ATPase activity associated with the magnesium-protoporphyrin IX chelatase enzyme of Synechocystis PCC6803: evidence for ATP hydrolysis during Mg$^{2+}$ insertion, and the MgATP-dependent interaction of the ChlI and ChlD subunits

Poul E. JENSEN$^1$, Lucien C. D. GIBSON$^2$ and C. Neil HUNTER$^3$

Krebs Institute for Biomolecular Research and Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

INTRODUCTION

Magnesium-protoporphyrin IX chelatase (Mg chelatase) catalyses the insertion of Mg$^{2+}$ into protoporphyrin IX, the first step unique to chlorophyll production. The Mg chelatase enzyme consists of three subunits, I (38–42 kDa), D (60–74 kDa) and H (140–150 kDa), in (bacterio)chlorophyll $a$-producing prokaryotes [1–3] and in higher plants [4–7]. The three subunits are designated BchI, BchD and BchH in bacteriochlorophyll-producing organisms, whereas in chlorophyll-producing organisms, they are designated ChlI, ChlD and ChlH. The enzyme-catalysed insertion of Mg$^{2+}$ into protoporphyrin requires hydrolysable ATP in addition to the metal ion and porphyrin substrate, and, in many respects, Mg chelatase shows similarities to the cobaltocchelatase that inserts Co$^{2+}$ into hydrogenobyrinic acid $a$-cadiamide, a tetrapyrrole precursor of vitamin $B_12$ [8]. This enzyme consists of a 140 kDa protein (CobN) binding the tetrapyrole, and a 450 kDa heteromeric protein that binds ATP and is composed of proteins CobS and CobT, of molecular masses 38 kDa and 80 kDa respectively [8]. Furthermore, there is significant sequence similarity between CobN and the ChlH of Mg chelatase, although no significant sequence similarity between ChlI/CobS and ChlD/CobT has been identified, apart from their respective molecular masses and an ATP-binding-site consensus in the former protein pair.

Earlier work on chloroplast extracts suggested that Mg$^{2+}$ chelation proceeds by a two-step reaction: an enzyme-activation step, followed by a Mg$^{2+}$-insertion step [9]. The activation step required the presence of hydrolysable ATP or 5'-[γ-thio]triphosphate (ATP[S]), and it was suggested that ATP might be required either for oligomerization or as an activator [9]. The Mg$^{2+}$-insertion step also appeared to require ATP, although at a lower concentration than needed for activation, and this could not be replaced by ATP[S] [9].

In continuous assays using purified subunits, it was shown that preincubation of the I- and D-subunits with ATP and MgCl$_2$ prior to mixing with the H subunit and protoporphyrin was necessary to overcome a lag period in product accumulation, which suggested an ATP- and Mg$^{2+}$-dependent interaction between the I and D subunits [10,11]. It has been proposed that ATP is required for the formation of a ternary complex between ATP and the I and D proteins [10]. More recently, it was
reported that the I and D subunits of the *Rhodobacter sphaeroides* Mg chelatase together form a highly active ATPase, and inhibitor studies indicated that ATP hydrolysis is required for reactions preceding the insertion of Mg into protoporphyrin IX [12]. This work appeared to demonstrate a role for ATP hydrolysis in providing energy to maintain I and D in this activated state to function in the metal chelation [12]. In the study by Hansson and Kannangara [12], protein phosphorylation was ruled out and the activation step was assumed to involve the formation of an I–D protein complex, although no such complex was demonstrated to exist.

In the present study, we have used a sensitive spectrophotometric method for the quantification of inorganic phosphate (P) released from the enzymic ATP hydrolysis by the overexpressed and highly purified Mg chelatase subunits from the cyanobacterium *Synechocystis* PCC6803. We have been able to observe the interaction of ChlI and ChlD directly, and to show that this requires the presence of both ATP and Mg. However, no significant ATP hydrolysis was identified with the I–D interaction; instead, ATP hydrolysis is clearly attributable to the process of Mg insertion, since there is a 7-fold increase in ATP hydrolysis when all subunits and substrates are present. Furthermore, factors that affect Mg insertion rates, such as increasing concentrations of either the H subunit or protoporphyrin IX, also affect ATPase rates in a similar manner.

