MgCH, magnesium chelatase; Proto, protoporphyrin IX; MgProto, magnesium protoporphyrin. This large enzyme complex comprises ChlH, I and D subunits, with I and D involved in ATP hydrolysis, and H the protein that handles the substrate and product. The 148 kDa ChlH subunit has a globular N-terminal domain attached by a narrow linker to a hollow cage-like structure. Following deletion of this ~18 kDa domain from the Thermosynechococcus elongatus ChlH, we used single particle reconstruction to show that the apo- and porphyrin-bound forms of the mutant subunit consist of a hollow globular protein with three connected lobes; superposition of the mutant and native ChlH structures shows that, despite the clear absence of the N-terminal ‘head’ region, the rest of the protein appears to be correctly folded. Analyses of dissociation constants shows that the ΔN159ChlH mutant retains the ability to bind protoporphyrin and the Gun4 enhancer protein, although the addition of I and D subunits yields an extremely impaired active enzyme complex. Addition of the Gun4 enhancer protein, which stimulates MgCH activity significantly especially at low Mg2+ concentrations, partially reactivates the ΔN159ChlH–I–D mutant enzyme complex, suggesting that the binding site or sites for Gun4 do not wholly depend on the N-terminal domain.

Key words: chlorophyll, chlorophyll biosynthesis, electron microscopy, magnesium chelatase, Synechocystis sp. PCC6803, Thermosynechococcus elongatus.

INTRODUCTION

Chlorophylls (Chls) are the essential cofactors for photosynthesis, and their biosynthesis involves a series of enzyme-driven reactions, the first committed step of which is catalysed by magnesium chelatase (E.C. 6.6.1.1; MgCH). This three-subunit enzyme (H ≈150 kDa; D ≈70 kDa; I ≈40 kDa) is situated at the branch-point between Chl and haem biosynthesis, and it catalyses the ATP-dependent insertion of a Mg2+ ion into the protoporphyrin IX (Proto) macrocycle [1,2]. H is the porphyrin-binding subunit [3,4]. The I subunit (BchI in purple phototrophic bacteria, Chl in cyanobacteria and plants) is an active ATPase [5,6] that associates with the D subunit [5,7]. The D subunit is not an ATPase, but the nucleotide binding site is required for allosteric regulation of MgCH [8]. I and D are members of the AAA+ superfamily, and cryo-EM of the BchI–D–MgATP complex showed that BchI and BchD form a double βββ ring [9]. The cyanobacterial MgCH from Synechocystis sp. PCC6803 has been analysed by steady-state [5,10–13] and transient [14] kinetic approaches, as has the thermostable enzyme from Thermosynechococcus elongatus [15]. The catalytic cycle is thought to involve the association of H-Pro and IDMgATP, forming a short-lived complex that couples the hydrolysis of ATP to the formation of magnesium protoporphyrin (MgProto).

The 2.1 Å (1 Å = 0.1 nm) structure of the BchI subunit from the purple phototroph Rhodobacter capsulatus [16] has been used to interpret models of the BchIΔLd complex calculated from single-particle cryo-EM reconstructions [9]. These models, obtained using subunits incubated with either ADP or ATP, show that the catalytic cycle of MgCH involves conformational changes in the ID units. Rather less is known about the H subunit, although a low-resolution structural model of BchH from Rba. capsulatus has been obtained through 3D reconstruction of negatively stained single particles [17], and a similar approach, augmented by SAXS, was used to determine a low resolution structure of the cyanobacterial ChlH subunit [18]. In contrast with the more open BchH structure, ChlH forms a more enclosed cage-like assembly, connected to an N-terminal ‘head’ region adjacent to a 5 nm-diameter opening in the structure. It was suggested that the more enclosed ChlH structure affords some protection for the labile MgProto product, given the potentially damaging combination of light and oxygen within cells of oxygenic phototrophs such as cyanobacteria and plants [18].

A more complex structure for the ChlH with respect to BchH could also be a consequence of interactions with the Gun4 protein [19,20], which stimulates Mg-chelatase at low magnesium concentrations [21]. The structure of the cyanobacterial Gun4 protein has been determined by X-ray crystallography [21,22], but there is currently no structural detail available for the ChlH–Gun4 complex.

The N-terminal ‘head’ domain of ChlH of T. elongatus was identified by binding NTA–Nanogold to the N-terminal His tag [18]. This domain, estimated with the ‘neck’ to have a molecular mass of ~17.6 kDa, is of interest given its location adjacent to the entrance to the lumen within ChlH and its connection to the main body of ChlH through a narrow linker region between Gly127

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and Phe156. We speculated that the head domain, together with a flexible linker, could be involved in gating access to the cavity within ChlH [18]. In the present study, we removed 159 residues from the N-terminus of ChlH from T. elongatus to find out whether this head domain is indeed at the N-terminus, and to determine whether it represents an autonomous region of ChlH, in the sense that the rest of the ChlH folds correctly in its absence. The low-resolution structures of the apo- and porphyrin-bound forms of the ΔN159ChlH subunit show that the N-terminal domain has been removed cleanly from the rest of the protein, and functional assays show that the remaining 130 kDa of this N-terminally truncated subunit still binds the porphyrin substrate, the product of the MgCH reaction, and the Gun4 enhancer protein. Only 10% of chelatase activity remains following removal of the head domain, but this value increases to 24% in the presence of saturating concentrations of Gun4. We discuss the possible functional roles of the head domain of ChlH.

