Purification and kinetic characterization of the magnesium protoporphyrin IX methyltransferase from *Synechocystis* PCC6803

Mark SHEPHERD, James D. REID and C. Neil HUNTER¹

Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, Firth Court, Western Bank, University of Sheffield, Sheffield S10 2TN, U.K.

Magnesium protoporphyrin IX methyltransferase (ChlM), catalyses the methylation of magnesium protoporphyrin IX (MgP) at the C₆ propionate side chain to form magnesium protoporphyrin IX monomethylester (MgPME). Threading methods biased by sequence similarity and predicted secondary structure have been used to assign this enzyme to a particular class of *S*-adenosyl-L-methionine (SAM)-binding proteins. These searches suggest that ChlM contains a seven-stranded β -sheet, common among small-molecule methyltransferases. Steady-state kinetic assays were performed using magnesium deuteroporphyrin IX (MgD), a more water-soluble substrate analogue of MgP. Initial rate studies showed that the reaction proceeds via

a ternary complex. Product (*S*-adenosyl-L-homocysteine; SAH) inhibition was used to investigate the kinetic mechanism further. SAH was shown to exhibit competitive inhibition with respect to SAM, and mixed inhibition with respect to MgD. This is indicative of a random binding mechanism, whereby SAH may bind productively to either free enzyme or a ChlM–MgD complex. Our results provide an overview of the steady-state kinetics for this enzyme, which are significant given the role of MgP and MgPME in plastid-to-nucleus signalling and their likely critical role in the regulation of this biosynthetic pathway.

Key words: chlorophyll biosynthesis, methylation, porphyrin.

INTRODUCTION

Chlorophyll biosynthesis is essential for the assembly and function of the photosynthetic complexes that harvest sunlight and convert it into chemical energy. An outline of the chlorophyll biosynthetic pathway is shown in Scheme 1, from which it can be seen that magnesium protoporphyrin IX methyltransferase (ChlM; E.C. 2.1.1.11) catalyses the second committed step in chlorophyll biosynthesis, entailing the conversion of magnesium protoporphyrin IX (MgP) to magnesium protoporphyrin IX monomethylester (MgPME). The analysis of the chlorophyll branch of this pathway has been facilitated by the overexpression of genes encoding these enzymes, often from bacterial sources, which has resulted in the production of the large amounts of protein needed for detailed functional studies [1–6]. Despite the successful overexpression of *bchM* encoding the methyltransferase from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* [7,8], no extensive kinetic work has been undertaken, possibly because this enzyme does not lend itself to continuous assays by spectroscopic methods.

Recent genetic studies of plastid-to-nucleus signal transduction have suggested that this enzyme fulfils a wider role in cellular metabolism. Feeding and inhibitor experiments have implicated the porphyrin product of the methyltransferase (MgPME) in plastid-to-nucleus signalling, leading to repression of the *lheb* genes in *Chlamydomonas reinhardtii* and cress seedlings [9]. It has been shown that feeding of MgPME to *C. reinhardtii* activates nuclear heat-shock genes through a light-responsive promoter element in dark-grown cultures [10,11]. Several *Arabidopsis* *gun* mutants, previously shown to be uncoupled in this signalling pathway [12], are proposed to affect the same plastid-to-nucleus signalling by perturbing levels of protoporphyrin, MgP and MgPME [13].

In order to begin to understand how both a substrate and a product of the methyltransferase are made available for

signalling as well as biosynthesis, it is important to understand the kinetics of methylation of MgP and, eventually, how this process is coupled to the one that precedes it in the pathway, that of insertion of Mg²⁺ into protoporphyrin by magnesium chelatase. Furthermore, by identifying possible conserved structural motifs, we can provide a greater insight into reaction chemistry.

We present here the first steady-state kinetic study of purified recombinant ChlM. The ChlM protein sequence was used as a template for the GenTHREADER program [14], to search the Protein DataBank (PDB) for proteins that are likely to contain similar structural motifs. Several proteins containing the *S*-adenosyl-L-methionine (SAM)-binding domain, a seven-stranded β -sheet, exhibit a significant similarity to ChlM. This suggests that ChlM belongs to this family, and binds to SAM in a similar way. Sequence motifs, previously identified as being conserved throughout the majority of methyltransferases [15,16], have also been identified in ChlM. The kinetic mechanism of ChlM from *Synechocystis* has been determined with the water-soluble analogue magnesium deuteroporphyrin IX (MgD). Steady-state kinetics demonstrate that the reaction proceeds through a ternary complex, and *S*-adenosyl-L-homocysteine (SAH) product-inhibition patterns indicate that substrate binding is not ordered. The significance of these data for the coupling of the methyltransferase and the chelatase, and for the availability of MgP and MgPME as signalling molecules, is discussed.

