchH*, *BchI*, and *BchD* were combined, the mixture was able to catalyze the insertion of Mg into protoporphyrin IX in an ATP-dependent manner. This was possible only when all three genes were expressed. The *bchH*, *-I*, and *-D* gene products are therefore assigned to the Mg chelatase step in bacteriochlorophyll biosynthesis. The mechanism of the Mg chelation reaction and the implications for chlorophyll biosynthesis in plants are discussed.

Photosynthetic organisms synthesize both chlorophyll and heme, the two major tetrapyrroles in nature. The biosynthetic pathways of these two porphyrins utilize a number of intermediates in common and the first step unique to chlorophyll production is the insertion of Mg into protoporphyrin IX (see Fig. 1). The enzyme catalyzing this insertion is known as magnesium-protoporphyrin IX chelatase and it lies at the branch point of the heme and the bacteriochlorophyll/chlorophyll biosynthetic pathways. Despite the importance of (bacterio)chlorophyll biosynthesis, there is relatively little known about the detailed enzymology and protein chemistry of this pathway, and in the case of Mg chelatase biochemical analyses have been confined to assays using intact cells of the photosynthetic bacterium *Rhodobacter sphaeroides* (1, 2), isolated plastids (3–6), and broken plastid systems (7–12). In these systems, ATP is absolutely required for magnesium chelatase activity (4). Furthermore, it has been demonstrated with extracts of pea (*Pisum sativum*) chloroplasts that two components, one soluble and the other with membrane affinity, participate in the enzymatic reaction and that there is an ATP requirement for the activation of these two components (10).

Recently, the analysis of this pathway in photosynthetic bacteria has provided a way forward (for a review, see ref. 13). This approach benefits from the availability of the genes for bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus* and *R. sphaeroides*, which are clustered on a small region of the genome, ≈45 kb long (14–17). The gene assignments have been based on the results of insertional mutagenesis, which have been correlated with the accumulation of biosynthetic intermediates, or by the measurement of enzymatic activities (15, 17–19). Positive identification of function has been lacking, but two recent publications describe the overexpression of the *bchM* gene from both *R. sphaeroides* and *R. capsulatus* in

Escherichia coli and demonstrate that the extracts of the *E. coli* transformants can convert Mg-protoporphyrin IX to Mg-protoporphyrin monomethyl ester (20, 21). Apart from positively identifying *bchM* as the gene encoding the Mg-protoporphyrin methyltransferase, this work opens up the possibility of extending this approach to other parts of the pathway. In this paper, we report the expression of the genes *bchH*, *-I*, and *-D* from *R. sphaeroides* in *E. coli*: extracts from these transformants, when combined *in vitro*, are highly active in catalyzing the chelation of Mg by protoporphyrin IX in an ATP-dependent manner. This is an important step forward since apart from identifying the role of three more *bch* genes—*bchH*, *-I*, and *-D*—it will allow the biochemistry of this reaction to be studied in detail. Moreover, the results have important implications for the study of chlorophyll biosynthesis in plants. Two genes have been identified, *ch42* from *Arabidopsis thaliana* (22) and olive from *Antirrhinum majus* (23), which share 49% and 39% identity with *bchI* and *bchH*, respectively, from *R. capsulatus*. In view of these homologies and the disruptive effects on chlorophyll biosynthesis caused by mutation of these plant genes, it seems likely that they also encode components of the Mg chelatase.