**MATERIALS AND METHODS**

**Protein production and purification**

The *Synechocystis* chlI, chlD and chlH genes were amplified by PCR using gene-specific primers, and cloned separately into the *NdeI* and *BamHI* sites of expression vectors pET9a and pET14b [13], yielding the plasmids pET9a-ChlI, pET9a-ChlD and pET9a-ChlH, from which non-tagged proteins were expressed, and pET14b-ChlI, pET14b-ChlD and pET14b-ChlH, from which histidine-tagged proteins were produced. Production of recombinant proteins was performed essentially as described by Jensen et al. [2]. Purification of the histidine-tagged ChlI, ChlD and ChlH subunits was performed as described previously [11], and the purity of the proteins was routinely found to be more than 95%, using SDS/PAGE with Coomassie Blue staining of the gels. Purification of non-tagged ChlI was achieved by a single ion-exchange step using Q-Sepharose, followed by elution with a NaCl gradient from 250 to 650 mM. Purity was found to be more than 95% on the basis of SDS/PAGE, with Coomassie Blue staining of the gels.

**Mg chelatase assay**

The standard stopped assay was performed as described previously [11]. Continuous assays were performed on a Shimadzu UV2101PC spectrophotometer, with a temperature-controlled cuvette holder set at 34 °C. The absorbance wavelength was set to 424 nm. The assay volume was 1.0 ml and contained 300 mM glycerol, 50 mM Mops/NaOH, pH 7.7, 16 mM MgCl₂, 1 mM dithiothreitol, 5 mM ATP, 4 μM protoporphyrin IX and amounts of protein as indicated in the Figure and Table legends. A standard curve was made by diluting known amounts of authentic Mg protoporphyrin IX (Porphyrin Products, Logan, U.T., U.S.A.) in assay buffer containing the same concentrations of ATP, MgCl₂ and chelatase subunits as found in the assays. For all assays, care was taken to avoid prolonged light exposure by keeping all materials covered in foil.

**ATPase assays**

Release of P, was measured using the EnzChek Phosphate Assay kit (Molecular Probes, Leiden, The Netherlands), in which P 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 (PNPase) [14]. This enzymic conversion of MESG results in a spectrophotometric shift in the maximum absorbance from 330 nm for the substrate to 360 nm for the product. The kit was used to determine P in both stopped and continuous assays. In stopped assays, the chelatase subunits were allowed to react as described above for Mg chelatase assays, with subunit and substrate concentrations as indicated in the Figure and Table legends. The assays were then stopped by heating the mixtures to 65 °C for 5 min. After cooling on ice, the volume was adjusted to 1 ml with a mixture of water, MESG, PNPase and reaction buffer according to the manufacturer’s instructions (Molecular Probes), and the assays were incubated for 30 min at 22 °C before A₅₉₀ was monitored. To correct for background absorbance, controls were prepared using reactions containing all the substrates, but without the chelatase subunits. From the difference between A₅₉₀ (with chelatase subunits) and A₅₉₀ (without chelatase subunits) the amount of P, was estimated from a standard curve prepared with known amounts of P. For the continuous assays, it was found that the conversion of MESG and P, into ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by the PNPase enzyme worked equally as well in chelatase buffer at 34 °C (results not shown); thus it was possible to monitor ATPase activity continuously during Mg chelatase.

The assays were performed in 1-ml quartz cuvettes in a split-beam spectrophotometer (Shimadzu UV2101PC) with temperature-controlled cuvette holders set at 34 °C. The reactions contained MESG and PNPase, at concentrations recommended by the manufacturer (Molecular Probes). The reference cuvette contained the same concentrations as the sample cuvette, except for the chelatase subunits. All the ingredients were mixed on ice, and then the reaction was initiated by adding the mixture to the cuvettes that had been prewarmed to 34 °C. ATPase activity was measured as the increase in absorbance at 360 nm. We found no significant effect on Mg chelatase activity of either the MESG substrate or the resulting products, and the continuous removal of P apparently did not affect the Mg chelatase rates obtained.