**EXPERIMENTAL**

**Construction of T. elongatus ΔN159chIH**

The truncated chlH gene was engineered by introducing a new Ndel site into the DNA at Met159 using the following primers: M159TOP, 5′-TGCCGCTTCCAAAGATCATATGCCTAACCTCC-3′ and M159BOT, 5′-GGAGTTTGGACATATGATCTTGGAAAGCGCA-3′. QuikChange® PCR was carried out according to the manufacturer’s protocol (Stratagene), using the T. elongatus cloned pET9aHis,ChlH as the template. The PCR product was digested with Ndel, gel purified and ligated with T4 DNA ligase (Promega). The resultant pET9aHis,ΔN159chIH construct was verified by DNA sequencing (GATC) prior to overexpression.

**Purification of recombinant ΔN159ChlH from T. elongatus**

The plasmid containing the gene encoding T. elongatus ΔN159ChlH was transformed into Escherichia coli and the cells were induced for protein production as described by Qian et al. [18], then harvested after 4 h expression. Initial purification revealed that most of the protein was in a highly aggregated state. Monomeric protein was produced by adding n-dodecyl-β-D-maltopyranoside (β-DDM) to the supernatant (and all buffers) to a final concentration of 0.05% and gently incubating for 1 min in a sonicating water bath prior to purification. The purification was performed as described in Qian et al. [18], using immobilized metal affinity chromatography, then ion-exchange chromatography on Q-Sepharose and finally HPLC gel filtration.

**Purification of recombinant T. elongatus ΔN159ChlH for MgCH assays**

The gene encoding ΔN159ChlH was overexpressed in E. coli strain Rosetta 2 pLysS (Novagen). Cultures in LB (500 ml) were grown to an OD₆₀₀ of 0.6 at 37°C then transferred to 20°C for 2 h with 0.4 mM IPTG. The purification was performed according to Qian et al. [18], using immobilized metal affinity chromatography, ion-exchange chromatography on Resource Q (GE Healthcare), and finally gel filtration on a Superdex 200 (GE Healthcare). Binding of deuteroporphyrin (ΔN) to ΔN159ChlH was performed using the method in Qian et al. [18].

**Recombinant T. elongatus Gun4 protein**

The gun4 gene from T. elongatus was cloned into pET14b by PCR with primers TEG4Ndel, 5′-TCCATATGGTCCACACAGAACCCCTAGC-3′ and TEG4BamHI, 5′-TGGATCCCTAGCGTCCAGGAGATGATTAGG-3′, using T. elongatus pGEX-4T-1Gun4 as the template. The PCR product was digested with Ndel and BamHI and ligated into pET14b cut with the same restriction enzymes. The resultant pET14bTEgun4 construct was verified by DNA sequencing (GATC) prior to overexpression. pET14bTEgun4 was transformed into E. coli strain Rosetta 2 pLysS for overexpression of the Gun4 proteins. Cultures in LB (500 ml) were grown to a D₆₀₀ of 1 at 37°C then transferred to 20°C overnight with 0.4 mM IPTG. The proteins were purified by metal affinity chromatography and buffer exchanged into 50 mM Tricine (pH 7.9), 0.2 M NaCl, 0.3 M glycerol prior to MgCH assays.

**Electron microscopy**

Purified proteins were adsorbed on to freshly glow-discharged carbon-coated copper grids and negatively stained as described in [18]. In order to obtain a suitable protein particle density on EM grids, the purified AN159ChlH protein was diluted using HPLC running buffer (50 mM Tricine/NaOH (pH 7.9), 0.3 M NaCl, 0.3 M glycerol, 1 mM DTT, 0.05% β-DDM). Carbon-coated (~159 Å thickness) 400 mesh copper EM grids (Agar Scientific) were glow-discharged for 30 s. The diluted protein solution (5 μl) was applied to a grid and left for 30 s before gentle blotting with filter paper. The grid then was washed twice with water and stained with 0.75% uranyl formate solution for 20 s. Excess stain solution was removed by blotting then drying in air. Data were collected using a Philips CM-100 microscope fitted with a 1K×1K Gatan Multiscan 794 CCD camera with magnification set at ×61000, which corresponds to 3.93 Å per pixel at the specimen level; the underfocus value was adjusted to −1.0 μm. Tilt pair images were recorded by turning the sample holder around the tilt axis by 0° and 10°.