MATERIALS AND METHODS

Construction of pET3a Derivatives Containing *bchH*, *bchI*, and *bchID*. The oligonucleotides 5'-GACATCTAGAGGA-GACGACCATATGCACGGTGAAGTCTC-3' and 5'-ACGGAAGCTTAGATCTTCACTCGGCGGCAAT-3' were used to amplify *bchH* by PCR and to introduce *Nde* I and *Bgl* II sites into the gene, which allowed cloning of the PCR fragment into pET3a (24), yielding plasmid pETBCHH. A *Bam*HI fragment containing most of the *bchI* gene was subcloned into pBluescriptII. This was used as the template in a PCR using the oligonucleotide 5'-CCCCGGTACCG-GAGACGACACATATGAAAAACCCTTCCCCTTC-3' and the pBluescriptII KS primer. The resulting 800-bp fragment was digested with *Kpn* I and *Bam*HI and ligated to the 2.3-kb *Bam*HI/*Hind*III fragment, which contains the *bchD* gene. The resulting ≈3-kb fragment was cloned into pBluescriptII, yielding plasmid pBBCHID. This plasmid therefore contains the *bchI* and *-D* genes in the same arrangement as on the *R. sphaeroides* chromosome with a *Nde* I site preceding the *bchI* gene, enabling cloning into pET3a. A deletion of *bchD* was effected by digesting pBBCHID with *Pst* I and the ≈3.95-kb fragment was religated yielding plasmid pBBCHI. These plasmids were then digested with *Nde* I and subjected to partial digestion with *Bam*HI. The resulting *Nde* I/*Bam*HI fragments of ≈3.1 kb in the case of *bchID* and ≈1.05 kb in the case of *bchI* were cloned into pET3a, yielding plasmids pETBCHID and pETBCHI. Plasmids for overexpression were transformed into *E. coli* BL21(DE3) (25). *E. coli* BL21(DE3) strains containing pET3a and its derivatives were grown at

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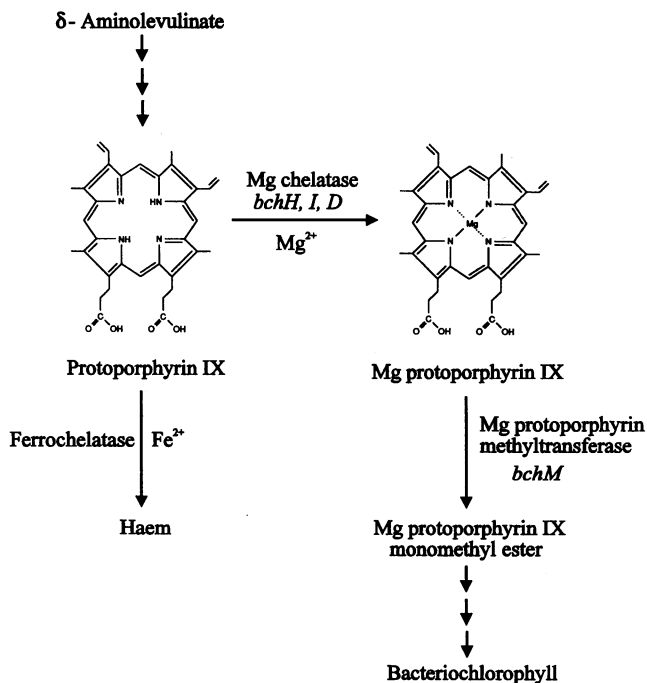


FIG. 1. Pathway of (bacterio)chlorophyll biosynthesis showing the branchpoint at the two possible chelation steps.

37°C in 100 ml of LB medium containing 100 $\mu\text{g}\cdot\text{ml}^{-1}$ of ampicillin until the A_{600} of the cultures reached 0.6–1. Protein expression was induced by the addition of isopropyl β -D-thiogalactopyranoside to the cultures at a final concentration of 0.4 mM. After 4 hr, the cells were harvested and the cell pellets were stored at -20°C .

Mg-Protoporphyrin Chelatase Assays. Cell pellets were thawed, resuspended in 2 ml of 0.1 M Tricine, pH 7.9/0.3 M glycerol/25 mM MgCl_2 /4 mM ATP/4 mM dithiothreitol and disrupted by sonication for 3×30 sec on ice. The cell debris was removed by centrifugation at $20,000 \times g$ for 15 min at 4°C . Protein concentrations of the samples were estimated with the Bio-Rad DC protein assay kit.

Enzyme assays were carried out in a vol of 1 ml of the buffer described above containing 1 mg of each protein extract, 1.5 μM protoporphyrin, 50 mM phosphocreatine, and 10 units of creatine phosphokinase. Incubations were carried out in the dark for 1 hr at 30°C , after which time the absorbance spectrum of each sample between 370 and 650 nm was recorded on a Beckman DU640 spectrophotometer and the fluorescence emission spectrum of each sample between 500 and 650 nm was recorded on a SPEX FluoroMax spectrofluorimeter using an excitation wavelength of 420 nm.