The sensitivity of the assay was 2 n mole P (2 nmol of P in a 1-ml volume). Assuming a reaction time of 30 min, this corresponds to a rate of 0.07 nmol/min, but the observed rates were usually significantly higher than this.

**Affinity column chromatography**

A 1-ml Ni²⁺-agarose affinity column was prepared according to the manufacturer’s instructions (Novagen, Madison, WI, U.S.A.), except for the final equilibration, which was performed in binding buffer (5 mM imidazole/100 mM NaCl/20 mM Tris/HCl (pH 7.9)] containing 10 mM MgCl₂ and 5 mM ATP. Cell pellets from 20 ml of induced *Escherichia coli* culture were resuspended in 1.5 ml of this buffer, mixed, sonicated and then microcentrifuged to remove debris. The supernatant was applied on to the column by gravity, and unbound proteins were washed first with 10 ml of BB containing 10 mM MgCl₂ and 5 mM ATP, and subsequently with 8 ml of wash buffer (WB) with a higher concentration of imidazole [60 mM imidazole/100 mM NaCl/20 mM Tris/HCl (pH 7.9)] containing 10 mM MgCl₂ and 5 mM ATP. Finally, the bound proteins were eluted with elution buffer [500 mM imidazole/500 mM NaCl/20 mM Tris/HCl (pH
chelatase hydrolyses ATP. Incubating the three subunits without the protoporphyrin substrate allowed ATP hydrolysis at a rate of 0.31 nmol of P\(_i\)/min, and performing the experiment in the presence of protoporphyrin resulted in a 7-fold increase in the rate of P\(_i\) release to 2.10 nmol of P\(_i\)/min (Table 1). Substitution of the protoporphyrin IX with either NMPP, which is an inhibitor of ferrochelatase [17] and Mg chelatase [18], or Mg proto- porphyrin did not result in an increased ATPase activity (Figure 1A). The inhibitory effect of NMPP on both the Mg chelatase and ATPase activity was explored further. With 0.15 \(\mu\)M NMPP in combination with 2 \(\mu\)M protoporphyrin IX, 41% inhibition of the Mg chelatase activity was observed and, when assayed for ATPase activities under the same conditions, 42% inhibition of the Mg\(^{2+}\)-chelation-dependent ATPase activity was shown. Thus the increase in ATPase activity is clearly dependent on the protoporphyrin substrate, which allows the metal ion to be inserted, and this strongly suggests that ATP hydrolysis has a role in the insertion of Mg\(^{2+}\) into protoporphyrin. Lowering the concentration of all three protein subunits in the assay by 50% resulted in a similar decrease in ATPase activity (results not shown).

Since the enzymic phosphate detection system used in the present study requires Mg\(^{2+}\) ions for optimal performance, the stopped ATPase assay was used to demonstrate that Mg\(^{2+}\) in excess of ATP is required for the ATPase activity of the ChlII subunit and the ChlII/ChlD and ChlII/ChlD/ChlH mixtures (results not shown).

Figure 1(B) shows representative traces from continuous ATPase and Mg chelatase assays recorded at an absorbance of 360 nm or 424 nm respectively. A lag period of 6–8 min is clearly seen prior to the linear phase of each of the reactions, and the maximum rate of ATP hydrolysis clearly coincides with the maximum rate of Mg chelation.

The significant ATPase activity observed in experiments with all three subunits in combination, but without the protoporphyrin substrate, suggests that one or more of the three subunits, either singly or in combination, possesses ATPase activity. An analysis of the ATPase activity of either individual subunits or different double combinations of the subunits revealed that ChlII alone has significant ATPase activity (Table 1). In the standard 1-ml assays, 3.6 \(\mu\)g of ChlII was typically used; however, when concentrations of up to 10.8 \(\mu\)g of ChlII alone were used, ATPase activity still could not be detected, and at higher ChlII concentrations the protein precipitated. This precipitation of ChlII was due to the presence of MgCl\(_2\) and ATP in the assay, since we routinely kept ChlII in a stock solution of 5 mg/ml, in the absence of MgCl\(_2\) and ATP, without visible precipitation. ChlII alone had ATPase activity close to the detection limit of the assay (Table 1). ChlII and ChlD in combination had almost the same ATPase activity as the three subunits in combination without protoporphyrin, suggesting that only ChlII contributes to the activity. ChlII in combination with ChlII did not give a significantly different activity from that obtained with ChlII alone. ChlD in combination with ChlII resulted in precipitation of the protein, and it was not possible to estimate the ATPase activity of this combination.

