Magnesium chelatase from *Rhodobacter sphaeroides*: initial characterization of the enzyme using purified subunits and evidence for a BchI–BchD complex

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The enzyme magnesium-protoporphyrin IX chelatase (Mg chelatase) catalyses the insertion of Mg into protoporphyrin IX, the first committed step in (bacterio)chlorophyll biosynthesis. In the photosynthetic bacterium *Rhodobacter sphaeroides*, this reaction is catalysed by the products of the *bchI*, *bchD* and *bchH* genes. These genes have been expressed in *Escherichia coli* so that the BchI, BchD and BchH proteins are produced with N-terminal His₆ affinity tags, which has led to the production of large amounts of highly purified, highly active Mg chelatase subunits from a single chromatography step. Furthermore, BchD has been purified free of contamination with the chaperone GroEL, which had proven to be a problem in the past. BchD, present largely as an insoluble protein in *E. coli*, was purified in 6 M urea and refolded by addition of BchI, MgCl₂ and ATP,

yielding highly active protein. BchI/BchD mixtures prepared in this way were used in conjunction with BchH to determine the kinetic parameters of *R. sphaeroides* Mg chelatase for its natural substrates. We have been able to demonstrate for the first time that BchI and BchD form a complex, and that Mg²⁺ and ATP are required to establish and maintain this complex. Gel filtration data suggest that BchI and BchD form a complex of molecular mass 200 kDa in the presence of Mg²⁺ and ATP. Our data suggest that, *in vivo*, BchD is only folded correctly and maintained in its correct conformation in the presence of BchI, Mg²⁺ and ATP.

Key words: bacteriochlorophyll, chlorophyll, histidine tag, refolding, tetrapyrrole.

INTRODUCTION

In all photosynthetic organisms, the first step unique to the synthesis of (bacterio)chlorophyll is the insertion of Mg into protoporphyrin IX. The enzyme which catalyses this step, magnesium-protoporphyrin IX chelatase (Mg chelatase), lies at the branch point of (bacterio)chlorophyll and haem biosynthesis, and is therefore of fundamental importance in the biosphere, since it is responsible for directing tetrapyrroles into the Mg branch of the pathway.

The photosynthetic bacterium *Rhodobacter sphaeroides* has often been used as a model system for the study of chlorophyll biosynthesis, and the Mg chelatase enzyme from this bacterium has been studied [1–3]. However, these studies were limited by the fact that enzyme activity could only be measured using whole cells, and only recently have there been any reports of measurable enzyme activity using crude extracts of broken cells [4,5]. Studies on the plant enzyme have largely been conducted using whole chloroplasts or crude chloroplast extracts [6–8], although recently the components of the enzyme have been partially purified from pea chloroplasts [9].

The first identification of the protein components of any Mg chelatase was carried out by producing the BchI, BchD and BchH proteins from *R. sphaeroides* in *Escherichia coli* and demonstrating that cell extracts containing these proteins could be combined to form an active enzyme [10]. Subsequently, the same approach was used to identify the protein components (ChlI, ChlD and ChlH) from *Synechocystis* [11] and *Chlorobium vibrioforme* [12]. A similar approach has been used to identify the

Mg chelatase proteins from tobacco [13]. In all of the organisms studied so far, Mg chelatase consists of three proteins: BchI/ChlI (36–42 kDa), BchD/ChlD (60–74 kDa) and BchH/ChlH (130–150 kDa). The respective I, D and H subunits from the various different species exhibit high levels of amino acid similarity.

The availability of purified subunits of the *Synechocystis* Mg chelatase has allowed us to investigate the biochemical and enzymological properties of this enzyme, and to begin to understand its mechanism and catalytic properties [14]. It is also important to study Mg chelatases from different organisms in order to highlight the common properties, and to investigate the differences, for this enzyme from different sources. With this in mind, we expressed the genes encoding the *R. sphaeroides* Mg chelatase subunits in *E. coli* [10]. However, until now, highly purified subunits of the *R. sphaeroides* enzyme have not been available. In a previous report the *R. sphaeroides* BchI and BchH proteins were purified, but BchD was only partially purified and was heavily contaminated with GroEL [15]. This is not ideal, since both GroEL and Mg chelatase utilize ATP, and therefore any studies on ATP utilization by Mg chelatase using GroEL-contaminated BchD will be somewhat unreliable.

In the present study, we have incorporated His₆ affinity tags at the N-termini of the BchH, BchI and BchD proteins, which has facilitated the rapid purification of these proteins to homogeneity, and we show that affinity-purified BchD is completely free of GroEL. BchD expressed in *E. coli* is present mainly in the insoluble fraction of the cells, and the small amount of BchD present in the soluble fraction consists primarily of high-

Abbreviations used: DTT, dithiothreitol; Mg chelatase, magnesium-protoporphyrin IX chelatase; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; MgP, magnesium protoporphyrin IX.

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molecular-mass aggregates, suggesting that the protein is not folded correctly in *E. coli*. The protein can be purified from the insoluble fraction of the cells in 6 M urea and added to the Mg chelatase assay to yield protein with a significantly higher specific activity than BchD purified from the soluble fraction. Furthermore, the specific activity of the soluble BchD is increased significantly by pre-incubation in 6 M urea prior to addition to the assay. BchD purified in 6 M urea is refolded on addition of BchI, MgCl₂ and ATP, which provides a useful starting point for the study of this enzyme.

We have used BchD prepared and refolded in this way to investigate some of the basic properties of the enzyme. The relative concentrations of the three proteins required for optimal activity *in vitro* and the steady-state kinetic parameters of the enzyme for its natural substrates were determined. The facts that isolated BchD is misfolded and that unfolded BchD is refolded by BchI, Mg²⁺ and ATP suggested the possibility that the *in vivo* conformation of BchD is in a complex with BchI. We have demonstrated that BchI associates specifically with BchD in the presence of Mg²⁺ and ATP, and that this association is only maintained in the continued presence of Mg²⁺ and ATP. Gel filtration suggests that BchI and BchD form a complex of molecular mass 200 kDa in the presence of Mg²⁺ and ATP.