The time course was carried out by mixing together all the components in a 1-ml cuvette, except for protoporphyrin. Protoporphyrin was added and the absorbance spectrum was recorded immediately and at intervals thereafter.

HPLC Analysis of Pigments. To the incubation mixture 5 vol of acetone/ H_2O /32% NH_3 (80:20:1, vol/vol/vol) and 1 vol of hexane were added and mixed. The mixture was centrifuged at $20,000 \times g$ for 5 min and 40 μl of the lower phase was analyzed on a Waters 5- μm octadecylsilane column (8×100 mm) using a 10-min linear gradient from H_2O /triethylamine/acetonitrile (85:0.05:15, vol/vol/vol) to 100% acetonitrile at a flow rate of 2 ml/min. The eluate from the column was passed through a Shimadzu spectrofluorimetric detector (λ_{ex} , 420 nm; λ_{em} , 595 nm). Under these conditions Mg protoporphyrin eluted at 4.55 min.

SDS/PAGE. Fifty micrograms of each soluble protein extract was mixed with solubilization buffer and electrophoresed

on a 10% acrylamide gel by the method of Laemmli (26). Proteins were visualized by staining with Coomassie brilliant blue R250.

Protoporphyrin Solutions. Mg protoporphyrin IX was purchased from Protoporphyrin Products (Logan, UT). Protoporphyrin IX was purchased from Sigma and purified on a DEAE-Sepharose (acetate form) column (27) as described (28). Purified protoporphyrin was dissolved in 0.1 M NH_4OH . The concentration was measured in 2.7 M HCl using the millimolar extinction coefficient at 554 nm of 13.5 (29).

RESULTS

Our previous results had suggested that the three genes *bchH*, *-I*, and *-D* might encode Mg-protoporphyrin chelatase (19, 21). DNA fragments containing these genes were cloned into pET3a, yielding plasmids pETBCHH, pETBCHI, and pETBCHID (Fig. 2A); in the last case, *bchI* and *-D*, which are found together within the photosynthesis gene cluster, were cloned as a pair. It has not been possible to amplify and clone *bchD* separately so far, but the combination chosen has nevertheless allowed us to establish the requirement for *bchD* (see below).

The *E. coli* cultures containing the *bch* constructs were induced with isopropyl β -D-thiogalactopyranoside. We noticed that after 4 hr of induction the cells containing pETBCHH started to acquire a red color; the fluorescence emission of this pigment is at 633 nm (Fig. 2B), and we attribute this to protoporphyrin IX, since it is identical to the emission characteristics of authentic protoporphyrin IX. We also noted that the 410-nm absorbance was increased in incubations contain-