EXPERIMENTAL

Materials

All pigments were purchased from Porphyrin Products (Logan, UT, U.S.A.). The remaining chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Abbreviations used: ChlM, magnesium protoporphyrin IX methyltransferase; MgD, magnesium deuteroporphyrin IX; MgP, magnesium protoporphyrin IX; MgPME, magnesium protoporphyrin IX monomethylester; PDB, Protein DataBank; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

¹ To whom correspondence should be addressed (e-mail C.N.Hunter@sheffield.ac.uk).

Scheme 1 The chlorophyll biosynthetic pathway

(A) An outline of the chlorophyll biosynthetic pathway. The shaded area represents the region of the pathway under control of ChlM. (B) The methyltransferase reaction catalysed by ChlM. Abbreviations: ALA, δ -aminolaevulinic acid; proto, protoporphyrin IX; GGPP, geranyl-geranyl pyrophosphate; ChlM, magnesium protoporphyrin IX methyltransferase; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine.

Structurally biased sequence searches

The GenTHREADER program (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to search for proteins in the PDB, which contain a common fold and thus a common ancestry to ChlM. This program involves three steps; the alignment of sequences with the template, the calculation of pair potentials and solvation potentials, and the application of a neural network to express the findings in a single numerical value.

To produce the sequence alignment, the OWL redundant protein sequence data bank [17] was scanned using the program BLASTP [18], and the sequences were aligned using a simplified version of MULTAL [19], the multiple sequence alignment method. Insertions relative to the ChlM template were skipped and a sequence profile [20] was constructed using the BLOSUM 50 matrix [21]. The fold library used was based on the Brookhaven PDB [22], and the target sequences were aligned with ChlM using a global/local dynamic alignment algorithm.

Whereas GenTHREADER is more accurate at creating sequence-structure alignments than other fold-recognition methods [23,24], the average alignment accuracy is merely 46.2% where the correct fold is recognized. Therefore, on average, less than half of the template sequence is assigned the correct structural topology. Output sequences were aligned manually using Megalign. From this, the sequence of ChlM could be built into the alignment starting with the regions of high sequence identity (motifs I, post I and II), and the regions that most frequently corresponded to the conserved structural motifs in the output sequences.

Protein expression and purification

The plasmid pET9a-His₆-ChlM [25] was transformed into *Escherichia coli* BL21 (DE3) cells and the *chlM* gene was induced for 15 h at 20 °C using 0.4 mM isopropyl β -D-thiogalactoside. The cells were harvested by centrifugation and cells from 2 litres of culture were resuspended in 20 ml of chilled binding buffer [20 mM citrate/KOH (pH 5.8), 500 mM NaCl, 500 mM glycerol and 5 mM imidazole]. The cells were disrupted by sonication for 6 $\times$ 30 s on ice, and the cell debris was removed by centrifugation at 4 °C. The supernatant was loaded at 2 ml/min on to a 2.0 cm $\times$ 5.0 cm column packed with chelating Sepharose Fast-Flow resin (Amersham Biosciences) charged with 50 mM NiSO₄ and pre-equilibrated with 3 column vol. of binding buffer. The column was washed with 10 column vol. of binding buffer and 6 column vol. of binding buffer containing 60 mM imidazole (wash buffer) to remove any loosely bound contaminants. The His-tagged ChlM was eluted with binding buffer containing 250 mM imidazole (elution buffer). A 50 ml column of P-6 desalting gel (Bio-Rad) was equilibrated with 50 mM citrate/KOH (pH 5.8), 300 mM glycerol and 200 mM NaCl, and used to remove imidazole from the buffer. A typical yield was 15 mg of protein from a 2 litre culture of *E. coli*.

HPLC gel-filtration chromatography

A Phenomenex S4000 column was equilibrated with 3 column vol. of running buffer (100 mM Tris/HCl, pH 7.5, 100 mM NaCl and 100 μ M SAM), and was calibrated at 1 ml $\cdot$ min⁻¹ with the

following markers: Blue dextran (which eluted in the void volume, V_0), thyroglobulin (669 kDa), apoferritin (443 kDa), sweet-potato β -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). ChlM (10 μ M) was incubated in running buffer containing 100 μ M MgD for 2 min at 30 °C, and 20 μ l of this was applied to the column at a flow rate of 1 ml $\cdot$ min⁻¹. Eluted protein was detected using a photodiode array detector, monitoring absorbance at 295 nm.

