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 ChII and ChID subunits

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Insertion of Mg^{2+} into protoporphyrin IX catalysed by the three-subunit enzyme magnesium-protoporphyrin IX chelatase (Mg chelatase) is thought to be a two-step reaction, consisting of activation followed by Mg^{2+} chelation. The activation step requires ATP and two of the subunits, ChII and ChID (I and D respectively), and it has been speculated that this step results in the formation of an I–D–ATP complex. The subsequent step, in which Mg^{2+} is inserted into protoporphyrin, also requires ATP and the third subunit, H, in addition to ATP-activated I–D complex. In the present study, we examine the interaction of the I and D subunits of the Mg chelatase from the cyanobacterium *Synechocystis* PCC 6803. We demonstrate the purification of an I–D complex, and show that ATP and Mg^{2+} are absolute requirements for the formation of this complex, probably as MgATP. However, ATP may be replaced by the slowly hydrolysable analogue, adenosine 5′-[γ -thio]triphosphate, and, to a minor extent, by ADP and the non-hydrolysable ATP analogue,

adenosine 5′-[β , γ -imido]triphosphate, all of which suggests that ATP hydrolysis is not necessary for the formation of the ChII–ChID complex. A sensitive continuous assay was used to detect ATPase activity during Mg^{2+} chelation, and it was found that the maximum rate of ATP hydrolysis coincided with the maximum rate of Mg^{2+} insertion. The rate of ATP hydrolysis depended on factors that determined the rate of Mg^{2+} chelation, such as increasing the concentration of the H subunit and the concentration of protoporphyrin. Thus ATP hydrolysis has been identified as an absolute requirement for the chelation step. The I subunit possessed strong ATPase activity when assayed on its own, whereas the D subunit had no detectable activity, and when the I and D subunits were assayed in combination, the ATPase activity of the I subunit was repressed.

Key words: ATP analogues, chlorophyll biosynthesis, enzyme activation, protein–protein interaction, tetrapyrrole.

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 ChII, ChID and ChIH. 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 cobalto-chelatase that inserts Co^{2+} into hydrogenobyrinic acid *a,c*-diamide, a tetrapyrrolic precursor of vitamin B₁₂ [8]. This enzyme consists of a 140 kDa protein (CobN) binding the tetrapyrrole, 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 ChIH of

Mg chelatase, although no significant sequence similarity between ChII/CobS and ChID/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

Abbreviations used: AMP-PCP, adenosine 5′-[β , γ -methylene]triphosphate; AMP-PNP, adenosine 5′-[β , γ -imido]triphosphate; ATP[S], adenosine 5′-[γ -thio]triphosphate; BB, binding buffer; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; Mg chelatase, magnesium-protoporphyrin IX chelatase; NMPP, *N*-methylprotoporphyrin IX; PNPase, purine nucleoside phosphorylase; WB, wash buffer.

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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^{2+} 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_i) 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 ChII and ChID directly, and to show that this requires the presence of both ATP and Mg^{2+} . However, no significant ATP hydrolysis was identified with the I-D interaction; instead, ATP hydrolysis is clearly attributable to the process of Mg^{2+} insertion, since there is a 7-fold increase in ATP hydrolysis when all subunits and substrates are present. Furthermore, factors that affect Mg^{2+} -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-ChII, pET9a-ChID and pET9a-ChIH, from which non-tagged proteins were expressed, and pET14b-ChII, pET14b-ChID and pET14b-ChIH, 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 ChII, ChID and ChIH 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 ChII 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 MgCl_2 , 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, UT., U.S.A.) in assay buffer containing the same concentrations of ATP, MgCl_2 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_i was measured using the EnzChek Phosphate Assay kit (Molecular Probes, Leiden, The Netherlands), in which P_i reacts with 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to form ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine in a reaction catalysed by purine nucleoside phosphorylase (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_i 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 A_{360} 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 A_{360} (with chelatase subunits) and A_{360} (without chelatase subunits) the amount of P_i was estimated from a standard curve prepared with known amounts of P_i . For the continuous assays, it was found that the conversion of MESG and P_i 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^{2+} chelation. 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_i apparently did not affect the Mg chelatase rates obtained. The sensitivity of the assay was 2 μM P_i (2 nmol of P_i 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 Ni^{2+} -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 (BB) [5 mM imidazole/100 mM NaCl/20 mM Tris/HCl (pH 7.9)] containing 10 mM MgCl_2 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 MgCl_2 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)], also containing 10 mM MgCl_2 and 5 mM ATP. Finally, the bound proteins were eluted with elution buffer [500 mM imidazole/500 mM NaCl/20 mM Tris/HCl (pH

7.9)]. Fractions (1 ml) were collected from both the washing and elution steps, and 2–5 μ l of all the fractions were analysed for Mg chelatase activity, in the presence of the ChlH subunit or with both the ChII and ChlH subunits. Further aliquots of the same fractions (5 μ l) were also analysed using SDS/PAGE.