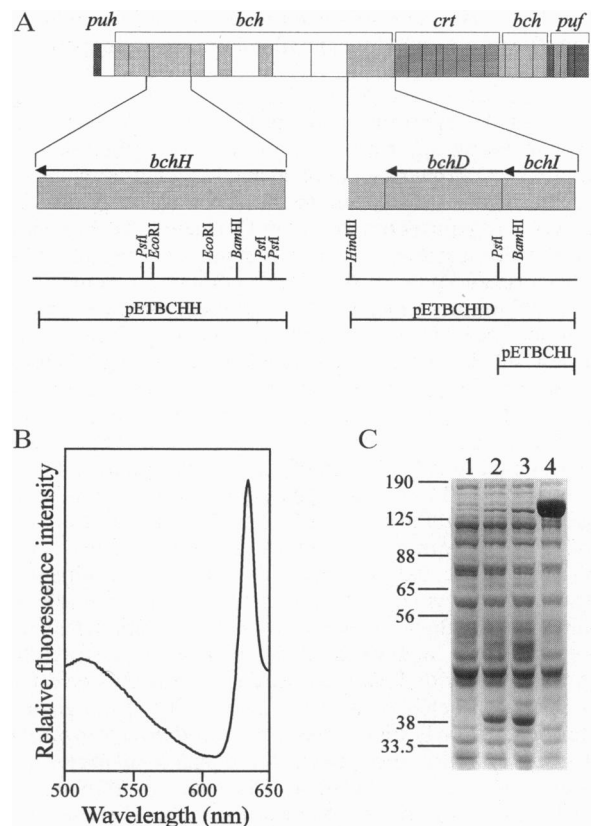


FIG. 2. (A) Map of the photosynthesis gene cluster, with the expanded areas showing the regions containing *bchH*, *-I*, and *-D* used for cloning into the expression plasmid pET3a. (B) Fluorescence emission spectrum of the pETBCHH cell-free extract showing the fluorescence maximum at 633 nm characteristic of protoporphyrin IX. (C) SDS/PAGE of the cell-free extracts prepared from *E. coli* strains containing *bch* genes. Lanes: 1, pET3a (control); 2, pETBCHI; 3, pETBCHID; 4, pETBCHH. Sizes of molecular mass markers (kDa) are indicated.

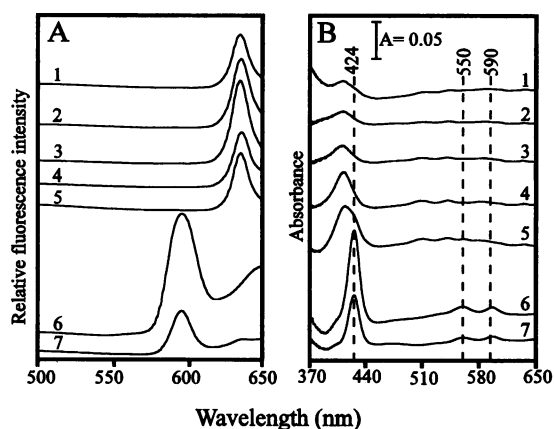


FIG. 3. Magnesium-protoporphyrin chelatase assays. Fluorescence emission spectra (A) and absorbance spectra (B) of incubations containing extracts as follows: trace 1, pET3a; trace 2, pETBCHID; trace 3, pETBCHI; trace 4, pETBCHH; trace 5, pETBCHH + pETBCHI; trace 6, pETBCHH + pETBCHID. Trace 7, pET3a extract incubated with Mg protoporphyrin; the fluorescence emission spectrum was recorded on a 1:10 dilution of this incubation mixture.

ing pETBCHH extract (see Fig. 3B, traces 4 and 5). We suggest that this pigmentation is due to the BchH protein sequestering endogenous protoporphyrin IX; we will comment on the significance of this in the *Discussion*. The cells from the respective inductions were disrupted and the crude extracts were centrifuged; the resulting cell-free supernatants were used for all subsequent work, since these contained all the Mg chelatase activity, suggesting that the BchH, -I, and -D proteins accumulated in a soluble form. The same extracts used for Mg chelatase assays were analyzed by SDS/PAGE (Fig. 2C). The BchH and -I proteins are clearly visible on the gel, having apparent molecular masses of ≈ 140 and ≈ 38 kDa, respectively; no BchD protein can be seen at the expected molecular mass of ≈ 70 kDa, but the omission of *bchD* from the experiment has such a decisive effect on the activity of the enzyme that there must be some BchD protein present (see below).

The cell-free extracts were mixed in various combinations to test the requirement for the *bchH*, -I, and -D gene products. In each case, protoporphyrin IX was the substrate, and the formation of Mg protoporphyrin IX was established by absorbance and fluorescence spectroscopy. Fig. 3 shows the fluorescence emission and absorbance spectra of the *E. coli* extracts after 60 min of incubation with protoporphyrin IX; in contrast to the substrate, Mg protoporphyrin IX has a characteristic emission maximum at 595 nm (Fig. 3A, trace 7), and this is clearly present in the BchH, -I, and -D sample. All

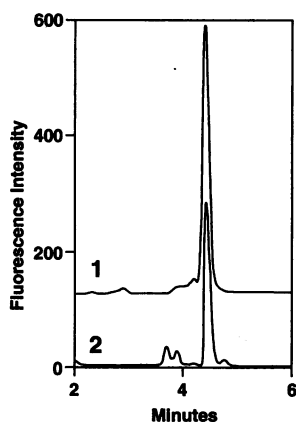


FIG. 4. HPLC identification of product formed in Mg chelatase assays. Trace 1, authentic Mg protoporphyrin IX; trace 2, product formed from incubation of pETBCHH + pETBCHID extracts.