Spectroscopy

Fluorescence spectra were recorded on a SPEX Fluorolog spectrofluorimeter fitted with a temperature-controlled cuvette holder set to 30 °C. For composition of samples, refer to the legend of Figure 6 (see below). All ChlM samples were in 100 mM Tris/HCl, pH 7.5, 100 mM NaCl and 300 mM glycerol. Samples (1 ml) were incubated for at least 5 min at 30 °C before spectra were recorded. For tryptophan fluorescence emission measurements at 340 nm, the excitation wavelength was set to 280 nm, and 295 nm when SAH was present. Excitation and emission slit widths corresponding to 5 and 18 nm, respectively, were used. Additionally, an Oriol 340 nm bandpass filter was placed at the emission side of the cuvette holder.

Substrates and products

Porphyrin solutions were prepared each week by dissolving a small amount of porphyrin in buffer. Porphyrin concentrations were determined in 0.1 M HCl, using the ϵ_{398} of 433 000 M⁻¹ $\cdot$ cm⁻¹, after Mg²⁺ had been removed from the porphyrin by a 5 min incubation in 1 M acetic acid. SAM and SAH stock solutions were prepared daily in 0.1 M HCl and 0.1 M NaOH respectively. Their concentrations were determined using the ϵ_{256} value of 15 200 M⁻¹ $\cdot$ cm⁻¹ in 1 M HCl for SAM, and ϵ_{260} value of 16 000 M⁻¹ $\cdot$ cm⁻¹ at pH 7 for SAH [26].

ChlM assays

Reactions were carried out at 30 °C in 100 mM Tris/HCl, pH 7.5, 100 mM glycerol, 0.1 % Genapol X-080 (Fluka), 2 μ M ChlM and MgD and SAM concentrations as indicated in the relevant figure legends. The assay mixtures were incubated at 30 °C in the absence of SAM for 5 min to allow for thermal equilibration. The SAM was added, and 20 μ l aliquots were taken every 2 min over a period of 8 min, and quenched in 400 μ l of stop solution (acetone/water/33 % ammonia, 80:20:1, by vol.). These aliquots were spun down at 10 000 *g* for 5 min to form a pellet from any aggregated protein. Pigments were separated using reversed-phase HPLC [25]. Depending on the MgD concentration, 10–70 μ l of the soluble phase was loaded on to a Beckman ODS Ultrasphere column (150 mm $\times$ 4.6 mm). The pigments were separated by a 7 min linear gradient from 0 to 67 % solvent B at 2 ml/min (where solvent A is 0.005 % triethylamine in water and solvent B is acetonitrile), and then the gradient was paused for a further 5 min for the porphyrins to elute. Eluted porphyrins were detected using a Waters in-line fluorescence detector, using an excitation wavelength of 394 $\pm$ 5 nm and an emission wavelength of 580 $\pm$ 5 nm.

The peaks were integrated using Waters Millennium software. Known amounts of magnesium deuteroporphyrin IX monomethylester were analysed in the same way to produce a standard curve. The maximum rate during an assay was taken as the steady-state rate, and occurred at the beginning of the reaction.

Analysis of apparent dissociation constants for MgD binding to ChlM

Binding data were fitted to eqn (1), in which a single type of binding site is assumed:

$$F_{\text{obs}} = F_0 + F_{\text{max}} \frac{[L]_T + [E]_T + K_d - \sqrt{([L]_T + [E]_T + K_d)^2 - 4[L]_T[E]_T}}{2[E]_T} \quad (1)$$

where F_{obs} is the observed fluorescence, F_0 is the initial fluorescence, F_{max} is the maximum amplitude of fluorescence quenching, $[L]_T$ is the total ligand concentration, $[E]_T$ is the total concentration of ChlM protein (fixed at 0.5 μ M) and K_d is the apparent dissociation constant.

Analysis of kinetic data

Steady-state rates were estimated by fitting the time courses to a straight line. Steady-state rate data (v versus $[S]$; where v is the rate and $[S]$ is the substrate concentration) were fitted to the Michaelis–Menten equation (eqn 2) using unweighted non-linear regression (Sigmaplot 5; SPSS), and displayed in a double-reciprocal form to permit simple graphical analysis of inhibition data.

$$v = \frac{V_{\text{app}}[S]}{K_{\text{app}} + [S]} \quad (2)$$

where V_{app} is the apparent maximum rate and K_{app} is the apparent Michaelis constant. Error bars were plotted on the double-reciprocal plots to represent the S.E. when fitting the four-point steady-state rate data to a straight line.