**Data processing**

Single particles of ΔN159ChlH were picked semi-automatically from the electron micrographs using EMAN2 [23], with a square box of 56 pixels × 56 pixels corresponding to 22 nm × 22 nm. 20236 combined particles were then treated subsequently using the IMAGIC-5 software package [24]. All particles were band-pass filtered to suppress low spatial frequencies, initially according to the suggested values (low frequency cutoff 0.04; high frequency cutoff 0.8) in the IMAGIC-5 operation manual (http://www.imagescience.de/manuals/smi.pdf). The filtered particles were masked, normalized and centred for reference-free 2D classification; a set of 25 characteristic 2D classes was used for multi-reference alignment (MRA) and multivariate statistical analysis [25,26]. A few iterations were performed until a stable 2D classification was obtained. A total of 600 2D classes were selected for Euler angle assignment and subsequent 3D reconstruction. An initial 3D model was produced using the e2initialmodel.py programme in the EMAN2 software package. This 3D model was used to calculate the Euler angles of the averaged 2D classes; a new model was produced from the data set and re-projected so new Euler angles could be re-assigned. This iteration was continued until a stable 3D model was obtained.
Similar treatments were applied for the 3D reconstruction of porphyrin-bound ΔN159ChlH. Here, 18,659 particles were picked from 220 electron micrographs.

**Binding of substrate and product to ΔN159ChlH**

Tryptophan quenching assays and calculations of $K_d$ values for the $D_{ox}$ and magnesium deuteroporphyrin (Mg$D_{ox}$) ligands were performed as described previously [3].

**Labelling proteins**

Gun4 was reduced in 10 mM DTT for 10 min at room temperature before buffer exchange into 20 mM NaH$_2$PO$_4$ and 150 mM NaCl, pH 7.4, using a Zeba Spin column (Thermo Scientific) and then labelled with a 10-fold excess of Alexa Fluor 532 C$_5$ maleimide (Life Technologies), dissolved in dry DMF, for 2 h at room temperature while being protected from the light. The reaction was quenched by the addition of 10 mM DTT for 15 min.

Wild-type (WT) ChlH and ΔN159ChlH were desalted into PBS titrated to pH 8.3 with 0.2 M pH 9 sodium bicarbonate, using a Zeba Spin column. Protein was labelled with a 3-fold excess of Atto 488 NHS ester dissolved in dry DMF for 1 h at room temperature while being protected from light. The reaction was quenched with the addition of 10 mM Tris/HCl (pH 7.4) for 15 min.

**Monitoring protein association via FRET**

**RESULTS**

**Choice of the ΔN159ChlH truncation, and purification of the recombinant T. elongatus ΔN159ChlH protein**

The N-terminal domain had been identified previously by attaching a 5 nm diameter NTA–Nanogold particle to the N-terminal His$_6$ tag, and imaging the labelled molecules using EM [18]. The molecular mass of the N-terminal ‘head’ domain of ChlH, connected to the rest of ChlH by a narrow linker, was estimated to lie within the range 14.1–17.3 kDa [18]. Thus, the sequence MG$^{127}$SFSLAQIG$^{135}$ QSKSV1ANFMKKRKEKSG$^{153}$ AG$^{154}$ FQDAMLK (Figure 1A) is likely to contain the linker region. It was noted in [18] that Gly$_{127}$ is conserved in all MgCh H subunits, and that between three and five glycine residues are generally found in this region of ChlH proteins, consistent with a degree of flexibility in the linker and a possible gating role for the ‘head’ region. Truncation using Met$_{126}$ as the start codon conserved the linker, but this construct did not yield any recombinant protein; using Met$_{160}$ instead for the truncated ChlH mutant designated ΔN159ChlH did yield protein, found in both the soluble and insoluble fractions of disrupted E. coli cells. Expression trials (results not shown) were performed to limit the aggregation of truncated protein and, following purification using immobilized metal affinity chromatography then ion-exchange chromatography, we monitored the aggregation state of the ΔN159ChlH protein using HPLC gel filtration. It was found that exchanging ΔN159ChlH into buffer containing the detergent β-DDM maintained nearly 580 ± 5 nm filter, detected the production of product MgD$_{ox}$ over a period of 1–2 h. Steady-state rates ($v_{ss}$) were calculated using the plate reader software (MARS Data analysis suite version 2.41) taking the steepest gradient over a 10 min time interval. Calibration was performed using known concentrations of MgD$_{ox}$ in the presence of standard concentrations of subunits (ChlI, D and H) and the data were fitted to a second order polynomial. Non-linear regression was performed using Igor Pro. The concentration of porphyrins was determined by visible spectroscopy in 0.1 M HCl with the molar absorption coefficient of 433 000 M$^{-1}$·cm$^{-1}$ at 399 nm. The concentration of ATP in water was determined using the molar absorption coefficient of 15 400 M$^{-1}$·cm$^{-1}$ at 260 nm. Concentrations of proteins were determined according to their absorbance at 280 nm with the following molar absorption coefficients: ChlI, 15 640 M$^{-1}$·cm$^{-1}$; ChlD 30 230 M$^{-1}$·cm$^{-1}$; ChlH 15 4600 M$^{-1}$·cm$^{-1}$; ΔN159ChlH 148 080 M$^{-1}$·cm$^{-1}$; Gun4 67 380 M$^{-1}$·cm$^{-1}$.

