Heterologous Expression of the *Rhodobacter capsulatus* BchI, -D, and -H Genes That Encode Magnesium Chelatase Subunits and Characterization of the Reconstituted Enzyme*

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Magnesium chelatase inserts Mg$^{2+}$ into protoporphyrin IX in the chlorophyll and bacteriochlorophyll biosynthetic pathways. In photosynthetic bacteria, the products of three genes, *bchI*, *bchD*, and *bchH*, are required for magnesium chelatase activity. These genes from *Rhodobacter capsulatus* were cloned separately into expression plasmids pET3a and pET15b. The pET15b constructs produced NH$_2$-terminally His$_6$-tagged proteins. All proteins were highly expressed and were purified to near homogeneity. The BchI and BchH proteins were soluble. BchD proteins were insoluble, inactive inclusion bodies that were renatured by rapid dilution from 6M urea. The presence of BchI in the solution into which the urea solution of BchD was diluted increased the yield of active BchD. A molar ratio of 1 BchI:1 BchD was sufficient for maximum renaturation of BchD. All of the proteins were active in the magnesium chelatase assay except His-tagged BchI, which was inactive and inhibited in incubations containing non-His-tagged BchI. Expressed BchH proteins contained tightly bound protoporphyrin IX, and they were susceptible to inactivation by light. Maximum magnesium chelatase activity per mol of BchD occurred at a stoichiometry of 4 BchI:1 BchD. The optimum reaction pH was 8.0. The reaction exhibited Michaelis-Menten kinetics with respect to protoporphyrin IX and BchH.

Expression of the three *R. sphaeroides* genes in *Escherichia coli* showed that all three gene products, plus ATP, Mg$^{2+}$, and protoporphyrin IX, were required for magnesium chelatase activity (4, 5). An enzyme activation step involving the BchI and BchD subunits was observed (5) that was similar to that reported for the pea magnesium chelatase (6). However, very low expression of the BchD protein limited further characterization of magnesium chelatase (5). Heterologous expression of individual subunits and reconstitution of magnesium chelatase activity has recently been described for tobacco (7), the cyanobacterium *Synechocystis* sp. PCC 6803 (8), and the green bacterium *Chlorobium vibrioforme* (9). However, characterization of the reaction using recombinant proteins has been limited due to low levels of expression and poor recovery of activity from these systems.

We now report the cloning and high level expression of the magnesium chelatase genes from *R. capsulatus*, the purification of the gene products, and characterization of the reconstituted enzyme. Portions of this work were previously published in abstract form (10).

EXPERIMENTAL PROCEDURES

Construction of Plasmids for Expression of bchI, bchD, and bchH—The plasmid pRPS404 (11), which contains a 44-kilobase pair region of the *R. capsulatus* photosynthetic gene cluster, was used as a PCR template throughout. A modified T-vector was constructed between the EcoRI and BamHI sites of pBluescript (KS) (Stratagene, La Jolla, CA) essentially as described in Ref. 12, and this vector was used to subclone the bchI and bchD genes as described below. The oligonucleotides 5’-GGATCATCTTGCGGAAACTGT-3’ and 5’-ATGACTACC GCCGCT-GCTGACTTCAACCCCTGTG-3’ were used to amplify the bchID region of the photosynthetic gene cluster by PCR using Taq DNA polymerase (Stratagene). The 3-kilobase pair PCR product was then cloned directly into the T-vector, yielding plasmid pKSbchID. This cloning created an NdeI restriction site at the bchI translation start site. A region containing the bchI gene and the 5’ portion of bchD was then subcloned between the NdeI and BamHI sites of either pET3a (Novagen, Madison, WI) to create pBchI or pET15b (Novagen) to create pHisBchI. This cloning created an NdeI restriction site at the bchI translation start site. A region containing the bchH gene and the 5’ portion of bchD was then subcloned between the NdeI and BamHI sites of either pET3a (Novagen, Madison, WI) to create pBchH or pET15b (Novagen) to create pHisBchH. The oligonucleotides 5’-AGGGCCCATATGACGAGCAAGGCTAGTC-GGCCCTTG-3’ and 5’-ACCGTTGACGAGAGACCCTGCGG-3’ were used to amplify the bchD gene by PCR using Pfu DNA polymerase. These oligonucleotides introduced NdeI and BgII restriction sites. The PCR product was then either digested and cloned directly into pET3a to create pBchH or into the pCRBlunt (Invitrogen, Carlsbad, CA) vector to create pCRB-chH and then subcloned into pET15b to create pHisBchH.