EXPERIMENTAL

Production of recombinant proteins in *E. coli*

The *R. sphaeroides bchI* and *bchH* genes were excised from the plasmids pETBCHI and pETBCHH [10] with *NdeI* and *BamHI* and cloned separately into the expression vector pET14b [16], yielding plasmids pET14b-BCHI and pET14b-BCHH. The *bchD* gene was amplified by PCR using gene-specific primers, and cloned into the *NdeI* and *BamHI* sites of pET14b, yielding plasmid pET14b-BCHD. Growth of *E. coli* and induction of recombinant proteins was performed essentially as described in [10].

Purification of the BchI, BchD and BchH proteins

The cell pellets derived from 1 litre of culture were thawed at room temperature and resuspended in 50 ml of cold binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9) and sonicated for 6 × 30 s on ice. Cell debris was removed by centrifugation and the supernatants were processed on a Ni²⁺-agarose affinity column, according to the manufacturer's protocol (Novagen). The recombinant proteins were recovered by elution with imidazole buffer (500 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9). BchI and BchD purified from the soluble fraction were dialysed into 50 mM Tricine/NaOH, pH 7.9, 20% (v/v) glycerol and 1 mM dithiothreitol (DTT), pH 7.9, and stored at -20 °C. BchH was dialysed into 50 mM Tricine/NaOH, pH 7.9, 50% (v/v) glycerol and DTT, pH 7.9, and stored at -70 °C.

BchD was also purified from the *E. coli* insoluble fraction. The cell debris derived from 1 litre of cells was resuspended in binding buffer containing 6 M urea, incubated on ice for 1 h and then centrifuged (100 000 g; 1 h). The supernatant was processed on a Ni²⁺-agarose affinity column as above, except that all the column buffers contained 6 M urea. After elution, the protein was dialysed into 50 mM Tricine/NaOH (pH 7.9)/6 M urea, and stored at 4 °C.

Refolding of BchD

This was achieved by diluting 100 µl of BchD in 6 M urea (typically 8 µg) with 900 µl of 50 mM Tricine, pH 7.9, containing

BchI (typically 20 µg), MgCl₂ and ATP, so that the final concentrations of MgCl₂ and ATP in the refolding reaction were 20 mM and 4 mM respectively. The mixture was incubated at 34 °C for 5 min.

Gel filtration

Gel filtration was carried out using a Waters HPLC system fitted with a photodiode array detector and a Shodex KW-803 column (0.8 cm × 30 cm). The column was equilibrated with 50 mM Tricine/NaOH (pH 7.9)/100 mM NaCl and proteins were chromatographed in the same buffer at 20 °C at a flow rate of 0.8 ml/min. The column was calibrated with the following molecular mass markers: horse heart cytochrome *c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa), sweet-potato β-amylase (200 kDa) and glutamate dehydrogenase (330 kDa). To determine the native molecular masses of BchI, BchD and BchH, 50 µl aliquots of purified protein were loaded on to the column. Elution of proteins was monitored by recording absorbance spectra from 230 nm to 330 nm every 2 s. Chromatograms were derived from the accumulated absorbance scans using Millenium software (Waters).

Mg chelatase assays

The standard assay was performed in 100 µl of buffer containing 50 mM Tricine/NaOH, pH 7.9, 12 mM MgCl₂, 1 mM DTT, 4 mM ATP, 6 µM protoporphyrin IX and the amounts of protein indicated in the Figure legends. The assay mixtures were incubated for 30 min at 34 °C, the reactions stopped by addition of 900 µl of acetone/water/32% ammonia (80:20:1, by vol.), and the mixtures centrifuged at 15000 g for 5 min. A 1 ml portion of the aqueous phase was analysed on a SPEX FluoroLog spectrofluorimeter (SPEX Industries Inc.). The excitation wavelength was set to 420 nm and the emission spectrum was recorded between 550 and 650 nm. The fluorescence intensity response of magnesium protoporphyrin IX (MgP) was linear from 1 to 500 pmol when quantified by integration of the peak area of known amounts of authentic MgP (Porphyrin Products, Logan, UT, U.S.A.). The amount of enzymically formed MgP in each assay was quantified from this standard curve.

Continuous assays were performed on a SPEX FluoroLog spectrofluorimeter with a fitted temperature-controlled cuvette holder set at 34 °C. The excitation wavelength was set to 420 nm and the emission wavelength was 596 nm. The assay volume was 1.0 ml and contained 50 mM Tricine/NaOH, pH 7.9, 10 mM MgCl₂, 1 mM DTT, 4 mM ATP, 6 µM protoporphyrin IX and the protein concentrations indicated in the legend to Figure 2. A standard curve was made by diluting known amounts of authentic MgP (Porphyrin Products) in assay buffer containing the same concentrations of ATP, MgCl₂ and Mg chelatase subunits as in the assays.

Measurement of steady-state kinetic parameters

Kinetic parameters were calculated from continuous assays in which the BchD was refolded prior to addition to the assay. Assays (total volume 100 µl) were performed simultaneously and in triplicate using a Bio-Tek F2 Microplate reader fitted with a 420 ± 25 nm excitation filter and a 590 ± 17.5 nm emission filter. This filter combination was selective for MgP, and the response was linear from 0 to 50 pmol of MgP.

The apparent kinetic parameters were obtained by fitting the concentration dependence of the maximum reaction rate for each substrate, with the concentrations of the other two substrates

maintained at or close to saturation, to one of the following equations:

$$v/[E_t] = V_{\max}[S]/(K_m + [S]) \quad (1)$$

$$v/[E_t] = (V_{\max}[S])/(K_m + [S] + [S]^2/K_{si})$$

(simple substrate inhibition) (2)

where $[E_t]$ is the total enzyme concentration and K_{si} is the substrate inhibition constant (the concentration of substrate at which the rate is inhibited by 50%). The kinetic parameters were estimated using the Marquardt–Levenberg curve-fitting algorithm (SigmaPlot, Jandel Scientific, Madera, CA, U.S.A.).