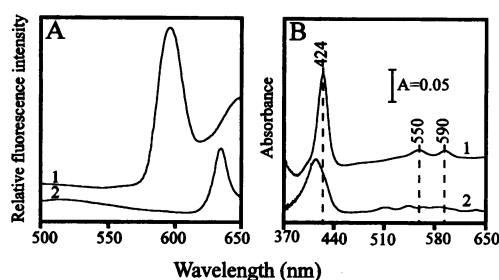


FIG. 5. Demonstration of an ATP requirement for the Mg chelatase reaction. Fluorescence emission spectra (A) and absorbance spectra (B) of incubations containing pETBCHH + pETBCHID extracts. Trace 1, with 4 mM ATP; trace 2, without ATP.

possible combinations were tested, and the only one that gave chelatase activity was the one with all three gene products present: no 595-nm emission peak was observed for any other combination. The spectrum is in perfect agreement with authentic Mg protoporphyrin. Fig. 3B shows the absorbance spectra of the same incubations used in Fig. 3A. Inspection of the protoporphyrin spectrum (Fig. 3B, trace 1) and Mg protoporphyrin spectrum (Fig. 3B, trace 7) shows that the absorbance behavior of these two tetrapyrroles differs substantially—first in the position of the Soret peak, and second in the extinction of the smaller maxima at 550 and 590 nm (30). The Mg chelatase activity of the BchH, -D, and -I proteins has catalyzed the conversion of protoporphyrin to Mg protoporphyrin, which results in a shift of the Soret peak from 410 to 424 nm and the appearance of the smaller maxima at 550 and 590 nm. When different extracts were tested, the only combination that showed any activity was that containing BchH, -I, and -D. However, it might be significant that the presence of the BchH and -I proteins imparts a 424-nm shoulder on the main peak of the protoporphyrin (Fig. 3B, trace 5); this might correspond to a protein-bound form of the substrate that could form prior to the insertion of Mg. The fluorescence emission spectrum of this species shows that it does not arise from the completed chelation reaction (Fig. 3A, trace 5). In addition, the identity of the product of the BchH, -I, -D incubation was verified by HPLC (Fig. 4). The retention times of authentic Mg protoporphyrin (Fig. 4, trace 1) and the product of the reaction (Fig. 4, trace 2) are identical, further confirming that the product is Mg protoporphyrin IX.

The results in Fig. 5 demonstrate the ATP requirement for the chelatase reaction, using both the fluorescence and absorbance assays. This requirement for ATP is absolute, since little or no product is formed in its absence. Fig. 6 shows a time course for this reaction, which allows us to obtain an estimate of the activity of this system. We calculate that it is ≈ 500 pmol of Mg protoporphyrin per 15-min incubation per mg of protein, which is consistent with the levels of activity measured by Walker and Weinstein (8) using lysed, fractionated chloroplasts. However, we have reason to believe that this underestimates the potential level of activity by a large margin; the reasons for this are discussed below.

DISCUSSION

The experiments described above demonstrate that magnesium chelatase consists of three components, the BchH, -I, and -D proteins. When mixed in crude suspension they are capable of associating to form an active chelatase, which we propose is formed from BchH, -I, and -D in a complex. This complex is capable of catalyzing the chelation of magnesium by protoporphyrin IX in an ATP-dependent manner.

