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## Five Glutamic Acid Residues in the C-Terminal Domain of the ChlD Subunit Play a Major Role in Conferring Mg<sup>2+</sup> Cooperativity upon Magnesium Chelatase

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### **S** Supporting Information

**ABSTRACT:** Magnesium chelatase catalyzes the first committed step in chlorophyll biosynthesis by inserting a Mg<sup>2+</sup> ion into protoporphyrin IX in an ATP-dependent manner. The cyanobacterial (*Synechocystis*) and higher-plant chelatases exhibit a complex cooperative response to free magnesium, while the chelatases from *Thermosynechococcus elongatus* and photosynthetic bacteria do not. To investigate the basis for this cooperativity, we constructed a series of chimeric ChlD proteins using N-terminal, central, and C-terminal domains from *Synechocystis* and *Thermosynechococcus*. We show that five glutamic acid residues in the C-terminal domain play a major role in this process.

Magnesium chelatase (EC 6.6.1.1) catalyzes the ATP-dependent insertion of a magnesium ion into protoporphyrin IX, the first committed step in chlorophyll biosynthesis.<sup>1,2</sup> The minimal catalytic unit of magnesium chelatase consists of three proteins; ChlH, ChlI, and ChlD. ChlH binds porphyrin and is likely to be the site of metal ion chelation.<sup>3–5</sup> ChlI and ChlD form a complex<sup>6,7</sup> and are members of the AAA<sup>+</sup> superfamily (ATPases Associated with various cellular Activities); ChlI catalyzes ATP hydrolysis, and the ATP binding site on ChlD regulates enzyme activity.<sup>8–10</sup>

Regulation of magnesium chelatase is particularly important as the enzyme forms part of a metabolic branch point; the porphyrin substrate for magnesium chelatase is also the substrate for ferrochelatase, the terminal enzyme in heme biosynthesis. Flux down the two pathways is likely to be strongly influenced by the relative rates of insertion of Fe<sup>2+</sup> and Mg<sup>2+</sup> by the two competing chelatases.<sup>11</sup> The regulatory enzymology of magnesium chelatase is complex and species-dependent, with the *Synechocystis* enzyme showing a complex cooperative response to free magnesium concentration, whereas the enzyme from the thermophile *Thermosynechococcus elongatus* shows a noncooperative response.<sup>8,12</sup> The magnesium chelatases from these two species have a high degree of sequence identity (82% for ChlI, 67% for ChlD, and 78% for ChlH), and hybrid chelatases composed of subunits from both species are active. Assays of these hybrid chelatases reveal that the *Synechocystis* ChlD protein is essential for magnesium cooperativity.<sup>12</sup>

This observation that the ChlD protein from *Synechocystis* is involved in magnesium cooperativity and the demonstration that the N-terminal AAA<sup>+</sup> nucleotide binding site allosterically controls chelatase activity lead to the view that the ChlD subunit acts as a regulatory hub for magnesium chelatase.<sup>9,10</sup> However, we do not know the basis for this control. The recent determination of the structure of the catalytic ChlH subunit<sup>13</sup> is an important step in understanding the structural basis for regulation and catalysis in magnesium chelatase mediated by ChlD.

To investigate how ChlD controls the response to magnesium, we built a series of chimeric ChlD proteins with domains from *Synechocystis* and *T. elongatus* subunits. There are three of these domains (Figure 1A and Figure S1): an AAA<sup>+</sup>-like N-terminal domain, a polyproline and acidic side chain rich central domain, and an integrin I-like C-terminal domain.<sup>14–16</sup> The sequence of the AAA<sup>+</sup> N-terminal domain is approximately 40% identical with that of the ChlI protein (Figure S2), but the AAA<sup>+</sup> N-terminal domain has not been shown to hydrolyze ATP in a manner that is independent of the rest of the protein. The role of the central domain is unknown, but the C-terminal domain contains an essential MIDAS motif.<sup>17</sup> MIDAS motifs frequently bind divalent metals and can be involved in protein–protein interactions, although these roles have not yet been demonstrated for the MIDAS motif in ChlD.<sup>18</sup>

Six chimeric variants of ChlD were generated (Figure 1). Detailed maps and a sequence alignment are presented in the Supporting Information (Figures S1 and S2). Genes for all six proteins are readily overexpressed in *Escherichia coli*, and the proteins can be purified by conventional chromatography (Figure S3). CD spectroscopy demonstrates that all six proteins are folded (Figure S3A).