ATPase assays

Release of P_i was measured using the EnzChek Phosphate Assay Kit (Molecular Probes), in which P_i reacts with 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to form ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine in a reaction catalysed by purine nucleoside phosphorylase [17]. This enzymic conversion of MESG results in a spectrophotometric shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product. Assays were performed in quartz cuvettes in a total volume of 1 ml containing 50 mM Tricine/NaOH, pH 7.9, 20 mM $MgCl_2$, 4 mM ATP, 60 mM urea, 1 mM DTT and the concentrations of MESG and purine nucleoside phosphorylase recommended by the manufacturer. The ATPase activity of BchI was measured using 8 μ g of protein; the BchI–BchD complex was prepared by refolding 12.8 μ g of BchD (10 μ l) with 8 μ g of BchI as described above, and the resulting mixture was assayed for ATPase activity. Product formation was monitored continuously in a split-beam spectrophotometer (Shimadzu UV2101PC) with temperature-controlled cuvette holders set at 34 °C. The reference cuvette contained the same components as the sample cuvette, except for the chelatase subunits. All the components were mixed on ice, and the reaction was started by adding the mixture to the cuvettes, which had been prewarmed to 34 °C. P_i formed during the incubation was quantified from a standard curve; KH_2PO_4 was used as the source for P_i to generate the correlation between concentration of P_i and A_{360} .

Other methods

SDS/PAGE and Western blotting were performed as described by Sambrook et al. [18]. Antibody–antigen complexes were detected using horseradish-peroxidase-conjugated goat anti-mouse antisera and the ECL detection reagents (Amersham). Protein concentrations were determined using the Bio-Rad protein assay, with BSA as standard. Porphyrin solutions were prepared as described [14]. Calculation of free Mg^{2+} in solution with ATP was performed using Winmaxchelator v1.70 [19], adjusted for pH, temperature and ionic strength using the constants given by Brooks and Storey [20].

RESULTS

Purification of His-tagged Mg chelatase subunits

In a previous study we cloned *bchI* and *bchD* into plasmid pET3a as one fragment, in the same arrangement as these genes are found on the *R. rubrum* chromosome [10]. *E. coli* cells containing this plasmid were induced with isopropyl β -D-thiogalactoside, and shown to accumulate large amounts of BchI. The presence of BchD in these cells was demonstrated, and BchD was shown to be essential for the Mg chelatase, although it was not possible to visualize BchD by SDS/PAGE [10]. BchD purified from cells containing this plasmid was heavily con-

Figure 1 Purification of His-tagged Mg chelatase subunits

(A) Affinity-purified Mg chelatase subunits were analysed by SDS/PAGE and compared with GroEL. Lanes contained approx. 2 μ g of purified proteins, as follows: 1, molecular mass markers (kDa); 2, BchH; 3, BchI; 4, BchD purified from the soluble fraction; 5, BchD purified from the insoluble fraction in 6 M urea; 6, GroEL. (B) An identical gel was blotted and probed with antibodies against GroEL.

taminated with GroEL [15]. As both Mg chelatase and GroEL utilize ATP, it was felt that studies on *R. rubrum* Mg chelatase would benefit greatly if BchD were purified to homogeneity. This was achieved by cloning the *bchD* gene into pET14b, which allowed the production of large amounts of BchD protein with an N-terminal His₆ tag in *E. coli*. Most of the BchD protein was present in the insoluble fraction of the cells, and this was purified by solubilization in buffer containing 6 M urea, followed by affinity chromatography in the presence of 6 M urea. It was also possible to purify a small amount of BchD protein from the soluble fraction. The *bchH* and *bchI* genes were recloned into pET14b so that the BchH and BchI proteins could be produced with His₆ tags at their respective N-termini, and tagged BchH and BchI proteins were purified by affinity chromatography.

Figure 1(A) shows SDS/PAGE analysis of the His₆-tagged Mg chelatase proteins purified by affinity chromatography. BchH, BchI and both the soluble and urea-solubilized forms of BchD were at least 95% pure after a single chromatography step. Also shown is GroEL, which runs very slightly ahead of BchD. This is expected, as the calculated molecular mass of BchD is 58.5 kDa, and the presence of the His tag is expected to increase the molecular mass to 60.7 kDa, whereas the calculated molecular mass of a GroEL monomer is 57.3 kDa. An identical gel was blotted and probed with antibodies raised against GroEL (Figure 1B). This demonstrates that neither the soluble nor insoluble forms of purified BchD contain GroEL.

Assay and refolding of purified BchD

The yield of pure BchD protein from the soluble fraction was approx. 2 mg/litre, whereas it was possible to purify approx. 80 mg of BchD from the insoluble fraction derived from 1 litre of cells. Numerous attempts were made to refold the protein purified in urea, prior to addition to the assay, without success. However, it was discovered that direct addition of BchD in urea to the Mg chelatase assay mixture, so that the urea was diluted 100-fold, yielded highly active BchD protein. When this was done, the

Table 1 Refolding of BchD

A portion of 500 ng of BchD in 6 M urea was refolded by 10-fold dilution in buffer containing the indicated additions and incubation at 34 °C for 5 min. Where indicated, refolding reactions contained 10 mM MgCl₂, 4 mM ATP and 2 µg of Bchl. An aliquot of each reaction mixture was removed and analysed using the standard assay. Assays contained 50 ng of BchD, 200 ng of Bchl and 10 µg of BchH in a total volume of 100 µl.