We have previously found that a 2:1 molar ratio of ChlII:ChlD is optimal for Mg chelatase activity and, in cases where there is a molar excess of ChlII over ChlII, inhibition of the chelation reaction was observed [11]. Inspection of ATPase activities shows that a ChlII:ChlD ratio of 2:1 resulted in a reduction in ATPase activity, from 0.59 nmol of P\(_i\)/min with ChlII alone to 0.23 nmol of P\(_i\)/min with the ChlII–ChlD combination (Table 1). This might suggest that ChlD in some way modulates or inhibits the ATPase activity associated with ChlII; however, the basis for this modulation or inhibition of ATPase activity remains unclear.

**RESULTS**

**ATPase activity associated with the Mg chelatase enzyme complex**

ATP hydrolysis is an integral part of the magnesium chelation reaction and, as shown in Figure 1, the *Synechocystis* Mg

![Figure 1 ATPase activity associated with the *Synechocystis* Mg chelatase](image)

(A) Amounts of ChlII, ChlD and ChlH (4 \(\mu\)g, 3.6 \(\mu\)g and 27 \(\mu\)g respectively) were mixed with 16 mM MgCl\(_2\) and 5 mM ATP without protoporphyrin (− Protos), with 4 \(\mu\)M protoporphyrin (+ Protos) or with 4 \(\mu\)M NMPP. MESG and PNPass were added to the reaction mixture, and the reaction was started by adding the mixture into cuvettes prewarmed to 34 °C. The increase in \(A_{360}\) was measured. (B) Maximum rate of ATPase activity coincides with the maximum rate of Mg\(^{2+}\) insertion. The same amounts of the three chelatase subunits and substrate concentrations as in (A) were mixed with MESG and PNPass, and the reaction was started by adding the mixture into cuvettes prewarmed to 34 °C. \(A_{360}\) was measured to determine ATPase activity (upper trace), or \(A_{424}\) was measured to determine Mg chelatase activity (bottom trace).

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The rates shown are maximum rates of Pᵢ release and were obtained using the continuous assays, and the amount of subunits indicated together with 5 mM ATP and 16 mM MgCl₂. The I + D + H combination was assayed both without and with 4 μM protoporphyrin IX (PPix) or NMPP. The values given are means ± S.D., and n is the number of independent repeats. The proportions of subunits shown represent the molar proportions, and the specific activity is normalized to the total protein.

### Table 1 ATPase activity associated with the *Synechocystis* Mg chelatase subunits

The rates obtained in the continuous assay with ChlH alone were close to the detection limit of the Pᵢ detection assay. To rule out any inhibitory effects of the phosphate detection reagents on the ATPase activity, all three subunits were also tested individually using the stopped ATPase assay. In the stopped assay, the subunits were incubated in chelatase buffer with 5 mM ATP and 16 mM MgCl₂ for a fixed period of time, and subsequently the amount of phosphate produced was determined. The high ATPase activity observed with ChlH was also evident from the stopped assay ([133 ± 30 nmol/min per mg of protein (n = 3); values given as means ± S.D.], whereas the activity obtained with ChlD was close to the detection limit of the assay. The activity obtained with ChlH was higher than that with ChlD [2.8 ± 1.2 nmol/min per mg of protein (n = 5)], and in the continuous assay we obtained a value of 0.95 ± 0.34 nmol/min per mg (n = 3). It is therefore certain that ChlH possesses ATPase activity, whereas ChlD has no detectable ATPase activity when assayed alone; it is possible that ChlH might have weak ATPase activity. However, using ChlH protein that had been purified only by the affinity step (involving histidine-tagging), and not purified subsequently by ion-exchange chromatography, gave consistently higher ATPase activity measurements of approx. 20 nmol/min per mg of protein. This higher ATPase activity associated with the ChlH preparation before the ion-exchange step did not correlate with higher activity in Mg chelatase assays, and therefore the observed ATPase activity is most probably due to contamination with ATPases originating in *E. coli*. We cannot rule out that the residual ATPase activity observed with the ChlH preparation used in the present study was caused by contaminating ATPases, which persist during both the affinity-purification and the subsequent ion-exchange steps.