The observation that the *E. coli* strain overexpressing *bchH* acquires a red color due to endogenous protoporphyrin IX provides a clue as to the role played by this subunit *in vivo*. We suggest that BchH binds protoporphyrin IX prior to the

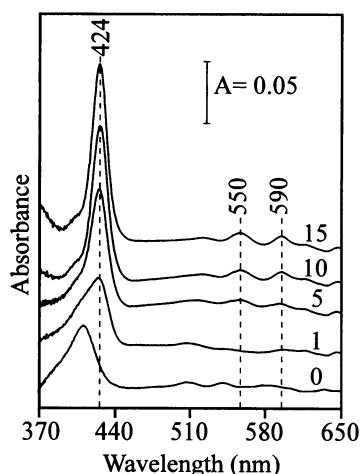


FIG. 6. Time course of conversion of protoporphyrin to Mg protoporphyrin by combined pETBCHH + pETBCHID extracts. Absorbance spectra were recorded at indicated time intervals (min) after addition of protoporphyrin to the incubation mixture.

insertion of the magnesium atom and that this protein, when overproduced in *E. coli*, sequesters protoporphyrin IX. In a system unaccustomed to the presence of large amounts of such a protein, and without the regulatory mechanisms to cope with the effective removal of protoporphyrin IX, the amount of heme would be reduced since protoporphyrin IX is the normal substrate for ferrochelatase in *E. coli*. A reduction in the cellular levels of heme may stimulate δ -aminolevulinic acid synthesis, through a reduction in the normal levels of feedback inhibition on this step of the pathway by heme (31), and this will be reflected in an increased flux along the pathway to protoporphyrin IX. A protoporphyrin IX-BchH complex can be isolated from this overproducing strain of *E. coli* (data not shown) and this will provide important information on the role of this protein in the catalysis of Mg chelation.

Inspection of Fig. 2 reveals that the three chelatase subunits are not overproduced by the same amount and, notwithstanding possible effects such as an incapacity to bind the protein stain, BchD is present in the lowest amount. We believe that the activities reported here are limited by the amount of BchD present. Future studies need to investigate the stoichiometry of the subunits by using purified components.

The requirement for ATP is consistent with measurements of chelatase activity conducted on extracts of chloroplasts, and it has been proposed that ATP might activate the enzyme prior to catalysis of Mg insertion (10). It is interesting to note that the enzyme cobaltochelatase, which has been purified from *Pseudomonas denitrificans* (32), has several aspects in common with the Mg chelatase reported here, including a requirement for ATP. Cobaltochelatase catalyzes the insertion of cobalt into the corrin ring during coenzyme B₁₂ biosynthesis; it is a complex enzyme consisting of a monomeric 140-kDa protein and a heteropolymeric 450-kDa protein composed of 37- and 70-kDa subunits. The 140-kDa protein is the product of the *cobN* gene and it binds the tetrapyrrole substrate, whereas the 450-kDa CobS/T complex contains an ATP binding site. The parallels with the Mg chelatase are extremely persuasive: BchH, -I, and -D have molecular masses of approximately 140, 70, and 38 kDa, respectively, and we have evidence that BchH, like CobN plays a role in binding tetrapyrrole. Furthermore, there is 31% identity between BchH/CobN (23), although there are no significant homologies for BchI/CobS and BchD/CobT, apart from the fact that both BchI and CobS contain an ATP-binding site consensus. Finally, it is interesting to note that a protein complex of 450 kDa might be susceptible to centrifugation to the extent that it could behave as a membrane-bound component in a differential centrifugation experiment; if a complex such as the cobaltochelatase

also exists for the Mg chelatase, it could explain the sedimentation behavior of the Mg chelatase activity observed in chloroplast extracts (8).

In a previous paper (21) on the overproduction and assay of BchM, the methyltransferase enzyme that follows the chelation reaction (see Fig. 1), we have suggested that the genes *ch42* from *Arabidopsis* (22) and olive from *Antirrhinum* (23) might encode components on the Mg chelatase. The present work further strengthens this proposed function; since the protein sequence deduced from *ch 42* has 49% identity with BchI, and that from olive has 39% identity with BchH, it is reasonable to propose that both plant genes encode subunits of the Mg chelatase. An interesting question remains; is there an equivalent of the BchD protein in other photosynthetic organisms? If there is, it will further strengthen the relevance of the bacterial enzyme to the study of Mg chelatase in plants.

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