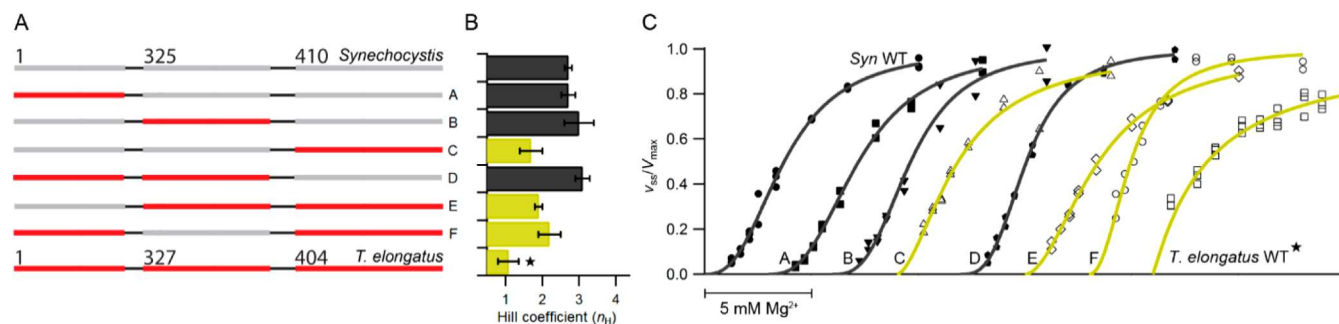
All six chimeric proteins are active in the presence of ChlI and ChlH from *Synechocystis* (Figure 1B,C). Chelatase activities are generally slightly lower than those of the *Synechocystis* enzyme (Table S1). These six chimeric constructs all show a cooperative response to free magnesium, but some of them show a substantially reduced cooperativity (i.e., Hill coefficient) when compared with that of the wild type.

The differences in behavior between these constructs allow us to dissect the functional contribution of individual domains