The high ATPase activity observed with *Synechocystis* ChlI protein is in sharp contrast with the recently published data on the *R. sphaeroides* Mg chelatase subunits, in which BchI was reported to have no ATPase activity when tested on its own [12]. This prompted us to purify non-histidine-tagged ChlI which, when assayed for ATPase activity using the continuous assay, gave rates of 150 ± 19 nmol/min per mg ChlI (n = 2), which is within the range obtained with its histidine-tagged counterpart (Table 1). This rules out any possible effect of the N-terminal histidine-tag on increasing the ATPase activity associated with ChlI.

When molar proportions of the three Mg chelatase subunits (I : D : H) of 2 : 1 : 4 were employed, the level of the H subunit was limiting [11]. Thus, by increasing the proportion of the ChlH subunit over the other two subunits, the Mg chelatase activity can be increased. Figure 2(A) shows the effect on both Mg chelatase and ATPase activity when the amount of the H subunit was increased from 6.75 to 432 μg in the presence of constant amounts of ChlI and ChlD (1 μg and 0.9 μg respectively), which corresponds to a molar ratio of ChlI : ChlD of 2 : 1. It is clear that the increase in ATPase activity correlates closely with the increase in Mg chelatase activity. Furthermore, if the ATPase activity was in part attributable to ChlI, one would expect the ATPase activity to become increasingly out of step with the rate of chelation with increasing amounts of ChlH, since an increasing proportion of the added ChlH molecules could participate in ATP hydrolysis not related to Mg²⁺ insertion. This is not the case, and supports the notion that ChlH probably has no significant ATPase activity on its own. The ratio of mol of ATP hydrolysed/mol of Mg²⁺ protoporphyrin formed in Figure 2(A) is between 30 and 40.

Figure 2(B) shows the increase in ATPase and Mg chelatase with increasing protoporphyrin concentration. As seen in Figure 2(A), the increase in ATPase activity correlates with the increase in Mg chelatase activity, and the ratio of mol of ATP hydrolysed/mol of Mg²⁺ protoporphyrin formed is, as in Figure 2(A), between 30 and 40.

To examine the role of ChlI in the Mg chelatase complex further, an experiment was performed where ChlD and ChlH were kept at limiting concentrations (3.6 μg of ChlD and 27 μg of ChlH, corresponding to a ChlD : ChlH ratio of 1 : 4), and the concentration of ChlI was varied. The increase in both Mg chelatase and ATPase activity was linear up to approx. 4 μg of ChlI, corresponding to proportions of I : D : H of 2 : 1 : 4 (Figure 2C). Above 4 μg of ChlI, the Mg chelatase rate reaches saturation and a slight inhibition of Mg chelatase activity is seen. The ATPase activity, however, continues to increase, which suggests that ChlI protein not participating in Mg²⁺ chelation exhibits Mg²⁺-independent ATPase activity. The inhibition of Mg chelatase activity with high ChlI : ChlD ratios could be due to the increasing concentration of ADP released by ChlI, since ADP is a strong inhibitor of the *Synechocystis* Mg chelatase (see Figure 6). The data show that increasing the concentration of

<table>
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<th>Subunit</th>
<th>Amount of subunit per assay (μg)</th>
<th>Proportions of subunits</th>
<th>Maximum rate (nmol Pᵢ/min)</th>
<th>Specific activity (nmol/min per mg)</th>
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<td>I + D + H</td>
<td>4 + 3.6 + 27</td>
<td>2:1:4</td>
<td>0.31 ± 0.05</td>
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<td>I + D + H + PPix</td>
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<td>2.10 ± 0.19</td>
<td>60.70 ± 5.49</td>
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<td>I + D + H + NMPP</td>
<td>4 + 3.6 + 27</td>
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<td>0.36 ± 0.03</td>
<td>10.40 ± 0.87</td>
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<tr>
<td>I</td>
<td>4 + 3.6 + 27</td>
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<td>0.59 ± 0.19</td>
<td>147.5 ± 47.5</td>
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<tr>
<td>D</td>
<td>10.8</td>
<td>1</td>
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<td>1</td>
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<tr>
<td>H</td>
<td>69</td>
<td>1:3:1:4</td>
<td>Precipitation</td>
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<td>I + D</td>
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<td>1:4</td>
<td>Precipitation</td>
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ATPase activity associated with the Synechocystis magnesium chelatase