In eqn (1), $F_{obs}$ is the observed fluorescence, $F_0$ is the initial fluorescence, $F_{min}$ is the minimum amplitude of fluorescence quenching and $K_d$ is the apparent dissociation constant.

In eqn (2), $v_{ss}$ is the observed steady-state rate, $v_0$ is the steady-state chelatase rate in the absence of Gun4, $v_{lim}$ is the limiting steady-state rate reached at saturating Gun4, $n_d$ is the concentration of binding sites for Gun4, i.e. ([ChlH]×(number of sites/ChlH)), and $K_{app}$ is the apparent dissociation constant for the active ChlH–Gun4 complex.

\[
F_{obs} = F_0 - F_{min} - \frac{[Gun4] + [ChlH] + K_d - \sqrt{([Gun4] + [ChlH] + K_d)^2 - 4[ChlH][Gun4]}}{2[ChlH]} \tag{1}
\]

\[
v_{ss} = v_0 + v_{lim} - \frac{[Gun4] + n_d + K_{app} - \sqrt{([Gun4] + n_d + K_{app})^2 - 4n_d[Gun4]}}{2n_d} \tag{2}
\]
all the truncated ChlH in a monomeric state. It is likely that removal of the N-terminal domain and the linker exposes a hydrophobic patch, which can be shielded by detergent molecules. Subsequently, therefore, 0.05% β-DDM was added to the supernatant and to all buffers used for ΔN159ChlH ChlH.

Recombinant His6-tagged ΔN159ChlH from T. elongatus was purified using a three-step procedure involving immobilized metal affinity chromatography, ion-exchange chromatography, and gel filtration. For protein analysed by single particle analysis (see next section), an additional HPLC gel filtration step was included. The SDS/PAGE analysis of ΔN159ChlH, monitored through each of the purification steps, shows a high level of purity after gel filtration (Figure 1B). As expected, removal of the N-terminal domain reduces the molecular mass of ΔN159ChlH, in comparison with the ChlH standard, which has a predicted molecular mass of 148 kDa.

**Single particle analysis of the ΔN159ChlH and porphyrin-bound ΔN159ChlH proteins**

Figures 2(A) and 2(B) show typical raw data for the clearly monomeric ΔN159ChlH and DIX-bound ΔN159ChlH proteins. Approximately 70 particles could be identified within a single 1K × 1K micrograph, and in total 20236 and 18659 particles were picked for further analysis (see Experimental section). The top two rows of Figures 2C and 2D show examples of averaged 2D classes from ΔN159ChlH and porphyrin-bound ΔN159ChlH proteins, and for comparison the bottom two rows display the reprojections from the stable 3D models, which are consistent with the corresponding 2D classes. Supplementary Figure S1 shows the distribution of Euler angles used for our image analyses of the ΔN159ChlH and porphyrin-bound ΔN159ChlH protein samples. A total of 600 2D averaged classes was selected for ΔN159ChlH and porphyrin-bound ΔN159ChlH, and compared with WT ChlH (apo-ChlH, 75 classes; substrate-bound ChlH, 92 classes; [18]); the absence of the ‘head’ region presumably allows a better 3D distribution of the protein orientations.

The reconstructed 3D models of the ΔN159ChlH and DIX-bound ΔN159ChlH protein are displayed in Figure 2E, twice rotated about the z-axis by 90°. The threshold values for the volume of these models were adjusted to their predicted molecular mass of 130 kDa. The ΔN159ChlH and porphyrin-bound ΔN159ChlH structures, calculated to 23 and 27 Å, respectively (Supplementary Figure S2), both show a hollow globular protein with three connected lobes enclosing a large open lumen. There are no significant differences between these two structures at these low resolutions.

Figure 3 shows the superposition of the ΔN159ChlH mutant on to the previously published T. elongatus ChlH model [18]. The choice of handedness and best fit was judged by eye, but we emphasize that the absolute hand of the two structures is unknown. The two models are not identical in their globular regions, which may be the result of a slight alteration in folding or it may be attributed to the experimental error associated with negative staining. However, the superposition shows that the main globular regions of the truncated and WT ChlH proteins are of a similar size and that the gaps and apertures in the structure are approximately in the same positions. The most striking and significant difference is the clear absence of the WT ‘head’ region from the ΔN159ChlH mutant.

**Porphyrrin binding studies and the effect of Gun4 on ΔN159ChlH in MgCh assays**

Given the apparent preservation of the rest of the ChlH structure, following removal of the N-terminal ‘head’ domain, it was of interest to see whether the porphyrin binding and catalytic functions had been retained in the ΔN159ChlH mutant. The fluorescence of ChlH tryptophan residues is quenched upon binding of porphyrins, enabling dissociation constants between protein, substrate and product to be determined [3]. Figure 4 displays plots of integrated fluorescence as a function of tetrapyrrole concentration, with the data fitted to a single substrate binding model [3]. The calculated Kᵢ values of ΔN159ChlH for DIX substrate and MgDIX product were 1.06 ± 0.09 and 1.64 ± 0.08 μM, respectively; these values are consistent within the dissociation constants for WT T. elongatus ChlH of 1.48 ± 0.3 μM for DIX [18] and 2.12 ± 0.2 μM for MgDIX (see Supplementary Figure S3).