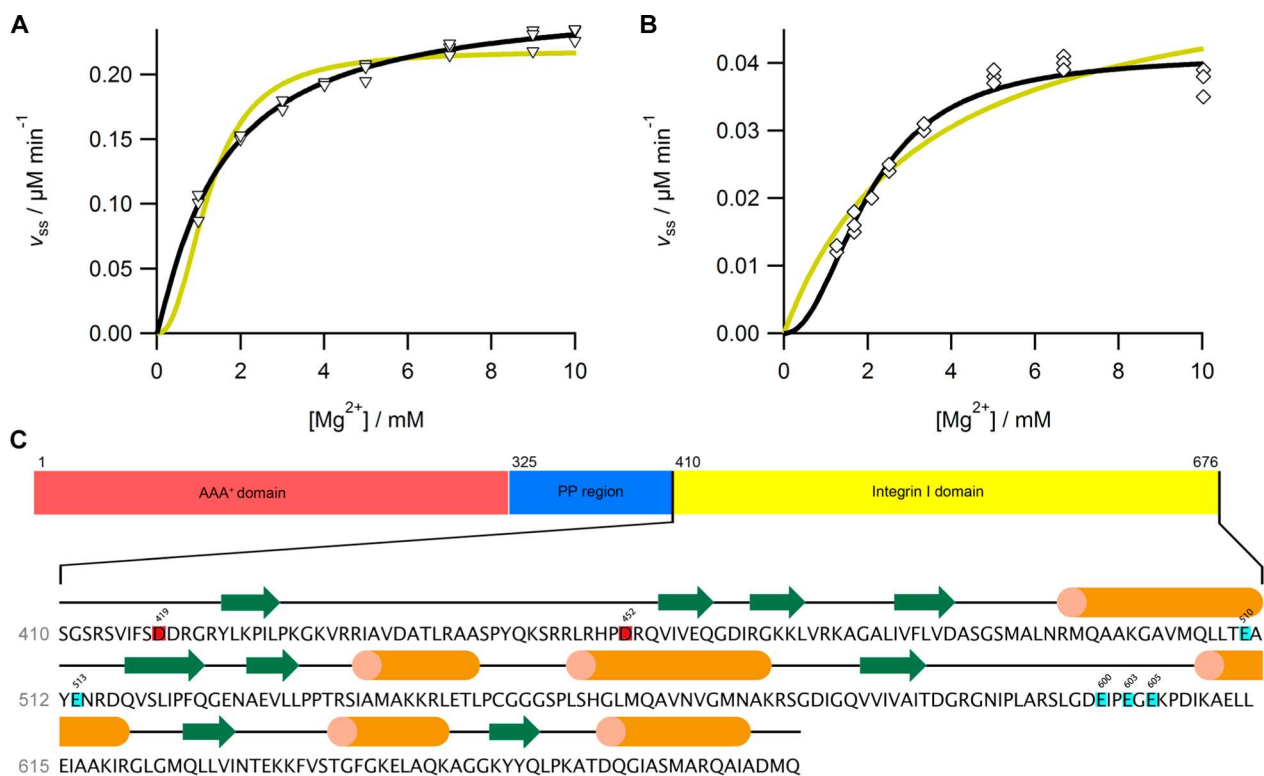
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**Figure 1.** (A) Diagram of ChlD chimeras compared with the wild-type proteins from *Synechocystis* and *T. elongatus*. Amino acid numbers show the boundaries of each domain. (B) Histogram of the corresponding Hill coefficients ( $n_H$ ) of constructs when assayed under saturating conditions (5 mM MgATP<sup>2-</sup> and 8  $\mu$ M DIX) in 50 mM MOPS/KOH and 1 mM DTT at  $I = 0.1$  M, pH 7.7, and 34 °C with 0.1  $\mu$ M ChlD, 0.2  $\mu$ M *Synechocystis* ChlI, and 0.4  $\mu$ M *Synechocystis* ChlH [(★) data from *T. elongatus* previously collected at 45 °C<sup>12</sup>]; yellow bars correspond to constructs with a *T. elongatus* C-terminal domain. (C) Mg<sup>2+</sup> dependence of the steady-state rate of magnesium chelation with chimeras A–F and *Synechocystis* and *T. elongatus* wild-type ChlD; each trace is offset by 3 mM Mg<sup>2+</sup> for the sake of clarity. Assay conditions and coloring as in panel B. The lines are theoretical and described by the Hill equation (supplementary eq 1), with characterizing parameters in Table S1.