Figure 2 Correlation between ATPase and Mg chelatase activities

(A) The effect of increasing ChlH concentration. Amounts of ChlI and ChlD (1 μg and 0.9 μg respectively, resulting in a molar ratio of 2:1) were incubated with increasing amounts of H, as shown, and these proteins were mixed with 16 mM MgCl₂, 5 mM ATP and 4 μM protoporphyrin IX, as indicated. (B) The effect of increasing protoporphyrin concentration. Amounts of ChlI, ChlD and ChlH (4 μg, 3.6 μg and 27 μg respectively) were mixed with 16 mM MgCl₂, 5 mM ATP and various concentrations of protoporphyrin IX, as indicated. (C) The effect of increasing ChlI concentration. Amounts of ChlD and ChlH (3.6 μg and 27 μg respectively) were incubated with increasing amounts of ChlI as indicated, and subsequently were mixed with 16 mM MgCl₂, 5 mM ATP and 4 μM protoporphyrin. Owing to the observed precipitation of protein when the ratio of ChlI to ChlD was below 2:1, the points with less than 4 μg of ChlI were obtained with correspondingly less ChlD.

ChlI beyond a ChlI:ChlD ratio of about 2 to 1 results in no significant increase in Mg chelatase activity. Furthermore, a ChlI-dependent increase in ATPase activity is seen, which confirms that ChlI is indeed an ATPase.

Figure 3 ChlI and ChlD interaction in the presence of ATP and Mg²⁺

Histidine-tagged ChlD protein was mixed with non-histidine-tagged ChlI protein in the presence of different nucleotides and MgCl₂, as described in the Materials and methods section. Aliquots (2 μl) of each of the eight wash fractions (13–20), and 2 μl of each of the six eluted fractions (21–26) were assayed for Mg chelatase activity by mixing with 40 μg of ChlH. The resulting activity is displayed in each histogram above its corresponding Coomassie-Blue-stained SDS-polyacrylamide gel. The following combinations of nucleotide and MgCl₂ were analysed using this system: (A) ATP + MgCl₂; (B) ADP + MgCl₂.

Co-purification of a ChlI–ChlD complex in the presence of ATP and Mg²⁺

A close interaction between the ChlI and ChlD subunits in the presence of ATP and MgCl₂ has been suggested from the continuous Mg chelatase assays, in which a lag in the onset of product formation was observed unless the ChlI and ChlD subunits were preincubated with ATP and MgCl₂ before mixing with the ChlH subunit and protoporphyrin [11]. To verify that a physical interaction between ChlI and ChlD actually occurs, and to analyse the nucleotide and metal ion requirements for this interaction, a convenient assay was developed in which histidine-tagged ChlD was mixed with non-histidine-tagged ChlI. The experiments were performed by resuspending E. coli cell pellets containing the respective subunits in buffer containing 5 mM ATP and 10 mM MgCl₂, and subsequently mixing them. After disruption of the cells by sonication and removal of the extract by centrifugation, the supernatant was applied to a Ni²⁺-resin that retains the histidine-tagged proteins. If a physical interaction...
occurs between histidine-ChlD and ChlI, this should result in the retention of both subunits on the resin. The wash and eluted fractions were analysed for Mg chelatase activity in assays with saturating amounts of ChlH protein (to test for 'I–D activity') or the ChlH–ChlD protein complex (to test for 'D-activity'), or by SDS/PAGE (to observe co-purification of the ChlI and ChlD proteins). As seen in Figure 3(A), a physical interaction between ChlI and ChlD was indeed observed, which was dependent on the presence of ATP and MgCl₂. The ChlI–ChlD interaction is apparent from the Mg chelatase activity obtained when the eluted fractions were assayed with ChlH, and also from the gel of the corresponding fractions, which reveals co-purification of non-histidine-tagged ChlI and histidine-tagged ChlD. In a separate experiment in which a histidine-tagged version of ChlII was mixed with a non-tagged version of ChlID in the presence of ATP and MgCl₂, a similar result was obtained (results not shown). Substitution of ATP for ADP (but keeping MgCl₂ constant) resulted in co-purification of less of the non-tagged subunit, as seen also in the 5-fold lower Mg chelatase activity when the fractions were assayed in the presence of ChlH protein, and from an inspection of the corresponding gel (Figure 3B). This suggests that ATP is necessary for establishing the ChlI–ChlD interaction, and that ADP only supports the ChlI–ChlD interaction to a minor extent.