RESULTS

Identification of a putative conserved core domain for ChlM

The structure of ChlM has not yet been determined. To determine whether ChlM belongs to the well-characterized family of small-molecule methyltransferases, the GenTHREADER program [14] was used to search the PDB for proteins that are likely to contain similar structural motifs. Searches for structural homologues of ChlM using GenTHREADER yielded 10 protein sequences, at the 90 % confidence level (results not shown). This means that there is a 90 % chance that each of these 10 proteins from the PDB has a common fold and therefore common ancestry to ChlM. All were SAM-binding proteins containing a seven-strand mixed β -sheet characteristic of small-molecule methyltransferases. This domain, together with the bound SAM, is highlighted in black in Figure 1(A) within the structure of the conserved β -sheet motif of glycine-N-methyltransferase [27].

Because the sequence alignments from GenTHREADER are inherently poor (see the Experimental section), the multiple sequence alignment in Figure 1(B) had to be constructed manually. This would prove quite difficult with 10 protein sequences that contain little sequence identity, so six of them were selected at random to produce Figure 1(B). Firstly, where any of the conserved methyltransferase motifs were present, they were stacked on top of one another using the program Megalign. The β -sheet regions were also aligned, and are denoted by arrows in Figure 1(B). Motif I is clearly present in all of the protein sequences in the alignment, and motifs post I and II are present

Figure 1 The β -sheet methyltransferase motif and alignment of ChIM with putative structural homologues

(A) The structure of glycine-*N*-methyltransferase [27] highlighting the conserved β -sheet motif and bound SAM, which both appear in black. Proteins that have been identified as potential structural homologues to ChIM all contain a mixed β -sheet of this type ($\uparrow\uparrow\uparrow\uparrow\downarrow$). (B) Multiple alignment of ChIM with protein sequences obtained through a single cycle of GenTHREADER [14]. The

Figure 2 SDS/PAGE demonstrating the purification of proteins prepared from isopropyl β -D-thiogalactoside-induced *E. coli* containing the *chlM* gene

Lane 1, markers; lane 2, sonicated cells; lane 3, supernatant after removal of cell debris; lane 4, unbound fraction; lane 5, pure ChlM eluted with 250 mM imidazole; lane 6, pure ChlM after buffer exchange to remove imidazole; lane 7, markers.

in most of the proteins in Figure 1(B), including ChlM. A sequence that resembles motif III appears to be present in ChlM starting at Leu-174. This motif is present in most of the other enzymes, but is difficult to align, as the alignment of β -strand number 5 takes precedent in Figure 1(B). Hence, motif III is not marked on the sequence alignment. However, considering the presence of other methyltransferase motifs I, post I and II, and the SAM-binding domain, we conclude that ChlM is a member of the small-molecule methyltransferase family. The significance of these methyltransferase motifs (I, post I, II and III), particularly their structural relevance, is discussed below.

Steady-state kinetics

ChlM was purified in a single-step procedure using a Ni^{2+} -affinity column (see the Experimental section). Analysis by SDS/PAGE indicated that ChlM was greater than 95% pure after this step (Figure 2, lanes 5 and 6). Figure 3 shows the trace obtained when HPLC gel-filtration chromatography was performed on ChlM under the conditions described in the figure legend. The peak obtained corresponds to a protein of approx. 20 kDa, which demonstrates that ChlM migrates as a monomer (the predicted molecular mass of ChlM is 26 kDa). Previous assays of MgP methyltransferase activity have concentrated on the biological substrate, MgP [1,7]. However, such hydrophobic porphyrins tend to aggregate in aqueous solution [28]. The present work describes the use of a more water-soluble analogue, MgD, which lacks the two vinyl groups on the A and the B rings seen in the structure in Scheme 1(B).