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1 The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
The magnesium chelatase genes in these plasmids were sequenced to check for errors using a Dye-Deoxy terminator kit (PE Applied Biosystems, Foster City, CA) with the Applied Biosystems model 377 sequencer. The first subclone of bchI, which was used to make the expression clones, was sequenced completely, and the final bchI expression clone was sequenced in from the 5′-end to verify that they were in frame. The first bchD and bchH subclones were sequenced 600 base pairs in each end, and the final bchD and bchH expression clones were sequenced in from the 5′-end to verify that they were in frame. No errors were detected.

Expression of BchI, BchD, and BchH in E. coli—E. coli BL21 (DE3) pLysS (Novagen) strains containing the expression plasmids were grown at 25 or 37°C in 1 liter of LB medium (13) containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol until the A₆₀₀ of the cultures was 0.8. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. After 4 h, the cells were harvested by centrifugation. For the expressed pET3a constructs, the cells from 1 liter of culture were resuspended in 20 ml of 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 4 mM DTT. The suspension was then frozen at −20°C, thawed once, and then lysed completely by passage through a French pressure cell (SLM, Urbana, IL) at 20,000 p.s.i. For expression from pET15b constructs, the cells from 1 liter of culture were resuspended in 20 ml of 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 15 mM MgCl₂, and 5 mM imidazole. This suspension was frozen at −20°C, thawed once, and then lysed by passage through a French pressure cell at 20,000 p.s.i.

Purification of the Expressed Proteins—The BchH protein was purified essentially as described previously for the protein from R. sphaeroides (5).

The BchD protein was expressed as inclusion bodies, and these were purified as described in the pET system manual (Novagen). The inclusion bodies were then solubilized in 6 M urea, 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 4 mM DTT. The solubilized protein was loaded onto a RESOURCE Q (Amersham Pharmacia Biotech) cation exchange chromatography column that was pre-equilibrated in the same buffer, and then the protein was eluted with this buffer containing a linear gradient of 0–1 M NaCl, in 10 column volumes. The BchD protein eluted at 0.4 M NaCl.

The Bch expression lysate was centrifuged at 30,000 × g for 30 min, and then polyethylene glycol-8000 (molecular weight, 8000) was added to the soluble supernatant to a concentration of 10% (w/v). The mixture was placed on ice for 30 min and then centrifuged for 30 min at 10,000 × g, the pellet was discarded, and polyethylene glycol-8000 was added to a final concentration of 25% (w/v). After standing for 2 h on ice, the solution was centrifuged at 30,000 × g for 30 min. The supernatant was discarded, and the precipitate was washed once with 30% (w/v) polyethylene glycol-8000 and then redissolved in 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 4 mM DTT. The BchH protein was then purified by cation exchange chromatography as described above for BchD but without urea in the buffers. BchH eluted at 0.3 M NaCl.

The HisBchH and HisBchI proteins were purified on a Ni²⁺-chelating column as described in the pET system manual, except that the eluted proteins were immediately desalted by Sephadex G-25 (Amersham Pharmacia Biotech) chromatography into 6% glycerol, 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 4 mM DTT.

The HisBchD protein was expressed as inclusion bodies, and these were purified as described in the pET system manual (Novagen). The solubilized protein was then further purified on a Ni²⁺-chelating column under denaturing conditions in 6 M urea as described in the pET system manual. After the protein was eluted, the buffer was changed to 6 M urea, 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 4 mM DTT.