To characterize the interaction between ΔN159ChlH and Gun4, a FRET experiment was performed. Here, ChlH was labelled with Atto 488 NHS ester, and Gun4 with Alexa Fluor 532 maleimide.

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**Figure 1** The proposed head, linker and main regions of ChlH; purification of the T. elongatus ΔN159ChlH mutant lacking the head and linker regions

(A) 3D structural model of the apo-ChlH MgCH subunit from T. elongatus, calculated at 30 Å resolution [18]; the indigo circle delineates the N-terminal head domain, connected to the rest of ChlH (blue circle) by a narrow linker (black circle). Amino acids predicted to comprise the three parts of ChlH are shown to the right of the model. The glycine residue highlighted in bold after the first methionine residue in the linker sequence is conserved in all known ChlH/BchH proteins. The methionine residue in red in the linker sequence was chosen as the initiation codon for the ΔN159ChlH. (B) SDS/PAGE of purified recombinant ΔN159ChlH, 1, Protein markers; 2, supernatant fraction from cell extract; 3, flow-through from binding supernatant to Ni²⁺ affinity column; 4, pooled eluted fractions containing ΔN159ChlH; 5, pooled eluted fractions containing ΔN159ChlH following anion-exchange chromatography; 6, ΔN159ChlH fractions from size-exclusion chromatography; 7, ΔN159ChlH fractions following HPLC size-exclusion chromatography; 8, WT ChlH standard. The arrow indicates the ΔN159ChlH band, estimated as 135 kDa.
Figure 2  EM raw data and classifications with 3D reconstruction of *T. elongatus* ΔN159ChlH

(A) Electron microscopy of purified ΔN159ChlH with the white boxes indicating single proteins. Scale bar = 50 nm. (B) Electron micrograph of purified D9-bound ΔN159ChlH. (C) Gallery of selected 2D averaged classes (top two rows) and their corresponding reprojections (bottom two rows) from 3D models of ΔN159ChlH. The box size is 22 nm × 22 nm. (D) Gallery from porphyrin-bound ΔN159ChlH. (E) 3D reconstruction of ΔN159ChlH without (green) and with (red) bound porphyrin, rotated through 90°. The 3D models were generated using Chimera [33].
Figure 3  Superposition of the 3D structures of T. elongatus ΔN159ChlH and WT ChlH

Superposition of the WT ChlH structure [18] (blue wire mesh model) on to the 3D model for the ΔN159ChlH (green solid – without porphyrin) rotated through 90° successively about the z-axis.

Figure 4  Substrate and product binding curves for T. elongatus ΔN159ChlH

Quenching of ΔN159ChlH protein fluorescence of; top, deuteroporphyrin IX; bottom, MgD IX. 0.1 μM of purified ΔN159ChlH was incubated with 0–15 μM substrate or product and incubated at 34 °C. Using the excitation wavelength of 295 nm, fluorescence emission scans were recorded at 34 °C. A.U., arbitrary units. Both curves show single-site binding. The Kₐ values are 1.06 ± 0.09 and 1.64 ± 0.08 μM, respectively.

Figure 5  Substrate and product binding curves for T. elongatus ΔN159ChlH

Quenching of ΔN159ChlH protein fluorescence of; top, deuteroporphyrin IX; bottom, MgD IX. 0.1 μM of purified ΔN159ChlH was incubated with 0–15 μM substrate or product and incubated at 34 °C. Using the excitation wavelength of 295 nm, fluorescence emission scans were recorded at 34 °C. A.U., arbitrary units. Both curves show single-site binding. The Kₐ values are 1.06 ± 0.09 and 1.64 ± 0.08 μM, respectively.

DISCUSSION

The H subunit binds the Proto substrate and the MgProto product of the reaction catalysed by MgCH, which requires the formation of a transient H–I–D complex and the hydrolysis of 14 MgATP²⁻ molecules [12]. Thus, interactions with the I and/or D subunits are part of ChlH function, as is the formation of a complex with the Gun4 protein [19,20], and with the next enzyme in the pathway, MgProto methyltransferase [27–30]. Nonetheless, it is not obvious why ChlH is such a large protein, comprising 1326 amino acids and 148 kDa for the T. elongatus subunit, for example. It was proposed in [18] that the hollow ‘caged’ structure of ChlH encloses the MgProto product, protecting it from photodestruction and channelling it to the methyltransferase. Thus, the need for a protective enclosed structure could account for the extra size of ChlH relative to its BchH counterpart from anaerobic photosynthetic bacteria where harmful light/oxygen combinations are less likely. However, even the BchH subunits from the purple photosynthetic bacteria such as Rba. capsulatus consist of 1189 residues and 129 kDa, so this large three-lobed structure, determined by single particle reconstruction at a resolution of 25 Å [17], is required for its core catalytic functions. These require interactions with porphyrins and the I and/or D subunits, but not, for example, with Gun4, since no homologue of this enhancer protein has been found in purple phototrophs.