ChlM catalyses a two-substrate, two-product reaction, and it was of interest to distinguish between a ping-pong mechanism, and a random or ordered ternary complex mechanism. This can be achieved by measuring the rate of product formation as a function of each substrate concentration, at a number of fixed concentrations of the other substrate. A system with a ping-pong mechanism should show a constant $V_{\text{app}}/K_{\text{app}}$ for the variable

Figure 3 Gel filtration of ChlM

A Phenomenex S4000 column was equilibrated with 3 column vol. of running buffer (see text). ChlM ($10 \mu\text{M}$) was pre-incubated for 2 min at 30°C in running buffer containing $100 \mu\text{M}$ MgD, and $20 \mu\text{l}$ of this was loaded on to the column. Flow rate, $1 \text{ ml} \cdot \text{min}^{-1}$.

substrate at every concentration of the other substrate, yielding a double-reciprocal plot containing a series of parallel lines, whereas ternary complex mechanisms produce lines that intersect at a point to the left of the $1/v$ axis. A kinetic analysis of the reaction was conducted by varying the concentrations of SAM and MgD in turn. Turnover was followed using the HPLC quenched assay with fluorescence excitation set to 394 nm, and the emission wavelength fixed at 580 nm.

The concentration of SAM was varied at several different fixed concentrations of MgD. These data are shown as a double-reciprocal plot in Figure 4(A). Figure 4(B) shows the corresponding experiment whereby MgD was varied in the presence of several fixed concentrations of SAM. The plots of v versus S demonstrate a simple hyperbolic dependence of initial rate upon ligand concentration (results not shown). The fitted parameters have been used to define the theoretical lines in Figure 4. Therefore, no bias has been introduced with respect to data at low substrate concentrations. Error bars are present to represent the S.E. when fitting the four assay time points to a straight line, the slope of which defines the steady-state rate. The lines in Figure 4(A) intercept the $1/v$ axis at approximately the same point. Clearly V_{app} does not change with $[\text{MgD}]$, implying that the $K_{\text{m}}^{\text{MgD}}$ is less than $100 \mu\text{M}$. Further data on MgD binding to ChlM are discussed below (see also Figure 6). The inset in Figure 4(B) shows the plot of apparent V_{max} versus $[\text{SAM}]$. This provides an estimate for $K_{\text{m}}^{\text{SAM}}$ of $38 \pm 17 \mu\text{M}$. The reciprocal plots in Figure 4 are clearly not parallel, which rules out the possibility of a ping-pong mechanism. The ternary complex is likely to be formed either by a random or ordered addition of substrates.

Product inhibition kinetics using SAH

Figures 5(A) and 5(B) show the double-reciprocal plots for SAH inhibition with respect to SAM and MgD respectively. As before,

seven β -strands of the SAM-binding domains were stacked on top of one another, and aligned with the predicted β -strand regions of ChlM. Protein names: PIMT, protein isoaspartyl methyltransferase; PAMT, putrescine aminopropyltransferase; Cmaa1 and Cmaa2, mycolic acid cyclopropane synthase; ErmC', erythromycin resistance methyltransferase C'; GNMT, glycine-N-methyltransferase. Species names: TM, *Thermotoga maritima*; MT, *Mycobacterium tuberculosis*; BS, *Bacillus subtilis*; SP, *Synechocystis* PCC6803; RN, *Rattus norvegicus*. β -Strands are shown as arrows, and conserved methyltransferase motifs I, post I and II have been boxed with a solid line. Regions where four or more residues are chemically similar have a black background. The $\beta 7$ region of protein isoaspartyl methyltransferase is aligned with the other $\beta 6$ regions because in this protein, $\beta 7$ precedes $\beta 6$. The program Megalign was used to produce the figure.

Figure 4 Lineweaver–Burk plots, fitted with parameters derived from v versus S plots, for the methylation of MgD

The symbols represent steady-state rates derived from stopped assays with four time points; $k_{\text{cat}} = 3.5 \text{ min}^{-1}$. (A) $[\text{SAM}]$ was varied at several fixed concentrations of MgD: (●) 100 μM , (○) 250 μM and (▼) 500 μM . (B) $[\text{MgD}]$ is varied at several fixed concentrations of SAM: (●) 50 μM , (○) 500 μM and (▼) 800 μM . The inset shows the plot of apparent V_{max} (V_{app}) versus $[\text{SAM}]$. This gives approximate values of $K_{\text{m}}^{\text{SAM}} = 38 \mu\text{M}$, and $k_{\text{cat}}/K_{\text{m}}^{\text{SAM}} = 1500 \text{ M}^{-1} \cdot \text{s}^{-1}$. The value at 25 μM SAM is not shown in the double-reciprocal plot to improve the clarity of the data. Error bars in the Lineweaver–Burk plots indicate the S.E. of the initial [product] versus time data (results not shown). Error bars in panel (B) inset represent S.E. in estimating V_{app} values.

kinetic parameters were obtained from non-linear regression analysis of the untransformed data. These data indicate a competitive inhibition pattern with respect to SAM, and mixed inhibition with respect to MgD. This is indicative of a random-order mechanism (Scheme 2), whereby SAH may bind to either free enzyme or a ChlM–MgD complex. As the lines in Figure 5(B) intersect on the $1/S$ axis and to the left of the $1/v$ axis, this implies that SAH binds to free enzyme and to a ChlM–MgD complex with equal affinity. These data are consistent with the reaction scheme shown in Scheme 2.