Additions	BchD activity (pmol of MgP·min ⁻¹ ·µg of BchD ⁻¹)
None	0
MgCl ₂	0
ATP	0
MgCl ₂ + ATP	0
Bchl	0
Bchl + MgCl ₂	0
Bchl + ATP	0
Bchl + MgCl ₂ + ATP	139.1 ± 7.5

apparent specific activity with respect to BchD was 45.5 ± 4.3 pmol of MgP·min⁻¹·µg of BchD⁻¹, whereas the specific activity of the BchD protein purified from the soluble fraction was 2.7 ± 0.4 pmol of MgP·min⁻¹·µg of BchD⁻¹. Furthermore, it was demonstrated that denaturing the soluble BchD in 6 M urea prior to addition to the assay increased the specific activity of this protein to 27.3 ± 2.5 pmol of MgP·min⁻¹·µg of BchD⁻¹. This suggests that the soluble BchD is largely misfolded or aggregated and that only a small fraction of the protein is correctly folded and active. Furthermore, it appears that unfolded BchD is refolded in the presence of other components of the Mg chelatase assay. We demonstrated that refolding of BchD requires Bchl, Mg²⁺ and ATP (Table 1). ADP did not substitute for ATP in the refolding (results not shown). Optimization of the refolding conditions indicated that BchD was most effectively refolded in the presence of 20 mM MgCl₂ and 4 mM ATP (results not shown).

Gel filtration analysis of Mg chelatase subunits

Purified His-tagged BchH, Bchl and soluble BchD were analysed by gel filtration. The His-tagged BchH protein was eluted at a molecular mass of 137 kDa, which is very similar to the calculated molecular mass for the His-tagged protein of 131.3 kDa and to the molecular mass of 133 kDa observed in SDS/polyacrylamide gels. This shows that His-tagged BchH behaves as a monomer and has the same properties as the non-His-tagged BchH protein purified by Willows et al. [15].

His-tagged Bchl was eluted from the gel filtration column with an apparent molecular mass of 49 kDa. The predicted molecular mass of His-tagged Bchl is 38.4 kDa, and the molecular mass of the protein observed on an SDS/polyacrylamide gel is 40 kDa. Non-His-tagged Bchl was reported to have an apparent molecular mass of 50 kDa in gel filtration [15] and was shown to be a dimer by dynamic light scattering. Our data show that the apparent native molecular mass of His-tagged Bchl is very similar to that of the non-tagged protein.

Most of the soluble BchD protein was eluted in the void volume of the column, suggesting that the protein is present as high-molecular-mass aggregates (results not shown). This could explain why the activity of this BchD was increased so dramatically after treatment with 6 M urea. No evidence was found for a 550 kDa molecular mass species, such as that reported by

Willows et al. [15] for BchD. Interestingly, there was a small peak in the elution profile of soluble BchD corresponding to a molecular mass of 56 kDa, which is very similar to the calculated molecular mass of a BchD monomer (60.7 kDa) and to the molecular mass of 60 kDa exhibited by BchD on an SDS/polyacrylamide gel.

Optimization of the proportions of BchH, Bchl and BchD in enzyme assays

The next objective was to determine optimum proportions of the three proteins to use for kinetic measurements (Figure 2). This was carried out by conducting a series of protein titration experiments in which one of the proteins was present at a saturating concentration, one was present at a limiting concentration and the concentration of the third was varied.

In the first series of experiments, BchH was present at a saturating concentration, Bchl was limiting and the concentration of BchD in the reaction was varied, as shown in Figure 2(A). All the components of the assay were mixed in a cuvette, and BchD was added to give the indicated concentrations and a final concentration of urea of 60 mM. Product formation was monitored using the continuous assay and the maximum rates were plotted (Figure 2A, trace 1). This experiment was then repeated, except that BchD was refolded prior to addition to the assays by mixing increasing concentrations of BchD with constant concentrations of Bchl, MgCl₂ and ATP. An aliquot of each refolding mixture was then added to the rest of the reaction components, and the maximum reaction rate was measured using the continuous assay (Figure 2A, trace 2). The final concentrations of BchH, Bchl and urea were the same as before (Figure 2A, trace 1). This experiment demonstrated that the maximum rate achieved was virtually the same whether the BchD was refolded prior to addition to the assay or added directly to the assay mixture. The inset in Figure 2(A) shows the increase in fluorescence at 595 nm in the assays containing 27.2 nM BchD, measured over a period of 30 min. Trace 1 is the assay to which BchD was added directly, and trace 2 is the assay in which BchD was refolded first. This shows that the maximum rates obtained were virtually identical; however, if BchD was added directly to the reaction mixture, the maximum rate was not reached for 10–12 min, whereas when BchD was refolded prior to addition to the assay the lag was greatly reduced and the maximum rate was reached within 10 min. The long lag period seen for trace 1 was presumably due to BchD refolding and associating with Bchl. This suggested that, for kinetic measurements, it would be more practical to refold BchD prior to addition to the assay, so that the maximum rate is reached quickly and the kinetics of the Mg chelation reaction can be measured without any interference from the effects of the refolding of BchD. In both experiments the optimum BchD/Bchl molar ratio was approx. 1:1. In the assays where BchD was present in molar excess over Bchl, inhibition occurred. This ratio was confirmed and the inhibitory effect of excess BchD was also observed using standard assays (results not shown). The specific activity of the BchD, calculated from the assays containing 13.6 nM BchD (i.e. limiting BchD), was 1900 pmol of MgP·h⁻¹·µg of BchD⁻¹.

The converse experiment, in which the concentration of BchD in the assays was kept constant and the concentration of Bchl was increased, was carried out by refolding BchD with increasing concentrations of Bchl and adding an aliquot of the Bchl/BchD mixture to the rest of the reaction components. The final concentration of Bchl in the assays was varied as shown in Figure 2(B), and saturation was reached at a Bchl/BchD molar

Figure 2 Optimization of the proportions of BchH, Bchl and BchD in assays

Assays were performed in a total volume of 1 ml. Formation of MgP was measured using the continuous assay and the maximum rates were plotted. (A) BchD titration. Assays contained 769 nM BchH, 27.8 nM Bchl and the indicated concentrations of BchD. ○, BchD added directly to the assay; ●, BchD refolded prior to addition to the assay. Inset: fluorescence traces of assays containing 27.2 nM BchD. Trace 1, BchD added directly to the assay; trace 2, BchD refolded prior to addition to the assay. (B) Bchl titration. Assays contained 769 nM BchH, 8.5 nM BchD and the indicated concentrations of Bchl. (C) BchH titration. Assays contained 13.6 nM BchD, 55.6 nM Bchl and the indicated concentrations of BchH.

ratio of approx. 5:1. The specific activity of the Bchl, calculated from the assays containing 11 nM Bchl (i.e. limiting Bchl), was 1500 pmol of MgP · h⁻¹ · μg of Bchl⁻¹.