The single particle/SAXS study of the T. elongatus ChlH protein [18] revealed that the majority of the protein forms a hollow globular structure, which is attached to an N-terminal ‘head’ domain through a narrow linker. The present work also from T. elongatus were assayed for in vitro MgCH activity by continuously monitoring fluorescence from the MgDIX product at 580 nm. The assays were performed using recombinant H, I and D subunits from T. elongatus, at 45 °C [15]. Figure 5(B) shows the steady-state rates of MgCH activity for WT ChlH (●) and ΔN159ChlH (○) in the presence and absence of Gun4. As expected from previously published work [21], the steady-state rate of WT MgCH increases up to 1.6-fold with increasing concentration of Gun4. ΔN159ChlH protein has 10% activity compared with WT, which in presence of saturating concentrations of Gun4 is increased to ~24% of WT activity. The Kₐ for the Gun4–ΔN159ChlH MgCH complex is ~8-fold lower compared with WT, indicating that saturation of the MgCH complex occurs at a lower concentration of Gun4. Removal of the N-terminal head region of ChlH appears to enhance Gun4 binding to the core chelatase complex, possibly by making the binding site more available.

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showed that porphyrin binding and catalytic activity can be separated to some extent; N-terminal deletion of 565 residues (42% of the sequence) from the Synechocystis ChlH abolished enzymatic function, leaving porphyrin binding unaffected. Thus, normal catalytic function requires the N-terminal 565 residues, and the purpose of the present study was to narrow down the search for a catalytically essential region of ChlH by removing 159 residues from the N-terminus. Ideally, removal of this domain would leave the rest of ChlH unaffected structurally, if not functionally, and indeed the low-resolution structure of the ΔN159ChlH mutant and comparison with WT ChlH show that the N-terminal domain has been removed cleanly from the rest of the protein, leaving the rest of the structure apparently intact. This observation provides confirmation that that the head domain is indeed encoded by the ~17.6 kDa N-terminal fragment. Given that ΔN159ChlH appears to maintain its hollow globular structure with or without bound porphyrin and the protein appears to be correctly folded, ChlH appears to consist of two autonomous domains, joined by a narrow hinge region. We speculated that this Gly^{127–Phe^{156}} region controls the binding of ChlH to other subunits or to the membrane, or it is involved in mobility of the head domain, possibly opening or closing the cavity within ChlH [18].

Analysis of dissociation constants shows that ΔN159ChlH is able to bind Proto and MgProto with normal affinities; the FRET data in Figure 5(A) indicate that the N-terminal truncation has not impaired the binding of Gun4 to this subunit. Chelatase assays with the H, I and D subunits show that the loss of the N-terminal domain has severely decreased MgCH activity, so this delineates the porphyrin binding and catalytically important parts of ChlH more precisely than achieved previously. The addition of Gun4 partly revives the MgCH activity of ΔN159ChlH, apparently compensating for the loss of the head domain to a limited extent. The ability of Gun4 to restore some activity to inactive ChlH has been noted before, when the gun-3 and cch mutations corresponding to A990V and P642L respectively in the Arabidopsis homologue [31] were introduced into the Synechocystis ChlH subunit, as A942V and P595L. Each of these mutations inactivates ChlH in MgCH assays, but the addition of Gun4 restored activity to 30–50% of WT (i.e. ChlH + Gun4) levels [32]. Gun4 does form a complex with ChlH [19,20], but this cannot be a result of interacting solely with the N-terminal domain, since it is absent from the ΔN159ChlH mutant. Nevertheless, it is possible that Gun4 binds adjacent to the N-terminal region perhaps promoting interaction of the I and D subunits with ChlH. Structural analysis of H–Gun4 complexes will help to clarify the functional role of Gun4 and its interaction with the ChlH subunit.

Figure 5 Gun4 is able to associate with and stimulate activity of ΔN159ChlH

(A) FRET association experiment between Gun4 and WT ChlH (●) or ΔN159ChlH (○). Assays contained 0.1 μM ChlH, in 50 mM Tricine/NaOH, 0.3 M glycerol and 200 mM NaCl, pH 7.9, at 45 °C. ChlH was labelled with Atto 488 and Gun4 labelled with Alexa Fluor 532. The quenching of emission of Atto 488 was monitored at 524 nm. A.U. – arbitrary units. The curves can be described by eqn (1) with the concentration term, [ChlH], held at 0.1 μM with characterizing parameters: WT ChlH $K_0 = 0.009 \pm 0.003 \mu M$ and $\Delta$N159ChlH $K_0 = 0.016 \pm 0.004 \mu M$. (B) Assembly titrations of MgCH between Gun4. Assays contained 0.1 μM ChlH, 0.2 μM ChlI, 0.4 μM ChlD in 50 mM Mops/KOH, 0.3 M glycerol, 1 mM DTT, 10 mM free Mg$^2+$, J = 0.1 with KCl and 8 μM D$_2$O, pH 7.7, at 45 °C. The curves can be described by eqn (2), $K_0$ was held at 0.4 (i.e. one binding site on ChlH for Gun4) based on the fit in (A) with characterizing parameters WT ChlH $K_{app} = 0.39 \pm 0.14 \mu M$ and $\Delta$N159ChlH $K_{app} = 0.05 \pm 0.03 \mu M$.