Determination of an apparent dissociation constant for MgD with ChlM

Scheme 2 implies that a ChlM–MgD complex exists, so it was of interest to ascertain whether such a complex could form, and to determine an apparent dissociation constant for this process. The basis of the experiment was the observation that the formation of a ChlM–MgD complex is accompanied by partial quenching of protein fluorescence at 340 nm. Protein solutions were excited at 280 nm (295 nm when SAH was present) and emission was

Figure 5 Lineweaver–Burk plots, fitted with parameters derived from v versus S plots, for the product inhibition by SAH

The symbols represent steady-state rates derived from stopped assays with four time points. (A) SAM was varied in the presence of several fixed concentrations of SAH: (●) no SAH added, (○) 100 μM and (▼) 300 μM SAH and varied [SAM]. [MgD] = 500 μM . (B) MgD was varied in the presence of several fixed concentrations of SAH: (●) 300 μM SAH, (○) 100 μM and (▼) no SAH and varied [MgD]. [SAM] = 500 μM . Error bars indicate the S.E. of the initial [product] versus time data (results not shown).

Scheme 2 The proposed reaction scheme for ChIM

The scheme depicts a random ternary mechanism, whereby both substrates may bind in either order. $K_m^{\text{SAM}} = 38 \mu\text{M}$, $K_m^{\text{MgD}} = 2.37 \mu\text{M}$, $k_{\text{cat}}/K_m^{\text{SAM}} = 1500 \text{ M}^{-1}\text{s}^{-1}$, $K_i \approx K_i'$.

Figure 6 Quenching of ChlM protein fluorescence by MgD

(A) Purified ChlM (0.5 μM) was incubated with 0–40 μM MgD for 5 min at 30 $^{\circ}\text{C}$. Fluorescence emission scans were recorded at 30 $^{\circ}\text{C}$ with an excitation wavelength of 280 nm, and a plot of ChlM fluorescence against MgD was produced. The line is defined by a single-site binding model (eqn 1) with a K_d value for MgD binding of $2.37 \pm 0.26 \mu\text{M}$. (B) The procedure was repeated using 0–30 μM MgD in the presence of 500 μM SAH, and an excitation wavelength of 295 nm. This yielded a K_d value of $4.74 \pm 1.76 \mu\text{M}$. The inset in (A) shows a series of spectra illustrating the quenching of ChlM fluorescence by MgD.

recorded between 320 and 360 nm. The inset in Figure 6(A) shows a typical family of spectra. Emission peaks were integrated between 325 and 355 nm and plotted against [MgD] (Figure 6A). These data are consistent with a single-site binding model with a K_d value of $2.37 \pm 0.26 \mu\text{M}$. No shifts in the absorption or emission maxima of the MgD ligand are apparent in the protein bound state. The experiment was repeated in the presence of 500 μM SAH, using an excitation wavelength of 295 nm (Figure 6B). This yielded a K_d value of $4.74 \pm 1.76 \mu\text{M}$, implying that SAH only slightly affects the binding of MgD to ChlM. The addition of SAM or SAH alone did not result in quenching of ChlM fluorescence.

DISCUSSION

By considering the position of the three methyltransferase motifs (Figure 1B) and other conserved residues in the sheets, we could assign putative β -sheet regions in ChlM. It is therefore likely that ChlM contains an 'AdoMet [SAM]-dependent methyltransferase fold', as reviewed in Cheng and Roberts [29]. The high degree of sequence similarity around the three conserved methyltransferase motifs is unsurprising when one considers their position in the

β -sheet. Motifs I, post I and II (Figure 1B) constitute segments of strands 1, 2 and 4 respectively (Figure 1A). These strands are positioned adjacent to the bound SAM cofactor and are an intrinsic part of the substrate-binding pocket.