Figure 2(C) indicates that a Bchl/BchD molar ratio of 4:1 in the refolding and subsequent assay gave a good rate, and

Figure 3 Optimization of MgCl₂ concentration

Standard assays were performed with 490 nM BchH, 55.6 nM Bchl, 13.6 nM BchD, either 4 mM ATP (○) or 8 mM ATP (●) and the indicated total concentrations of MgCl₂.

therefore this ratio was chosen for further experiments. In the next series of experiments, the optimum molar ratio of BchH to Bchl/BchD was investigated by refolding BchD with Bchl at a Bchl/BchD ratio of 4:1 and assaying with increasing concentrations of BchH, as shown in Figure 2. In this experiment, Bchl/BchD became saturated at a BchH concentration of 490 nM, which is equivalent to a BchH/Bchl/BchD molar ratio of approx. 36:4:1. The specific activity of the BchH, calculated from assays containing limiting BchH, was 80 pmol of MgP · h⁻¹ · μg of BchH⁻¹.

The experiments described in Figure 2 indicated that the most useful way of performing enzyme assays was to refold BchD by dilution in Bchl, MgCl₂ and ATP, and to initiate the assays by adding this mixture to the rest of the assay components. They also demonstrated that the combination of 490 nM BchH, 55.6 nM Bchl and 13.6 nM BchD gave a good rate of product formation, and therefore all subsequent assays were performed in this way and with these concentrations of the three proteins.

Optimization of the MgCl₂ concentration

In a previous study on the Mg chelatase from *Synechocystis* [14], we noted that the enzyme requires free Mg for chelation. Before conducting experiments to determine kinetic parameters, it was important to investigate whether the *R. sphaeroides* enzyme has a similar requirement. Two series of assays were performed, one containing 4 mM ATP and a second containing 8 mM ATP, and the total MgCl₂ concentration was varied as shown in Figure 3. The experiment clearly demonstrated that the *R. sphaeroides* enzyme requires free Mg for chelation, since no chelation took place when the concentration of Mg was lower than that of ATP, and very little chelation was seen when Mg and ATP were equimolar. In assays containing 4 mM ATP, the optimum total Mg concentration was between 12 and 18 mM, and in assays containing 8 mM ATP the optimum was 20 mM Mg.

Steady-state kinetic parameters for protoporphyrin, Mg²⁺ and ATP

The kinetic parameters of the Mg chelatase enzyme for its natural substrates were determined using purified, refolded BchD. Figure 4(A) shows the dependence of the maximum reaction rate on protoporphyrin concentration. Some apparent inhibition was observed at protoporphyrin concentrations higher than 1 μM, and the data appeared to fit closely to an equation for simple

Figure 4 Measurement of steady-state kinetic parameters for Mg chelatase

Assays were performed with 490 nM BchH, 55.6 nM BchI and 13.6 nM BchD in a total volume of 100 μ L, and product formation was measured continuously in a microplate reader. A typical data set obtained by varying the concentration of each substrate is shown. (A) Dependence of the maximum rate on protoporphyrin concentration. The total MgCl_2 concentration in the assays was 12 mM and the ATP concentration was 4 mM. (B) Dependence of the maximum rate on Mg^{2+} concentration. The assays were performed with 1 μ M protoporphyrin and 4 mM ATP. (C) Dependence of the maximum rate on ATP concentration. The assays were performed with 1 μ M protoporphyrin and a free Mg^{2+} concentration of 8 mM.

substrate inhibition (see eqn. 2 in the Experimental section), giving a K_m of $0.17 \pm 0.06 \mu\text{M}$, a $V_{\max}$ of $34.7 \pm 0.95 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$ and a K_i of $14.2 \pm 7.0 \mu\text{M}$. Fitting the data up to 1 μM protoporphyrin to a rectangular hyperbola (Figure 4A, inset) gave a K_m of $0.15 \pm 0.05 \mu\text{M}$ and a $V_{\max}$ of

$32.1 \pm 1.0 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$. The dependence of the maximum rate on MgCl_2 concentration was measured in the presence of 1 μM protoporphyrin and 4 mM ATP, and the rate dependence is plotted as a function of the calculated free Mg^{2+} present (Figure 4B). The rate dependence followed the Michaelis–Menten relationship, and fitting the data to a rectangular hyperbola gave a K_m of $3.3 \pm 0.48 \text{ mM}$ and a $V_{\max}$ of $43.4 \pm 1.4 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$. The dependence of the maximum rate on ATP concentration was measured in the presence of 1 μM protoporphyrin. The concentration of MgCl_2 was varied so that the concentration of free Mg^{2+} in all the assays was constant at 8 mM. The rate dependence followed the Michaelis–Menten relationship, and fitting the data to a rectangular hyperbola (Figure 4C) gave a K_m of $0.12 \pm 0.02 \text{ mM}$ and a $V_{\max}$ of $22.1 \pm 0.7 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$.

Evidence for a BchI–BchD complex

The fact that refolding of BchD required BchI, MgCl_2 and ATP suggested that there is a specific BchI–BchD interaction that is promoted by Mg^{2+} and ATP. Figure 5 shows the results of an experiment to test this hypothesis, in which BchD was refolded with non-His-tagged BchI and the mixtures were passed over nickel affinity columns. One column was run with MgCl_2 and ATP in all the buffers, and a second column was run in which MgCl_2 and ATP were omitted after the initial wash step.