Assay for Magnesium Chelatase—The assay mixture contained, in 50–1000 µl, the following: 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, 4 mM DTT, 4 mM ATP, 20 mM phosphocreatine, 20 units/ml of rabbit muscle creatine phosphokinase, 4 µM protoporphyrin IX, and various amounts of recombinant proteins as described in the figure and table legends. The assay mixtures were incubated for 30 or 60 min at 30 °C and stopped by the addition of 9 ml of acetonitril:H₂O:32% (w/v) NH₃ (80:20:1, v/v/v) per ml of incubation mixture and analyzed by fluorescence spectroscopy with the excitation wavelength set at 418 nm and the emission wavelength set at 596 nm. The intensity of the emission at 596 nm was proportional to the concentration within the range of 1 nM to 1 µM. A standard curve was used to determine the amount of magnesium-protoporphyrin IX formed in the assay.

Antiserum to BchD and Immunoblotting—Antiserum to HisBchD was raised in a New Zealand White rabbit using standard procedures (14). The antiserum was cross-absorbed onto an E. coli lysate column.
For reconstitution of activity, BchD (or HisBchD), solubilized in 6 M urea, was added directly to the assay mixture containing the other two protein components. BchD, therefore, refolded to an active form in the reaction mixture during the incubation, as was recently reported for *C. vibrioforme* BchD (9). Although this method of assaying magnesium chelatase was useful for reconstitution experiments, it was unsatisfactory for enzyme kinetic studies because the proportion of BchD that is in the active form in the assay mixture could not be controlled. The factors that mediate renaturation of BchD were therefore examined as described below. Renatured BchD (or HisBchD) provided a consistent preparation for further study of the magnesium chelatase reaction.

Efficient *in vitro* renaturation of BchD required DTT and ATP (Table I). Moreover, the degree of renaturation was increased by the presence of BchI. A time course of the renaturation of BchD at 0 °C with BchI, ATP, and MgCl₂ indicated that BchD was maximally renatured at 90 min (data not shown). The concentration of BchD in the medium did not appear to have an effect on the renaturation efficiency, because approximately equal specific activity was obtained with the three concentrations tested (Table I). The slightly lower activity at higher BchD concentrations was caused by the higher concentrations of urea in the assay medium, which ranged from 30 to 120 mM in this experiment. A BchI:BchD molar ratio of 1:1 was sufficient for maximal recovery of active BchD (Table II). Neither further stimulation or inhibition of the renaturation was observed at higher BchI:BchD ratios. In the *R. capsulatus* chromosome, *bchI* is 5° to *bchD* and is part of the same transcription unit. This implies that BchI is translated before BchD, and it is proposed that its presence may aid in the folding of BchD to its active form *in vivo*.

Because BchD was solubilized in 6 M urea and added to assay medium by dilution from the urea solution, it was important to determine the effect of urea on the magnesium chelatase reaction. The reaction was progressively inhibited at increasing urea concentrations, with approximately 20% inhibition at 100 mM, 50% inhibition at 250 mM, and 90% inhibition at 800 mM (Fig. 5). Because the final urea concentration in the magnesium chelatase assays was usually 30–60 mM, its presence in the assay medium had only a minor effect on activity.

Reconstitution of *R. capsulatus* Magnesium Chelatase Mutants—*R. capsulatus* mutants ZY6 and DB350 contain inser- tional disruptions of the *bchH* and *bchI* genes, respectively (2, 17). Magnesium chelatase activity was absent in extract of either mutant alone, but was present in a mixture of the extracts from the two mutants (Table III). Activity was reconstituted in extract of ZY6 cells supplemented with recombinant BchH. Neither BchD, BchI, nor a combination of BchD and BchI reconstituted activity of ZY6 extract. Fractionated ZY6 extract from which BchD was removed by high speed centrifugation and ion exchange chromatography required addition of