**Figure 2.** (A) Mg<sup>2+</sup> dependence of the steady-state rate of magnesium chelation for the *Synechocystis* quintuple mutant (E510Q/E513Q/E600T/E603P/E605T) and (B) *T. elongatus* quintuple mutant ChlD (Q503E/Q506E/T594E/P597E/T599E). Assay conditions as in Figure 1B. The black lines are theoretical and are described by the Hill equation (supplementary eq 1), with an  $n_H$  value of  $1.1 \pm 0.1$  for the *Syn* quintuple mutant and an  $n_H$  of  $2.1 \pm 0.2$  for the *T. elongatus* quintuple mutant and characterizing parameters as in Table S4. The yellow lines allow comparison between the cooperative and noncooperative cases. In panel A, Hill coefficient  $n_H$  has been constrained to *Synechocystis* wild-type levels of cooperativity ( $n_H = 2.3$ ), and in panel B, Hill coefficient  $n_H$  has been constrained to *T. elongatus* wild-type levels of cooperativity ( $n_H = 1$ ); no other constraints were put upon the model. (C) Representation of the *Synechocystis* ChlD primary structure showing the distinct AAA', polyproline (PP), and integrin I domains. The expanded integrin domain below shows the predicted secondary structure elements (JPRED) within the C-terminal integrin I sequence, with  $\beta$  strands represented by green arrows and  $\alpha$  helices by orange cylinders. Residues with carboxyl side chains are highlighted; altering Asp (red) residues had no marked effect on magnesium cooperativity, while altering glutamic acid (blue) residues caused the marked differences in cooperativity seen in panels A and B and Table S4 and Figure S7.

to the complex cooperative response of the *Synechocystis* magnesium chelatase toward free magnesium.

At saturating concentrations of porphyrin and MgATP<sup>2-</sup>, the three chimeric ChlD proteins containing a *T. elongatus* C-terminal domain show a substantially reduced cooperative response to free magnesium (Figure 1B, constructs C, E, and

F). In contrast, constructs A, B, and D, with C-terminal domains derived from *Synechocystis*, show a cooperative response to magnesium (Figure 1B).

At low concentrations of ATP, we see similar behavior. All of the constructs with a *T. elongatus*-derived C-terminus are substantially less cooperative than the wild type (Figure S4A).

In contrast, constructs B and D with *Synechocystis* C-terminal domains show cooperative behavior essentially indistinguishable from that of the wild type. The magnesium chelatase activity of all six constructs was also measured at low concentrations of porphyrin (Figure S4B). When the concentration of porphyrin ( $1\ \mu\text{M}$ ) was below the  $K_m$ , ChlD proteins containing *T. elongatus*-derived C-terminal domains are all less cooperative than the wild type. The magnesium  $K_{0.5}$  for constructs A, C, and F is higher than the experimentally accessible range of free magnesium, so estimates of cooperativity are necessarily uncertain.

The ChlD subunit of *Synechocystis* magnesium chelatase plays a significant role in the cooperative response to free magnesium.<sup>9,12</sup> In line with our previous work, no magnesium cooperativity was observed with the *T. elongatus* ChlD protein, even under the broader range of experimental conditions used here (Figure S5).<sup>12</sup> The *Synechocystis/T. elongatus* ChlD chimeras generated in this study are at least slightly cooperative under the conditions tested, suggesting that magnesium cooperativity in magnesium chelatase requires a global response from all three domains of ChlD. However, the data in panels B and C of Figure 1 show a clear pattern in which chelatases with ChlD chimeras that include the *T. elongatus* C-terminal domain have significantly lower Hill coefficients, strongly suggesting that investigating potential  $\text{Mg}^{2+}$  interaction sites within this domain of the *Synechocystis* protein could provide structural insight into the cooperative response to free magnesium. This response is unlikely to involve the MIDAS motif (Figure S1), because it is present in all of the ChlD variants we examined.

Inspection of the C-terminal sequence highlighted four regions in which acidic residues (Asp and Glu) in *Synechocystis* are neutral residues in *T. elongatus* (Figure 2C and Figure S1). Some of these acidic residues, which potentially interact with a  $\text{Mg}^{2+}$  ion, were mutated to their respective counterparts (acidic to neutral in *Synechocystis* ChlD and neutral to acidic in *T. elongatus* ChlD) to determine the effect on magnesium chelatase magnesium cooperativity. A series of point mutations was generated, purified as wild-type protein, and shown to be folded by CD (Figure S7). Single-point mutations in both proteins (*Synechocystis* D419Q and D452Q and *T. elongatus* Q413D and Q452D) did not notably alter the cooperativity (Table S4 and Figure S8). Mutations in a double-glutamic acid motif in *Synechocystis* (E510Q E513Q) and the corresponding neutral motif in *T. elongatus* (Q503E Q506E) and a triple-glutamic acid motif in *Synechocystis* residues (E600T E603P E605T) and the corresponding neutral motif in *T. elongatus* (T594E P597E T599E) both produced marked changes in cooperativity (Table S4 and Figure S8). Remarkably, combining the mutations in these motifs (quintuple mutants) swapped the level of cooperativity in both proteins. The *Synechocystis* ChlD mutant lost cooperativity, from an  $n_H$  of 2.3 to 1.1, while the *T. elongatus* ChlD mutant became cooperative, from an  $n_H$  of 1 to 2.1 (in Figure 2A,B, compare black lines to yellow lines).