The fractions from both columns were analysed by SDS/PAGE and by enzyme assay (Figure 5). SDS/PAGE clearly showed that, when MgCl_2 and ATP were maintained in the buffer, BchI was retained on the column and BchI and BchD were co-eluted very sharply when the imidazole concentration was raised (Figure 5A, upper gel). In the absence of MgCl_2 and ATP, BchI was eluted during the wash step, whereas BchD was only eluted when the imidazole concentration was raised (Figure 5A, lower gel). Also, the elution pattern of BchD was slightly different, in that BchD began to elute immediately once the imidazole was applied, but was eluted more gradually from the column. There was some BchI in the fractions eluted from this column, but this is almost certainly because the BchI was still being eluted from the column at this point. In a control experiment we demonstrated that BchI alone does not bind to the affinity resin in the presence of MgCl_2 and ATP (results not shown), which confirms that, in the presence of MgCl_2 and ATP, BchI is retained on the column through a specific interaction with BchD.

The column fractions were assayed in the presence of BchH (BchI/BchD activity), BchH plus BchI (BchD activity) and BchH plus BchD (BchI activity). Figure 5(B) shows the activity profiles of the column fractions. The majority of the BchI/BchD activity from the column run in the presence of MgCl_2 and ATP was contained in a single fraction, which corresponds to the fraction containing the greatest amount of BchI and BchD proteins, as seen by SDS/PAGE. In the presence of MgCl_2 and ATP, essentially all of the BchD activity was contained in fraction 6, indicating that no BchD was eluted from the column at any other point. Similarly, all of the BchI activity was contained in the same fraction, demonstrating that none of the BchI was eluted from the column during the wash step in the presence of MgCl_2 and ATP. These results correlate precisely with those from SDS/PAGE, in which it can be seen that virtually all of the BchI and BchD protein is present in the same fraction.

In contrast, the omission of MgCl_2 and ATP from the wash and elution buffers resulted in a small amount of BchI/BchD activity in the eluted fractions, due to the small amount of BchI present in these fractions. However, it is clear that the BchI/BchD

Figure 5 Demonstration of a specific interaction between Bchl and BchD

His-tagged BchD was refolded by addition of partially purified non-His-tagged Bchl, MgCl₂ and ATP. The Bchl/BchD mixture was passed over a 1 ml nickel affinity column. The column was washed with buffer containing 10 mM MgCl₂ and 4 mM ATP, and then with buffer containing 10 mM MgCl₂, 4 mM ATP and 250 mM imidazole to elute the bound proteins (+MgATP column). This was then repeated, except that the washing and elution buffers contained no MgCl₂ or ATP (-MgATP column). In each case, four wash and four elution fractions were collected. The fractions were analysed by SDS/PAGE (**A**) and enzyme assays (**B**). (**A**) SDS/PAGE of column fractions. Upper gel, fractions from the +MgATP column; lower gel, fractions from the -MgATP column. Left-hand lane, Bchl/BchD mixture before loading; lanes 1–4, wash fractions; lanes 5–8, elution fractions. (**B**) Results of assays on column fractions. Fractions were analysed using the standard assay in the presence of 490 nM BchH (Bchl/BchD activity), 490 nM BchH plus 55.6 nM Bchl (BchD activity) and 490 nM BchH plus 13.6 nM BchD (Bchl activity). ■, Fractions from +MgATP column; □, fractions from -MgATP column.

activity in these fractions was significantly decreased compared with the fractions eluted from the column run with MgCl₂ and ATP. The gradual elution of BchD activity from this column immediately after the application of imidazole followed the elution pattern of the BchD protein seen on the SDS/polyacrylamide gel. In the absence of MgCl₂ and ATP, Bchl activity was eluted gradually during the wash step, which again precisely follows the elution of Bchl seen on the SDS/polyacrylamide gel. In a separate experiment, BchD was refolded by dilution with Bchl, MgCl₂ and ATP and the mixture chromatographed on an ion-exchange column. As long as MgCl₂ and ATP were present in the buffers, Bchl and BchD were also co-eluted from this column (results not shown).

Given this requirement for MgATP, it was of interest to see if any hydrolysis of ATP was connected with the formation of this

Figure 6 Gel filtration analysis of the Bchl-BchD complex

The oligomerization state of Bchl in the presence and in the absence of MgCl₂ and ATP was investigated. Untreated Bchl was chromatographed in the absence of MgCl₂ and ATP and compared with Bchl that was preincubated and chromatographed in the presence of 10 mM MgCl₂ and 4 mM ATP. The Bchl/BchD mixture (BchlD) formed by refolding BchD with Bchl, MgCl₂ and ATP was then chromatographed in the presence of 10 mM MgCl₂ and 4 mM ATP. The relative migration of molecular mass markers chromatographed in the presence of 10 mM MgCl₂ and 4 mM ATP is shown: 1, thyroglobulin (669 kDa); 2, apoferritin (443 kDa); 3, β -amylase (200 kDa); 4, alcohol dehydrogenase (150 kDa); 5, BSA (66 kDa); 6, carbonic anhydrase (29 kDa). To reduce 'noise' caused by the ATP in the running buffer, the data at 285 nm are presented.

Bchl-BchD complex. In continuous assays of P_i release, it was found that Bchl alone possessed significant levels of ATPase activity (6.5 ± 0.24 nmol of P_i · h⁻¹ · μ g of Bchl⁻¹). When Bchl and BchD were mixed, the rate was virtually identical (6.0 ± 0.54 nmol of P_i · h⁻¹ · μ g of Bchl⁻¹). The significance of this finding will be commented on in the Discussion section.

Further evidence for a Bchl-BchD complex was provided by gel filtration analysis of Bchl/BchD in the presence of MgCl₂ and ATP (Figure 6). BchD was refolded by dilution in buffer containing Bchl, MgCl₂ and ATP, then the mixture was concentrated and injected into a Shodex KW-803 gel filtration column with 10 mM MgCl₂ and 4 mM ATP in the running buffer. This was compared with Bchl that had been pretreated with MgCl₂ and ATP and chromatographed in the presence of MgCl₂ and ATP, and with untreated Bchl chromatographed in the absence of MgCl₂ and ATP. In the presence of MgCl₂ and ATP, the molecular mass of Bchl increased from approx. 50 kDa to approx. 100 kDa, but gave rise to an asymmetric peak. This could indicate that two Bchl molecules associate in the presence of MgCl₂ and ATP, but that the association is not stable, giving rise to the pronounced tail on the peak. The Bchl/BchD mixture gave rise to a species with a molecular mass of approx. 200 kDa.