![Figure 2: Spectra of HisBchH protein.](http://www.jbc.org/)

A, absorption spectrum of 166 μg of His-BchH in 1 ml of 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 1 mM DTT. Dashed line is the fully corrected fluorescence excitation spectrum of 66 μg of His-BchH in 1 ml (1 μM) of 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, and 1 mM DTT; solid line is the fluorescence excitation spectrum of 0.5 μM standard protoporphyrin IX. Emission was monitored at 635 nm.
were obtained from 100 mg of BchD, and the indicated amounts of HisBchI. Incubation was for 60 min BchD and BchI. The requirement for both proteins is consistent with the absence of the BchD in extracts of strain DB350 (Fig. 10). This ratio was also obtained when HisBchD was used (data not shown).

**DISCUSSION**

Three *R. capsulatus* genes, *bchI*, *bchD*, and *bchH*, have been cloned and overexpressed in *E. coli*, and the expressed proteins have been purified and reconstituted in vitro to yield magnesium chelatase activity. The characterization of this activity and of the individual subunits that we report here suggests that Mg$^{2+}$ chelation is a multistep reaction and that the individual subunits may have multiple roles in the assembly of active enzyme and in the reaction.

The *bchI*, *bchD*, and *bchH* genes were first shown to be involved in Mg$^{2+}$ chelation in *R. capsulatus* by insertional mutagenesis and analysis of the mutant phenotypes (2, 17). Genes homologous to *bchI*, *bchD*, and *bchH* have been described in several photosynthetic bacteria and plants. Reconstitution of active magnesium chelatase from the expressed genes was first reported for *R. sphaeroides* (18). In that report, *bchI* and *bchD* were co-expressed, but it was not possible to express active *bchD* alone. Reconstituted in vitro activity was limited by the amount of BchD present in the reaction mixture. The *bchI*, *bchD*, and *bchH* genes from the green bacterium *C. vibrioforme* were cloned, separately expressed, and recombined in vitro to yield magnesium chelatase activity (9). To reconstitute activity, it was necessary to treat the BchD protein, which was expressed as inclusion bodies, with 6 M urea to solubilize it and then add the solubilized BchD to assay mixtures containing BchI and BchH. In contrast, three genes from the cyanobacterium *Synechocystis* sp. PCC 6803, designated *chlI*, *chlD*, and *chlH*, respectively, were cloned and separately expressed to produce soluble proteins, and the proteins could be recombined in vitro to yield magnesium chelatase activity (8). Although the predicted *Synechocystis* sp. PCC 6803 ChlI and ChlH proteins are quite similar to the predicted BchI and BchH proteins (51% and 41% identity to the *R. capsulatus* proteins, respectively), ChlD is less similar to BchD (32% identity), and there are large gaps in the aligned sequences. All of these reports demonstrated that all three subunits are required for magnesium chelatase activity. However, further characterization of the activity and characterization of the individual subunits have not been reported.
Rhodobacter capsulatus Magnesium Chelatase

TABLE I

Requirements for BchD renaturation

BchD protein was solubilized in 6 M urea, 10 mM Tricine-NaOH, pH 8.0, and 2 mM DTT and then renatured by rapid dilution into 20 volumes of renaturation medium and maintained at 0 °C for 60 min. Complete renaturation medium contained 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl₂, 4 mM DTT, 4 mM ATP, an ATP regenerating system consisting of 20 mM phosphocreatine and 20 units/ml of creatine phosphokinase; and the indicated amount of BchI protein. For the magnesium chelatase assay, 10–50 μg of the renatured BchD-BchI mixture was supplemented with 4 μM protoporphyrin IX and 26 μM of BchH protein, additional BchI was added when needed to bring the final BchI content to the indicated level, the final volume was adjusted to 100 μl with complete renaturation medium, and the mixture was incubated for 30 min at 30 °C. ARS, ATP regenerating system.