Work by Kagan and Clarke [15] highlights that three conserved sequence motifs exist in several classes of methyltransferases. They found that the motifs I, II and III had consensus sequences of (V/I/L)(L/V)(D/E)(V/I)G(G/C)G(T/P)G, (P/G)(Q/T)-(F/Y/A)DA(I/V/Y)(F/I)(C/V/L) and LL(R/K)PGG(R/I/L)-(L/I)(L/F/I/V)(I/L) respectively. Also, motif post I, which is approx. 20 residues C-terminal to motif I, has a consensus sequence of hhXh(D/E) where h denotes a hydrophobic residue. Protein isoaspartyl methyltransferase ('PIMT'), an enzyme we expect to have the same fold as ChlM (Figure 1B), has previously been shown to contain motifs I, II and III [30].

In the crystal structure of *M.HhaI* DNA $m^5\text{C}$ methyltransferase, motif I has been shown to be involved in the binding of SAM [31]. The glycine residues form a tight loop responsible for correctly orientating the SAM in the hydrophobic binding pocket. In four of the proteins aligned in Figure 1(B) there is a charged residue flanked by hydrophobic residues. This sequence is motif post I, and the charged residue in ChlM is Asp-91. The corresponding residue in erythromycin-resistance methyltransferase C' (ErmC') is Glu-59, and has been shown to form hydrogen bonds with the two oxygen atoms on the ribose ring of SAM [16]. This provides an insight into the possible function of Asp-91 in ChlM.

ErmC' lacks motifs II and III, which explains the poor sequence identity in these regions [15]. Motif II is not present in some RNA and porphyrin precursor methyltransferases, but is almost as abundant as motif I [15]. The methyltransferases of porphyrin metabolism that lack this motif are involved in cobalamin biosynthesis, and could have evolved along a different lineage to ChlM. Where motif II is present, there is a central aspartic acid residue that is highly conserved. In some methyltransferase sequences this residue is preceded by an aromatic residue. In glycine-N-methyltransferase there are several aromatic residues in the SAM-binding pocket, acting to provide a hydrophobic environment [27]. It is thought that these large hydrophobic side chains stack alongside the adenine ring via non-ionic interactions. Tyr-128 in ChlM may be present to form non-polar interactions with SAM.

Motif III can be found in most of the sequences in Figure 1(B), albeit in varying positions and levels of completeness. Where motif III is present, there are often two central glycine residues. In ChlM, this motif starts at Leu-174, and the two conserved glycine residues are both present. It is unclear whether motif III is involved in SAM binding, as this motif is missing in enzymes of cobalamin biosynthesis and selected RNA methyltransferases [15]. The consensus does not appear to be essential for catalysis, as site-directed mutagenesis at several places in rat guanadinoacetate-N-methyltransferase motif III failed to alter k_{cat} or the K_m for SAM, although a 2-fold range of K_i values for SAH was observed [32].

ChlM is expected to differ from other members of the small-molecule methyltransferases in that it could possess a porphyrin-binding cleft; such a substrate-binding site has been observed in the structures of cobaltochelatase [33] and ferrochelatase [34].

In the experiments described here, the kinetic mechanism of purified ChlM was investigated. Assays were performed using MgD, a water-soluble analogue of the natural substrate MgP. It has already been shown that deuteroporphyrin, rather than protoporphyrin, can be used as a substrate for investigating substrate binding and steady-state kinetics for the preceding enzyme in the pathway, Mg chelatase [35].

The single peak in Figure 3 shows that the ChlM protein used was monomeric, and not contaminated by aggregated protein. The convergent v versus $[S]$ plots in Figure 4 demonstrate that the reaction proceeds via a ternary mechanism, and rules out the possibility of a ping-pong mechanism. The observation that SAH exhibits competitive inhibition with respect to SAM and mixed inhibition with respect to MgD (Figure 5) implies that a random-binding mechanism is operating. The point at which the lines in Figure 5(B) intersect suggest that SAH binds to free enzyme and a ChlM–MgD complex with similar affinity. The fluorimetric binding data in Figure 6(A) suggest that MgD binds to free enzyme, with a K_d value of $2.37 \pm 0.26 \mu\text{M}$. Furthermore, in the presence of $500 \mu\text{M}$ SAH, the K_d value is $4.74 \pm 1.76 \mu\text{M}$, implying that the presence of a ChlM–SAH complex does not significantly perturb the binding of MgD (Figure 6B). This is consistent with the SAH-inhibition kinetics in Figure 5(B). From these observations we conclude that catalysis by MgP methyltransferase proceeds via the binding of MgD to the free enzyme, leading to the formation of a ChlM–MgD complex with a K_d value of $2.37 \pm 0.26 \mu\text{M}$, or via the binding of SAM to the free enzyme (Scheme 2). The observation that SAM does not produce any fluorescence quenching does not imply that it cannot bind to free enzyme. This merely suggests that SAM does not induce a conformational change in the local environment of tryptophan residues in ChlM.