That the interaction was specific and required both the presence of nucleotide and MgCl₂ was verified by repeating the experiments, but omitting either ATP or MgCl₂, which resulted in only trace amounts of co-purified ChlI (Figure 4, lanes 2 and 3). Performing the experiment with AMP instead did not result in co-purification of the non-tagged subunit (Figure 4, lane 4). The formation of the complex of ChlI–ChlD was also explored in the presence of the two non-hydrolysable ATP analogues, adenosine 5’-[(β,γ-methylene]triphosphate (AMP-PCP) and adenosine 5’-[(β,γ-imido)triphosphate (AMP-PNP), and the slowly hydrolysable analogue ATP[S]. From Figure 4, it is clear that, out of the analogues tested, only ATP[S] supports a ChlI–ChlD interaction. AMP-PCP facilitates co-purification of a small amount of the non-histidine-tagged ChlII protein, and in the presence of AMP-PNP no detectable ChlI is observed. In order to verify that the D subunits that are eluted in the experiment in Figure 4 are active, the fractions were also assayed for Mg chelatase activity in the presence of 12 µg of ChlI and 40 µg of ChlH, and it was found that all possessed activity (results not shown). We therefore conclude that ChlI and ChlD form a complex in the presence of ATP, and that Mg²⁺ is an integral component in this interaction, probably as a MgATP complex. ATP[S] is the only one of the tested nucleotides that can substitute fully for ATP; in the presence of ADP and AMP-PNP, very little of the non-tagged protein was co-purified.

To test the stability of the formed I–D–MgATP complex, experiments were performed in which the I–D complex was established on the column in the presence of MgATP and washed (also in the presence of MgATP) to remove unbound proteins (i.e. the column was washed in BB with 5 mM ATP and 10 mM MgCl₂, and in WB with 5 mM ATP and 10 mM MgCl₂). The column was subsequently washed again with different combinations of nucleotide and MgCl₂, as indicated in Figure 5, before elution with imidazole buffer. Significant co-purification of the ChlI and ChlD subunits was only observed when the wash contained MgATP, ADP + MgCl₂ or ATP[S] + MgCl₂ (Figure 5, lanes 1, 2 and 7 respectively). Washing with MgCl₂ alone did not maintain the complex to any significant degree, nor did washing with AMP + MgCl₂. Washing with the two non-hydrolysable ATP analogues, AMP-PCP and AMP-PNP, revealed that only AMP-PNP could maintain the complex to some degree. Thus, despite the variations in the amount of D subunit present, qualitative inspection of the levels of I clearly demonstrate that the ChlI–ChlD complex is stable in the presence of MgATP or MgADP, and also in the presence of ATP[S] and, to a lesser degree, AMP-PNP.
Table 2 ATPase and Mg chelatase activities of the ChlI–ChlD complex

<table>
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<th>Subunit</th>
<th>Amount of subunit per assay (μg)</th>
<th>Rate (nmol/min)</th>
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<td></td>
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<tr>
<td>ID</td>
<td>7.6</td>
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<td>4 + 3.6</td>
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<td>I + D + H + PPix</td>
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<td>1.762 ± 0.07</td>
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</table>

Mg chelatase activity associated with the Synechocystis magnesium chelatase

In the present work, we have used a plausible explanation. In the present work, we have used a plausible explanation. In the present work, we have used a plausible explanation.
continuous assay that permits a more accurate estimation of rates in comparison with the stopped assay.