| Renaturation medium | Mg chelatase assay medium | Mg chelatase activitya |
|---------------------|---------------------------|-------------------------|
|                     | BchI | BchD | BchI:BchD | pmol μg⁻¹ BchD | % maximum |
| Experiment 1        |      |      |           |               |           |
| Complete            | 11.8 | 11.2 | 1.7       | 11.57 ± 0.35  | 100       |
| +5 μM protoporphyrin IX | 11.8 | 11.2 | 1.7       | 12.14 ± 0.47  | 105       |
| −BchI               | 11.8 | 11.2 | 1.7       | 4.41 ± 0.53   | 38        |
| Experiment 2        |      |      |           |               |           |
| Complete            | 6.2  | 2.5  | 4.0       | 13.80 ± 0.50  | 100       |
| −ARS                | 6.2  | 2.5  | 4.0       | 16.50 ± 0.25  | 120       |
| −ATP, −ARS          | 6.2  | 2.5  | 4.0       | 2.80 ± 0.15   | 20        |
| −ATP, −ARS, −MgCl₂  | 6.2  | 2.5  | 4.0       | 2.40 ± 0.30   | 17        |
| Experiment 3        |      |      |           |               |           |
| Complete            | 23.6 | 22.4 | 1.7       | 10.60 ± 0.80  | 100       |
| −ARS                | 23.6 | 22.4 | 1.7       | 8.45 ± 0.44   | 80        |
| −ATP, −ARS          | 23.6 | 22.4 | 1.7       | 2.22 ± 0.37   | 21        |
| DTT                 | 23.6 | 22.4 | 1.7       | 0.76 ± 0.12   | 7         |
| Experiment 4        |      |      |           |               |           |
| Complete            | 13.6 | 5.5  | 4.0       | 15.30 ± 1.20  | 100       |
| Complete            | 28.1 | 11.4 | 4.0       | 14.05 ± 1.20  | 92        |
| Complete            | 56.3 | 22.8 | 4.0       | 13.75 ± 1.80  | 90        |

a Activity values listed are means and standard deviations of duplicate (Experiments 1–3) or triplicate (Experiment 4) assays.

TABLE II

Effect of the BchI:BchD ratio on BchD renaturation

BchD protein solubilized and renatured as described in the legend to Table I. The renaturation medium contained the indicated ratio of BchI and BchD proteins. The renatured BchD-BchI mixture was then assayed for magnesium chelatase activity. All assays (100 μl) contained renaturation medium and 4 μM protoporphyrin IX, 11.2 μg of BchD, 33 μg of HisBchH protein, and additional BchI protein to bring the final amount to 42 μg. Assay mixtures were incubated for 60 min at 30 °C.

| BchD renaturation conditions | Mg chelatase activitya |
|-----------------------------|-------------------------|
|                            | BchI | BchD | BchI:BchD | pmol μg⁻¹ BchD | % maximum |
|                            | μg   | μg   | mol:mol   |               |           |
| 0.0                         | 11.4 | 0.0  | 0.0       | 3.6 ± 0.14    | 48        |
| 4.9                         | 11.4 | 0.7  | 0.7       | 6.5 ± 0.80    | 87        |
| 8.4                         | 11.4 | 1.2  | 1.2       | 6.5 ± 0.80    | 87        |
| 14.0                        | 11.4 | 2.0  | 2.0       | 6.8 ± 1.00    | 91        |
| 17.5                        | 11.4 | 2.5  | 2.5       | 7.5 ± 0.15    | 100       |
| 22.4                        | 11.4 | 3.2  | 3.2       | 7.2 ± 0.90    | 96        |
| 28.0                        | 11.4 | 4.0  | 4.0       | 7.5 ± 0.00    | 100       |
| 38.5                        | 11.4 | 5.5  | 5.5       | 7.1 ± 0.60    | 95        |

a Activity values listed are means and standard deviations of duplicate assays.