The *Synechocystis* ChlD C-terminal sequence was submitted to the Jpred 4.0 secondary structure prediction server, which predicted that the Glu residues highlighted in blue are exposed to solvent (Figure 2C); it is conceivable that they work in concert to bind and thus detect the concentration of magnesium. Subsequent conformational changes then control the rate of magnesium chelatase activity.

Sequence alignments of the magnesium chelatases currently studied in detail (Figure S1) show that the *Rhodobacter*

*sphaeroides* enzyme lacks this set of glutamate residues. Consistent with our observations, the *R. sphaeroides* chelatase is also not cooperative toward free magnesium.<sup>6</sup>

The data presented here further emphasize the regulatory role of the magnesium chelatase ChlD subunit. We show that while all three domains of the *Synechocystis* ChlD protein are involved in the cooperative response to free  $\text{Mg}^{2+}$ , the C-terminal domain plays a particularly significant role in this process. We used kinetic analysis and mutagenesis to identify five Glu residues in the C-terminal domain of *Synechocystis* ChlD, E510, E513, E600, E603, and E605, that appear to govern the major element of cooperativity and then show that the introduction of these residues into a native *T. elongatus* ChlD subunit exhibiting no cooperativity confers this property on this protein. Our previous study demonstrated that the AAA<sup>+</sup> site present in the N-terminal domain of ChlD is an allosteric regulator of metal ion chelation, as mutants in this site affect both  $\text{MgATP}^{2-}$  and  $\text{Mg}^{2+}$  binding.<sup>9</sup> Notably, these mutants also change the shape of the magnesium response curve; at the near saturating concentrations of  $\text{MgATP}^{2-}$  and porphyrin used, the wild-type *Synechocystis* enzyme showed a strongly sigmoidal response to free  $\text{Mg}^{2+}$  with a Hill coefficient of  $6.0 \pm 0.3$ , while ChlD variants with mutations of the arginine finger (R208A) and in the Walker B motif (E152Q) are much less cooperative toward  $\text{Mg}^{2+}$  with Hill coefficients of  $\sim 2.5$ . We also described an R289A mutant with at least 10-fold poorer interaction with magnesium. Possibly related to this, a cryo-EM analysis demonstrated large-scale conformational changes seen in the *R. capsulatus* BchID complex in the presence of various nucleotides.<sup>15</sup>

We have investigated the role of ChlD in handling magnesium in the intact cyanobacterial chelatase and propose a regulatory role for this subunit in the *Synechocystis* chelatase. In particular, five Glu residues in the C-terminal domain of *Synechocystis* ChlD play a major role in allowing  $\text{Mg}^{2+}$  to cooperatively regulate metalloporphyrin biosynthesis catalyzed by magnesium chelatase. We anticipate that the cyanobacterial ChlD subunit will undergo conformational changes during the Mg chelatase catalytic cycle and that the C-terminal domain of ChlD may be involved in the communication between the magnesium binding sites and the chelation active site in ChlH.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01080.

Experimental procedures, tables of kinetic values, and figures (PDF)

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A.A.B. and N.B.P.A. contributed equally to this work.

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**Notes**

The authors declare no competing financial interest.

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