Hansson and Kannangara [12] reported that the R. sphaeroides BchI has no significant ATPase activity, but combining BchI with BchD gave ATP hydrolysis at a rate of 117.9 nmol/min per mg of protein. With the Synechocystis subunits, almost the opposite result is obtained: the ChlII subunit hydrolysates ATP at a rate of 147.5 nmol/min per mg of protein, and when combined with the ChlD subunit in a 2:1 ratio, the rate is reduced to 30.3 nmol/min per mg of protein. This reduction is not solely due to normalization to an increased amount of protein, since the rate simply expressed in nmol/min is reduced from 0.59 nmol/min with 4 μg of ChlII alone to 0.23 nmol/min with the same amount of ChlII, but with ChlD present. Whether this apparent difference in ATPase activity arises from species differences or the fact that the BchD preparation used by Hansson and Kannangara [12] was contaminated with GroEL [10] is unclear. However, recent results from our laboratory on the ATPase activity of the BchII and BchD subunits of R. sphaeroides suggest that BchI is also an ATPase, and that a mixture of BchI and BchD does not possess a higher level of ATPase activity than BchI alone [19].

The existence of type-A and -B ATP-binding motifs (A/GX₃GKS/T and H/R/KX₉OXOODE respectively, where O specifies a hydrophobic residue and X specifies any residue) [20–22] in the I subunit of Synechocystis and other organisms is consistent with the I subunit being an ATPase, and it appears that at least one function of ChlII is to bind and hydrolyse MgATP at some stage in the catalytic cycle. The fact that ADP, ATP[S] and AMP-PNP can either mediate or maintain the ChlII–ChlD interaction suggests that they bind to the active site of the ChlII subunit, and thereby probably inhibit the ATPase activity associated with this subunit. Thus the ATPase activity exhibited by the ChlII subunit is normally harnessed to the Mg²⁺ insertion, since it is expected that ChlII and ChlD form a complex in vivo.

ADP has a strong inhibitory effect on Mg²⁺ chelation, whereas AMP only has a moderate effect. Although AMP has been suggested as a regulator of Mg chelatase, owing to its inhibitory effect on Mg chelatase activity in intact cucumber chloroplasts and chloroplast extracts [18,23], we did not observe significant inhibition of the Synechocystis Mg chelatase by AMP in vitro, and it seems unlikely that AMP is involved in regulation of the enzyme, at least from this source. It is far more likely that the availability of ATP, the build-up of ADP or the ATP/ADP ratio is involved in the regulation of the Mg chelatase enzyme.

In the experiments presented in this paper, the ratio of mol of ATP hydrolysed/molecule of Mg protoporphyrin formed was never less than 30. This high ratio probably does not relate to the situation in vivo. Estimation of rates for the two activities under steady-state conditions, as were used in the present study, might not reveal the true ratio of mol of ATP hydrolysed/mol of Mg protoporphyrin produced, since there might be some ‘slippage’ in the system, in which ATP is hydrolysed without doing any work (i.e. Mg²⁺ insertion). More accurate assessment of the stoichiometry would require single-turnover experiments.

In a recent paper we suggested a provisional model of the Mg chelatase cycle [11]. In this model we suggested that the I and D subunits interact with MgATP, and probably form a complex. The physical interaction between the two subunits has now been demonstrated by the work presented here. It is proposed that the H subunit with bound protoporphyрин IX reacts with this I–D–MgATP complex, and a short-lived complex consisting of all three subunits and at least two of the substrates is formed. Next, Mg²⁺ is inserted into protoporphyрин IX with concomitant hydrolysis of ATP resulting in another, probably short-lived, complex consisting of I, D, H, MgADP and Mg protoporphyrin. This complex dissociates into I–D–MgADP and H–Mg protoporphyrin, which then can be recharged with MgATP and protoporphyrin respectively, and participate in a new reaction cycle following removal of the reaction products. The hydrolysis of ATP in parallel with Mg²⁺ insertion has been demonstrated in the present work.

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