As an aid to rapid enzyme subunit purification by nickel-chelating column chromatography, the three R. capsulatus magnesium chelatase proteins were expressed as NH₂-terminally His-tagged fusion constructs. For two of the subunits, BchD and BchH, the His-tagged proteins were effective in reconstituting magnesium chelatase activity. However, His-tagged BchD did not reconstitute activity and, moreover, it acted as an inhibitor in incubations containing non-His-tagged BchI. The most likely explanation for the inhibition is that the His tag prevents catalysis in some way but still allows HisBchI to interact with BchH and/or BchD and blocks BchI from interacting constructively with these subunits.

The BchH and BchI proteins were expressed as soluble active proteins in E. coli, but BchD was expressed as insoluble inclusion bodies. The insoluble BchD was solubilized in 6 M urea and was refolded by dilution of the urea to yield active protein. Optimal refolding required ATP, DTT, and BchI. It has been shown that the presence of a reducing agent, such as DTT, is required for stabilizing magnesium chelatase (19–22) and that magnesium chelatase is sensitive to sulfhydryl reagents (21–24). The require-

![Figure 5: Effect of urea concentration on magnesium chelatase activity. Assays (50 μl) were done in triplicate and contained 1.7 μg of renatured BchD, 3.9 μg of BchI, and 33 μg of HisBchH in assay buffer with urea added. Incubation was for 30 min at 30 °C. Error bars indicate the S.D. about the mean. The line is the best fit exponential curve for the data points.](https://example.com/figure5.png)
ment of DTT for maintaining and reconstituting the activity of BchD, both in the refolding process and in the subsequent enzyme assay, suggests that it is this subunit that is most sensitive to oxidizing conditions. The ATP requirement is consistent with the hypothesis that ATP may help to refold and stabilize BchD by binding to its active site. It has been reported that pea magnesium chelatase undergoes irreversible loss of activity at room temperature in the absence of ATP (6, 25).

On the R. capsulatus chromosome, the bchD open reading frame is immediately downstream of the bchI open reading frame and is translated from the same transcript. The same organization of bchI and bchD occurs in C. vibrioforme (26). This conserved gene organization, together with the fact that BchI is required for optimal refolding of BchD in vitro, suggests that BchI may be essential for the efficient refolding and/or stabilization of BchD in vivo. The difference in the optimal stoichiometry of 1 BchI:1 BchD for refolding compared with 4 BchI:1 BchD for assay of magnesium chelatase activity suggests that the function of BchI in BchD refolding and magnesium chelatase activity may not be directly linked to one another. A lag phase in the kinetics of the reaction, as was

| Cell extract(s) added | Recombinant protein(s) added | Relative Mg-protoporphyrin IX formation |
|----------------------|-------------------------------|---------------------------------------|
|                      | ZY6 µg |  DB350 µg | BchH µg | BchD µg | BchI µg | %                      |
| **Experiment 1**     |        |          |        |        |        |                        |
| 0                    | 0      | 0        | 30.0   | 1.8    | 6.0    | 100                    |
| 250                  | 0      | 0        | 0.0    | 0.0    | 0.0    | 0                      |
| 0                    | 330    | 0        | 0.0    | 0.0    | 0.0    | 0                      |
| 250                  | 0      | 30.0     | 0.0    | 0.0    | 0.0    | 180                    |
| 250                  | 0      | 0        | 0.0    | 1.8    | 0.0    | 0                      |
| 250                  | 0      | 0        | 0.0    | 0.0    | 6.0    | 0                      |
| 40 (I) + 20 (D)      | 0      | 30.0     | 0.0    | 0.0    | 0.0    | 160                    |
| 40 (I)               | 0      | 30.0     | 0.0    | 0.0    | 0.0    | 0                      |
| 40 (I)               | 0      | 30.0     | 0.0    | 1.8    | 0.0    | 50                     |
| 20 (D)               | 0      | 30.0     | 0.0    | 0.0    | 0.0    | 5                      |
| 20 (D)               | 0      | 30.0     | 0.0    | 0.0    | 6.0    | 500                    |
| **Experiment 2**     |        |          |        |        |        |                        |
| 0                    | 0      | 0        | 10.0   | 1.8    | 5.2    | 100                    |
| 250                  | 330    | 0        | 0.0    | 0.0    | 0.0    | 50                     |
| 0                    | 330    | 10.0     | 0.0    | 0.0    | 0.0    | 0                      |
| 0                    | 330    | 0.0      | 1.8    | 0.0    | 0.0    | 0                      |
| 0                    | 330    | 0.0      | 0.0    | 5.2    | 0.0    | 0                      |
| 0                    | 330    | 0.0      | 1.8    | 5.2    | 500    | 50                     |