DISCUSSION

Properties of purified BchD, and formation of a Bchl-BchD complex

We have developed a system for the production in *E. coli* of His-tagged subunits of Mg chelatase from *Rhodobacter sphaeroides*. This is an improvement on our earlier system [15], in that all three proteins can be purified rapidly by affinity chromatography. In particular, the *bchD* gene has been expressed at a high level, making it possible to purify large amounts of BchD protein to homogeneity. In our previous system, *bchD* was expressed at a very low level [10], and attempts to purify BchD using this system

resulted in a preparation that was heavily contaminated with GroEL [15]. The fact that most of the BchD accumulated in an insoluble form could have presented problems; however, we discovered that it was possible to purify BchD from the insoluble fraction in 6 M urea and refold the protein by dilution of the urea with buffer containing BchI, MgCl_2 and ATP. Indeed, refolded BchD proved to be significantly more active in the Mg chelatase reaction than the small amount of BchD that could be purified from the soluble fraction. The soluble BchD consists of high-molecular-mass material, presumably aggregated protein, which can apparently be unfolded with urea and refolded in the Mg chelatase assay, thus increasing its activity 10-fold.

Taken together, these facts suggest that BchD does not fold correctly when in isolation. Indeed, BchD requires the presence of BchI, MgCl_2 and ATP during refolding, and this observation prompted the notion that BchI and BchD form a complex, a process that requires Mg^{2+} and ATP. Another observation, i.e. that preincubation of the BchI and BchD subunits from *R. sphaeroides* and *Synechocystis* with MgCl_2 and ATP abolished a lag phase in the Mg chelatase assay, provided indirect evidence that prior interaction of these two subunits was required for chelation to take place [14,15], and evidence has been presented for the interaction of the BchI and BchD subunits of the tobacco enzyme [13]. Our results have demonstrated directly for the first time that the BchI and BchD subunits of the *R. sphaeroides* Mg chelatase form a complex; Mg^{2+} and ATP are required for this process, and the continued presence of these cofactors is required to maintain the two proteins in the complex. We suggest that this is the normal conformation of BchI and BchD *in vivo*. Support for this is provided by two studies in which Mg chelatase activity was observed in cell-free extracts from *R. sphaeroides*. If the cells are lysed in the presence of MgCl_2 and ATP, a completely soluble Mg chelatase preparation can be obtained [4], whereas when the cells are lysed in the absence of MgCl_2 and ATP, BchD is found in the particulate fraction [5]. The hydrophobicity profile of BchD does not show any significant hydrophobic stretches, and the apparent membrane association of this protein in the absence of MgCl_2 and ATP is most probably due to dissociation of BchD from BchI and its subsequent aggregation. The lag phase seen in Mg chelatase assays has been suggested to be due to oligomerization of BchI and BchD. Our results suggest that this is the case, but this is really a consequence of setting up the *in vitro* assay with the individually purified proteins, and need not reflect an *in vivo* process, since it is likely that BchI and BchD exist as a complex in the cell. In a previous study [15], in which BchD was contaminated with GroEL, it appears that the BchD was folded, or at least kept in solution, by binding to GroEL. Such a GroEL–BchD complex could give rise to a high-molecular-mass complex, and indeed the native molecular mass of GroEL-contaminated BchD was reported to be 550 kDa [15]. Our data suggest that BchD, when purified to homogeneity, does not form a high-molecular-mass complex, but appears to aggregate in a non-specific manner.

The preliminary data reported herein show that BchI alone possesses significant ATPase activity, consistent with the presence of Type A and Type B ATP-binding motifs in the BchI protein. Formation of the BchI–BchD complex does not appear to be accompanied by ATP hydrolysis, since mixing BchI and BchD did not result in an increase in ATPase activity. This is in conflict with a recent report by Hansson and Kannangara [21] that BchI alone had very little ATPase activity, whereas a mixture of BchI and BchD possessed high levels of activity. This discrepancy may be explained, firstly, by the fact that the BchI used by these authors appears to have a significantly lower specific activity with respect to Mg chelatase activity than the BchI used in the

present study (see below). Secondly, since the BchD used by these authors was most probably present as a GroEL–BchD complex, and the release of proteins from GroEL is accompanied by ATP hydrolysis [22], it seems possible that mixing Mg^{2+} , ATP, BchI and GroEL–BchD would result in an elevated level of ATP hydrolysis compared with that observed with BchI alone.

Kinetics of the Mg chelation reaction in *R. sphaeroides*

The protein titration experiments showed that the *R. sphaeroides* Mg chelatase is quite similar to the *Synechocystis* enzyme with respect to the dependence of the maximum reaction rate on the concentrations of the individual subunits. The dependence of the rate on the concentrations of both BchH and BchI apparently followed Michaelis–Menten kinetics (results not shown), which was also demonstrated for the *Synechocystis* enzyme [14]. The two enzymes are analogous, in that high concentrations of the respective D subunits in the reaction cause inhibition and the respective H subunits appear to limit the reaction rate, since much higher concentrations of H subunits relative to I and D are required to achieve the maximum reaction rate. The optimum BchI/BchD ratio determined from the BchD titration was different from that determined from the BchI titration, which shows that it is not possible to draw conclusions about the stoichiometry of the Mg chelatase subunits *in vivo* from this type of experiment. However, these experiments provided useful working proportions of the three subunits for use in *in vitro* measurements and indicated that the presence of the His tags and affinity purification of the Mg chelatase subunits did not appear to affect the activity of the proteins. The specific activities of the different subunits that we measured compare very favourably with those of their non-His-tagged counterparts purified by Willows et al. [15]. We obtained a specific activity for BchH of $80 \text{ pmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$, compared with the previously reported value of $15 \text{ pmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$ [15]. Affinity-purified BchI had a specific activity of $1500 \text{ pmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$, as compared with the value of $19.8 \text{ pmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$ reported in [15]. The most dramatic improvement in specific activity in the present work was seen for BchD. A specific activity of $1900 \text{ pmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$ was obtained for BchD, whereas the specific activity of the BchD reported previously [15], and also as reported in [21], appears to be approx. $0.08 \text{ nmol of MgP} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$. This is probably due to the presence of GroEL in this preparation, resulting in a specific activity which is lower by five orders of magnitude.