REFERENCES

1. Gibson, L. C. D., Willows, R. D., Kannangara, C. G., von Wettstein, D. and Hunter, C. N. (1995) Magnesium-protoporphyrin chelatase of Rhodobacter sphaeroides: reconstitution of activity by combining the products of the chlh, -I and -D genes expressed in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 92, 1941–1944 CrossRef PubMed

2. Jensen, P. E., Gibson, L. C. D., Henningsson, K. W. and Hunter, C. N. (1996) Expression of the chlh, chlI and chlD genes from the cyanobacterium Synechocystis PCC6803 in Escherichia coli and demonstration that the three cognate proteins are required for magnesium-protoporphyrin chelatase activity. J. Biol. Chem. 271, 16662–16667 CrossRef PubMed

AUTHOR CONTRIBUTION

Nathan Adams, Christopher Marklew, Pu Qian, Amanda Brindley and Paul Davison performed the experiments. Nathan Adams, Christopher Marklew, Pu Qian, Amanda Brindley, Per Bullough and Neil Hunter designed the experiments, Nathan Adams, Christopher Marklew, Amanda Brindley, Per Bullough and Neil Hunter wrote the paper.

ACKNOWLEDGEMENT

We thank Dr Svetomir Tzokov for EM support.

FUNDING

C.N.H., P.O., A.B., N.A., P.B. and P.D. were supported by a grant [grant number BB/G021546/1] from the Biotechnology and Biological Sciences Research Council (UK). C.J.M. was supported by a doctoral studentship from the Biotechnology and Biological Sciences Research Council (UK).
3 Karger, G. A., Reid, J. D. and Hunter, C. N. (2001) Characterization of the binding of deuteroporphyrin IX to the magnesium chelatase H subunit and spectroscopic properties of the complex. Biochemistry 40, 9291–9299 CrossRef PubMed

4 Willows, R. D. and Beale, S. I. (1998) Heterologous expression of the Rhodobacter capsulatus Bchl, -O, and -H genes that encode magnesium chelatase subunits and characterization of the reconstituted enzyme. J. Biol. Chem. 273, 34206–34213 CrossRef PubMed

5 Jensen, P. E., Gibson, L. C. D. and Hunter, C. N. (1999) ATPase activity associated with the magnesium-protoporphyrin IX chelatase enzyme of Synechocystis sp. PCC6803: evidence for ATP hydrolysis during Mg2+ insertion, and the MgATP-dependent interaction of the ChlI and ChlD subunits. Biochem. J. 339, 127–134 CrossRef PubMed

6 Reid, J. D., Siebert, C. A., Bullough, P. A. and Hunter, C. N. (2003) The ATPase activity of the ChlI subunit of magnesium chelatase and formation of a heptameric AAA+ ring. Biochemistry 42, 6912–6920 CrossRef PubMed

7 Gibson, L. C. D., Jensen, P. E. and Hunter, C. N. (1999) Magnesium chelatase from Rhodobacter sphaeroides: initial characterization of the enzyme using purified subunits and evidence for a Bchl–BchD complex. Biochem. J. 337, 243–251 CrossRef PubMed

8 Adams, N. B. P. and Reid, J. D. (2013) The allosteric role of the AAA + domain of ChlD from the magnesium chelatase of Synechocystis species PCC 6803. J. Biol. Chem. 288, 28727–28732 CrossRef PubMed

9 Lundqvist, J., Eilmund, H., Wulf, R. P., Berglund, L., Eilmund, D., Emunsson, C., Hebert, H., Willows, R. D., Hansson, M., Lindahl, M. and Al-Karadaghi, S. (2010) ATP-induced conformational dynamics in the AAA + motor unit of magnesium chelatase. Structure 18, 354–365 CrossRef PubMed

10 Jensen, P. E., Gibson, L. C. D. and Hunter, C. N. (1998) Determinants of catalytic activity with the use of purified I, ChlD and H subunits of the magnesium protoporphyrin IX chelatase from Synechocystis sp. PCC6803. Biochem. J. 334, 335–344

11 Jensen, P. E., Reid, J. D. and Hunter, C. N. (2000) Modification of cysteine residues in the ChlII and ChlIIII subunits of magnesium chelatase results in enzyme inactivation. Biochem. J. 352, 435–441 CrossRef PubMed

12 Reid, J. D. and Hunter, C. N. (2004) Magnesium-dependent ATPase activity and cooperativity of magnesium chelatase from Synechocystis sp. PCC6803. J. Biol. Chem. 279, 26883–26889 CrossRef PubMed

13 Adams, N. B. P. and Reid, J. D. (2012) Nonequilibrium isotope exchange reveals a catalytically significant enzyme-phosphate complex in the ATP hydrolysis pathway of the AAA + ATPase magnesium chelatase. Biochemistry 51, 2029–2031 CrossRef PubMed