The SAM methyl group is bound to a charged sulphur atom, which renders the methylthiol moiety susceptible to nucleophilic attack either directly from the propionate carboxyl on ring C of the porphyrin or via an amino acid side chain with a methylated enzyme intermediate. The specificity constant for SAM ($k_{\text{cat}}/K_m^{\text{SAM}}$ is $1500 \text{ M}^{-1} \cdot \text{s}^{-1}$) is significantly lower than would be expected if the diffusional encounter with the enzyme was rate-limiting ($\geq 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ [36]). This relatively low value of k_{cat}/K_m implies that a quasi-equilibrium exists between enzyme–substrate complex and free substrate [37], provided that our active-site concentration is not much lower than our total enzyme concentration. We cannot be sure of this without an active-site titration, but Briggs–Haldane kinetics ($k_{\text{cat}}/K_m \geq 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ [36]) would require that less than 0.15% of our enzyme is active, which we consider unlikely. In order to determine the nature of the rate-determining step, e.g. enzyme isomerization, product release or methyl group transfer, it will be necessary to carry out detailed transient state kinetics.

Kinetic studies have been performed on MgP methyltransferase from *Euglena gracilis* [38], which imply the existence of a ternary complex. The K_m^{MgP} and K_m^{SAM} values were 20 and $230 \mu\text{M}$ respectively. Subsequent inhibition studies demonstrated that ChlM from *E. gracilis* operates via a random ternary mechanism [39]. In the case of our purified recombinant enzyme from *Synechocystis*, similar inhibition kinetics with SAH clearly demonstrate that SAH may bind to free enzyme or a ChlM–MgP complex, suggesting a random ternary mechanism. In contrast, steady-state kinetics performed on MgP methyltransferase from crude extracts of aetiolated wheat seedlings indicated that catalysis occurred via a ping-pong mechanism [40,41], implying that a methylated enzyme intermediate is present. Such an enzyme species has been demonstrated recently using radio-labelling techniques with sirohaem synthase, an enzyme in the cobalamin biosynthesis pathway that methylates uroporphyrinogen III [42]. In future, such techniques could be used to demonstrate the presence or absence of a covalent intermediate with ChlM from *Synechocystis*.

The enzyme-catalysed methylation of magnesium protoporphyrin IX (Scheme 1B) is particularly interesting, as both substrate and product of this reaction are implicated in plastid-

to-nucleus signalling [11,13]. Now that the reaction mechanism has been characterized at the level of steady-state kinetics, and is amenable to the application of single turnover studies (M. Shepherd, unpublished work), there will be continued interest in the function and regulation of this enzyme, particularly with respect to the interaction of ChlM with ChlH, the porphyrin-binding subunit of Mg chelatase. It is interesting that Hinchigeri et al. [43] have shown that MgP methyltransferase activity was stimulated upon the addition of cell free *E. coli* extracts containing overexpressed *bchH* from *R. capsulatus*. Thus, it is possible that a BchH/ChlH–MgP complex is the preferred substrate for the methyltransferase reaction. Karger et al. [35] have shown that BchH–MgD and ChlH–MgD complexes can form with K_d values of 2.4 and $0.22 \mu\text{M}$ for ChlH and BchH, respectively. Until the putative ChlH–MgP–ChlM interaction is studied in detail using both steady-state and single-turnover methods it will not be possible to examine the possibility of tight coupling between the Mg chelatase and MgP methyltransferase enzymes. This is not a recent idea, as a multienzyme complex for the conversion of protoporphyrin to MgPME was proposed many years ago [44].

In terms of regulation and signalling, the availability of MgP and MgPME for signalling roles [9–13] could hinge on the relative turnover rates and substrate-/product-binding affinities of the Mg chelatase and methyltransferase enzymes, and on the abundance of ChlH both as a component of the Mg chelatase [35,45,46] and as a potential substrate donor to the methyltransferase [1]. It has been observed that amounts of ChlH transcript vary considerably during a diurnal cycle [47–49]. Such variations may impinge on the coupling between Mg chelatase and MgP methyltransferase, with consequences for the availability of MgP and MgPME as signalling molecules.

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