The K_m values for protoporphyrin, Mg^{2+} and ATP that we obtained are very similar to those reported previously [15]; for comparison, the values are: protoporphyrin, $0.15 \mu\text{M}$ compared with $0.36 \mu\text{M}$ [15]; Mg^{2+} , 3.3 mM compared with 1.7 mM [15]; ATP, 0.12 mM compared with 0.16 mM [15]. The recent determination of these values for the purified, reconstituted Mg chelatase from *Synechocystis* [14] allows a comparison to be made with the present data for *R. sphaeroides*. The K_m values for Mg^{2+} and ATP for the *Synechocystis* enzyme are 4.9 mM and 0.49 mM respectively, which do not differ greatly from the *R. sphaeroides* data. However, in contrast with the *R. sphaeroides* enzyme, the *Synechocystis* enzyme [14] and the pea enzyme [9] exhibit a sigmoidal dependence on the concentration of Mg^{2+} . In addition, the pea enzyme exhibited a sigmoidal dependence on the concentration of ATP [9]. This suggests that there may be differences between Mg chelatases from bacteriochlorophyll- and chlorophyll-synthesizing organisms, possibly with respect to the interactions between the respective I and D subunits, Mg^{2+} and ATP. In all of the Mg chelatases, the I subunits have classical Type A and Type B ATP-binding motifs. The D subunits from

Synechocystis [11] and higher plants [13] also possess very distinct ATP-binding motifs, whereas *R. sphaeroides* BchD does not. The presence of additional binding sites for (Mg)ATP in the Mg chelatases of chlorophyll-synthesizing organisms may introduce co-operativity with respect to these substrates. This in turn could be a mechanism of regulation of these enzymes by the cellular concentration of these substrates. The observation that chelation can only proceed when the Mg^{2+} concentration exceeds the ATP concentration appears to hold true for each of the Mg chelatases, and emphasizes that Mg^{2+} plays at least two roles, one in forming a complex with ATP and the other as the inserting metal ion. In addition, ATP is required to play at least two roles. The present work shows for the first time that it is required to stabilize an I-D complex; furthermore, since refolding is not absolutely dependent on the presence of free Mg^{2+} (results not shown), ATP, in the form of MgATP, may have a structural role in the I-D complex. It seems likely that ATP is also used as an energy source during Mg^{2+} insertion. The formation of an I-D complex is likely to be a general feature of Mg chelatases, as this has also been demonstrated for the purified *Synechocystis* enzyme (P. E. Jensen, unpublished work). A detailed analysis of the kinetics of ATP hydrolysis is a necessary step in the characterization of this enzyme.

The K_m for protoporphyrin for *R. sphaeroides* Mg chelatase was estimated to $0.15 \mu M$, whereas the value for the *Synechocystis* enzyme is $1.25 \mu M$ [14] and the recently published estimate obtained with partially purified Mg chelatase from developing pea leaves is $0.008\text{--}0.014 \mu M$ [9]. Whereas *R. sphaeroides* and plants synthesize chlorophylls as the major tetrapyrroles, cyanobacteria such as *Synechocystis* synthesize large amounts of haem via ferrochelatase, since haem is an intermediate in the biosynthesis of the chromophore of phycobiliprotein, which is the major light-harvesting structure in these organisms [23]. These differences in K_m values for Mg chelatases from different sources may have some bearing on the allocation of pigments at this branch-point in tetrapyrrole metabolism.

It is apparent that Mg chelatase exhibits relatively low rates of product formation *in vitro*, which was measured over time-scales of tens of minutes. If we compare the reported V_{max} of *R. sphaeroides* ferrochelatase for protoporphyrin of $6600 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$ [24] with the V_{max} of Mg chelatase for protoporphyrin of $32.1 \text{ pmol} \cdot \text{h}^{-1} \cdot \mu\text{g}^{-1}$, it is apparent that the rate of metal insertion by ferrochelatase is much greater. We attribute this to the fact that insertion of Mg^{2+} into protoporphyrin is far more difficult than insertion of Fe^{2+} [25,26]. In photosynthetically growing *R. sphaeroides* cells, the requirement for bacteriochlorophyll could be up to 50-fold greater than the requirement for haem and, assuming that the two enzymes at this branch-point compete for the same pool of protoporphyrin, Mg chelatase must have developed a mechanism to achieve the eventual dominance of bacteriochlorophyll with respect to haem. We have already demonstrated that BchH binds protoporphyrin [10], which we have suggested could help to sequester protoporphyrin so that it is unavailable for ferrochelatase [27].

Another consideration is that the reported K_m of the *R. sphaeroides* ferrochelatase for protoporphyrin is $18 \mu M$ [24], whereas the K_m for protoporphyrin exhibited by Mg chelatase is $0.15 \mu M$. It is unlikely that free protoporphyrin accumulates within the cell, since this metabolite is extremely toxic, and we must assume that the cellular concentration of protoporphyrin is effectively very low, probably in the range of the K_m for protoporphyrin exhibited by Mg chelatase. At this concentration

of protoporphyrin, we can calculate that the two chelatase enzymes will turn over at approximately the same rate. This, coupled with the proposed sequestration of protoporphyrin by BchH, could explain how these bacteria are able to synthesize more bacteriochlorophyll than haem.

This could also provide a very sensitive mechanism to ensure that the cellular protoporphyrin concentration is not allowed to increase to dangerous levels, since any increase in the concentration of protoporphyrin would result in a rapid increase in the rate of turnover of ferrochelatase. This would lead to an increase in the cellular haem concentration, which has been shown to regulate δ -aminolaevulinic acid synthase by feedback inhibition [28]. This would, in turn, lead to a reduced flux through the pathway from ALA to protoporphyrin, thus reducing the cellular concentration of protoporphyrin.

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