FIG. 6. Immunoblot. The probe was antibody to purified R. capsulatus BchD protein. Lane 1 contains purified HisBchD protein; lane 2 contains extract of R. capsulatus strain ZY6, which has a disrupted bchH gene; and lane 3 contains extract of R. capsulatus strain DB350, which has a disrupted bchI gene. Migration positions of standard proteins with the indicated molecular masses (in kDa) are shown at the left of the image.

FIG. 7. pH optimum for magnesium chelatase. Assays (100 µl) were done in triplicate and contained 1.4 µg of renatured BchD, 4.8 µg of BchI, and 66 µg of HisBchH in assay buffer adjusted to the indicated pH and then incubated for 30 min at 30 °C. Error bars indicate the S.D. about the mean.

to oxidizing conditions. The ATP requirement is consistent with the hypothesis that ATP may help to refold and stabilize BchD by binding to its active site. It has been reported that pea magnesium chelatase undergoes irreversible loss of activity at room temperature in the absence of ATP (6, 25).

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FIG. 9. Concentration dependence on protoporphyrin IX and BchH. All assays (100 μl) contained 5.2 μg of BchI and 8.4 μg of BchD (2 μl of a 6 mM urea solution added to start the reaction). A, for protoporphyrin IX, the incubation contained 6 μg of HisBchH, and the free protoporphyrin IX concentration was varied from 0.5 to 4 μM. B, for BchH, the free protoporphyrin IX concentration was 4 μM, and HisBchH varied from 0.4 to 30 μg. Incubation was for 60 min at 30 °C. Results are plotted in double-reciprocal form. The lines are the best fit linear extrapolations for the data points.

FIG. 10. Optimal BchI:BchD stoichiometry. Assays (100 μl) in triplicate contained 10.8 μg of BchI, 33 μg of HisBchH, and an amount of renatured BchD sufficient to provide the indicated BchI:BchD molar ratio. Incubation was 30 min at 30 °C. Error bars indicate the S.D. about the mean.

The optimal stoichiometry of 4:1 for the BchI and BchD proteins suggests that they may form a complex in that ratio during an ATP-dependent activation step as shown in Reaction 2. For the purposes of this model, it is postulated that the complex has the composition BchI:BchD, although further work will be necessary to determine whether the 4:1 optimal component concentration ratio for enzyme activity actually indicates the existence of a complex with this ratio. It is not known whether this step requires ATP hydrolysis.

4 BchI + BchD + ATP + Mg2+ ⇌ BchIe•BchD

REACTION 2
The product of this activation is then able to use the protoporphyrin IX bound to the BchH protein as a substrate for ATP-dependent Mg\(^{2+}\) insertion as shown in Reaction 3. This reaction apparently requires ATP hydrolysis (6).

\[
\text{BchI}^*\text{BchD} + \text{BchH}^p + \text{ATP} + \text{Mg}^{2+} \leftrightarrow \text{Mg-protoporphyrin IX} + \\
\text{BchH} + \text{BchI}^*\text{BchD} + \text{ADP} + \text{Pi},
\]

REACTION 3

This reaction probably occurs in more than one step. The constancy of the reaction rate after the initial lag phase is completed suggests that the BchI*BchD complex, once it is formed, is stable during continued Mg\(^{2+}\) chelation cycles and does not dissociate in Reaction 3.

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