14 Viney, J., Davison, P. A., Hunter, C. N. and Reid, J. D. (2007) Direct measurement of metal-ion chelation in the active site of the AAA + ATPase magnesium chelatase. Biochemistry 46, 12788–12794 CrossRef PubMed

15 Adams, N. B. P., Marklew, C., J., Brindley, A. A., Hunter, C. N. and Reid, J. D. (2014) Characterization of the magnesium chelatase from Thermosynechococcus elongatus. Biochem. J. 457, 163–70 CrossRef PubMed

16 Fodje, M. N., Hansson, A., Hansson, M., Olsen, J. G., Gough, S., Willows, R. D. and Al Karadaghi, S. (2001) Interplay between an AAA module and an integrin I domain may regulate the function of magnesium chelatase. J. Mol. Biol. 311, 111–122 CrossRef PubMed

17 Sirivovski, N., Lundqvist, J., Rosenbäck, M., Eilmund, H., Al-Karadaghi, S., Willows, R. D. and Hansson, M. (2008) Substrate-binding model of the chlorophyll biosynthetic magnesium chelatase BchlH subunit. J. Biol. Chem. 283, 11652–11660 CrossRef PubMed

18 Qian, P., Marklew, C. J., Viney, J., Davison, P. A., Brindley, A. A., Soderberg, C., Al-Karadaghi, S., Bullough, P. A., Grossmann, J. G. and Hunter, C. N. (2012) Structure of the cyanobacterial magnesium chelatase H subunit determined by single particle reconstruction and small-angle X-ray scattering. J. Biol. Chem. 287, 4946–4956 CrossRef PubMed

19 Larkin, R. M., Alonso, J. M., Ecker, J. R. and Chory, J. (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signalling. Science 299, 902–906 CrossRef PubMed

20 Sobolik, R., Dührer, U., Komenda, J., Peter, E., Gardian, Z., Tichy, M., Grimm, B. and Wilde, A. (2008) Importance of the cyanobacterial GUN4 protein for chlorophyll metabolism and assembly of photosynthetic complexes. J. Biol. Chem. 283, 25794–25802 CrossRef PubMed

21 Davison, P. A., Schubert, H. L., Reid, J. D., Iorg, C. D., Heroux, A., Hill, C. P. and Hunter, C. N. (2005) Structural and biochemical characterization of GUN4 suggests a mechanism for its role in chlorophyll biosynthesis. Biochemistry 44, 7600–7612

22 Verdecia, M. A., Larkin, R. M., Ferrer, J.-L., Riek, R., Chory, J. and Noel, J. P. (2005) Structure of the Mg-Chelatase cofactor GUN4 reveals a novel hand-shaped fold for porphyrin binding. PLoS Biol. 3, e777–e789 CrossRef PubMed

23 Tang, G., Peng, L., Baldwin, P. R., Mann, D. S., Jiang, W., Rees, I. and Ludtke, S. J. (2007) EMAN2: An extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46 CrossRef PubMed

24 van Heel, M., Harausz, G., Ortlova, E. V., Schmidt, R. and Schatz, M. (1996) A new generation of the IMAGi image processing system. J. Struct. Biol. 116, 17–24 CrossRef PubMed

25 van Heel, M. and Stoffer-Meilicke, M. (1985) Characteristic views of E. coli and B. stearothermophilus 30S ribosomal subunits in the electron microscope. EMBO J. 4, 2389–2395 PubMed

26 van Heel, M. and Frank, J. (1981) Use of multivariate statistics in analysing the images of biological macromolecules. Ultramicroscopy 6, 187–194 PubMed

27 Shepherd, M., Reid, J. D. and Hunter, C. N. (2003) Purification and kinetic characterization of the magnesium protoporphyrin IX methyltransferase from Synechocystis PCC6803. Biochem. J. 371, 351–360 CrossRef PubMed

28 Shepherd, M. and Hunter, C. N. (2004) Transient kinetics of the reaction catalysed by magnesium protoporphyrin IX methyltransferase. Biochem. J. 382, 1009–1013 CrossRef PubMed

29 Shepherd, M., McLean, S. and Hunter, C. N. (2005) Kinetic basis for linking the first two enzymes of chlorophyll biosynthesis. FEBS J. 272, 4532–4539 CrossRef PubMed

30 Akaway, A., Reski, R., Yaronskaya, E. and Grimm, B. (2005) Cloning and expression of the tobacco CHLH sequence encoding Mg protoporphyrin IX methyltransferase and its interaction with Mg chelatase. Plant Mol. Biol. 57, 679–691 CrossRef PubMed

31 Mochizuki, N., Brusslan, J. A., Larkin, R., Nagatani, A. and Chory, J. (2001) Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc. Natl. Acad. Sci. U.S.A. 98, 2053–2058 CrossRef PubMed

32 Davison, P. A. and Hunter, C. N. (2011) Abolition of magnesium chelatase activity by the gun5 mutation and reversal by GUN4. FEBS Lett. 585, 183–186 CrossRef PubMed

33 Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. and Ferrin, T. E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 CrossRef PubMed