Other methods

SDS/PAGE was performed by the method of Sambrook et al. [15]. Protein concentrations were determined by using the Bio-Rad protein assay (Hemel Hempstead, Herts., U.K.) with BSA as the standard. Preparation and quantification of protoporphyrin IX and Mg protoporphyrin IX were performed as described by Jensen et al. [11]; for *N*-methylprotoporphyrin IX (NMPP), these procedures were performed as described by Brown et al. [16]. Quick desalting was performed using a PD-10 column according to the manufacturer's instructions (Pharmacia, Milton Keynes, U.K.).

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

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 protoporphyrin 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 μ M NMPP in combination with 2 μ 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 ChII subunit and the ChII/ChlD and ChII/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 ChII alone has significant ATPase activity (Table 1). In the standard 1-ml assays, 3.6 μ g of ChlD was typically used; however, when concentrations of up to 10.8 μ g of ChlD alone were used, ATPase activity still could not be detected, and at higher ChlD concentrations the protein precipitated. This precipitation of ChlD was due to the presence of $MgCl_2$ and ATP in the assay, since we routinely kept ChlD in a stock solution of 5 mg/ml, in the absence of $MgCl_2$ and ATP, without visible precipitation. ChlH alone had ATPase activity close to the detection limit of the assay (Table 1). ChII and ChlD in combination had almost the same ATPase activity as the three subunits in combination without protoporphyrin, suggesting that only ChII contributes to the activity. ChII in combination with ChlH did not give a significantly different activity from that obtained with ChII alone. ChlD in combination with ChlH 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 ChII:ChlD is optimal for Mg chelatase activity and, in cases where there is a molar excess of ChlD over ChII, inhibition of the chelation reaction was observed [11]. Inspection of ATPase activities shows that a ChII:ChlD ratio of 2:1 resulted in a reduction in ATPase activity, from 0.59 nmol of P_i /min with ChII alone to 0.23 nmol of P_i /min with the ChII–ChlD combination (Table 1). This might suggest that ChlD in some way modulates or inhibits the ATPase activity associated with ChII; however, the basis for this modulation or inhibition of ATPase activity remains unclear.

Figure 1 ATPase activity associated with the *Synechocystis* Mg chelatase

(A) Amounts of ChII, ChlD and ChlH (4 μ g, 3.6 μ g and 27 μ g respectively) were mixed with 16 mM $MgCl_2$ and 5 mM ATP without protoporphyrin (– Proto), with 4 μ M protoporphyrin (+ Proto) or with 4 μ M NMPP. MESG and PNPase 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 PNPase, 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).

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

The rates shown are maximum rates of P_i release and were obtained using the continuous assays, and the amount of subunits indicated together with 5 mM ATP and 16 mM $MgCl_2$. The I + D + H combination was assayed both without and with 4 μM protoporphyrin IX (PPix) or NMPP. The values given are means $\pm$ 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.

Subunit	Amount of subunit per assay (μg)	Proportions of subunits	Maximum rate (nmol P_i /min)	n	Specific activity (nmol/min per mg)
I + D + H	4 + 3.6 + 27	2:1:4	0.31 ± 0.05	3	8.96 ± 1.45
I + D + H + PPix	4 + 3.6 + 27	2:1:4	2.10 ± 0.19	3	60.70 ± 5.49
I + D + H + NMPP	4 + 3.6 + 27	2:1:4	0.36 ± 0.03	3	10.40 ± 0.87
I	4	—	0.59 ± 0.19	3	147.5 ± 47.5
D	10.8	—	0	1	—
	57.6	—	Precipitation	1	—
H	60	—	0.057 ± 0.020	3	0.95 ± 0.34
I + D	4 + 3.6	2:1	0.23 ± 0.05	4	30.26 ± 6.58
	4 + 7.2	1:1	Precipitation	1	—
I + H	4 + 27	1:2	0.50 ± 0.06	3	16.13 ± 1.94
D + H	3.6 + 27	1:4	Precipitation	2	—

The rates obtained in the continuous assay with ChIH alone were close to the detection limit of the P_i 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 $MgCl_2$ for a fixed period of time, and subsequently the amount of phosphate produced was determined. The high ATPase activity obtained for ChII was also evident from the stopped assay [133 ± 30 nmol/min per mg of protein ($n = 3$); values given as means $\pm$ S.D.], whereas the activity obtained with ChID was close to the detection limit of the assay. The activity obtained with ChIH was higher than that with ChID [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 ChII possesses ATPase activity, whereas ChID has no detectable ATPase activity when assayed alone; it is possible that ChIH might have weak ATPase activity. However, using ChIH 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 ChIH 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 ChIH 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* ChII 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 ChII which, when assayed for ATPase activity using the continuous assay, gave rates of 150 ± 19 nmol/min per mg ChII ($n = 2$), 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 ChII.

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 ChIH 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 ChII and ChID (1 μg and 0.9 μg respectively), which corresponds to a molar ratio of ChII:ChID 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 ChIH, one would expect the ATPase activity to become increasingly out of step with the rate of chelation with increasing amounts of ChIH, since an increasing proportion of the added ChIH molecules could participate in ATP hydrolysis not related to Mg^{2+} insertion. This is not the case, and supports the notion that ChIH probably has no significant ATPase activity on its own. The ratio of mol of ATP hydrolysed/mol of Mg^{2+} 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 Mg^{2+} protoporphyrin formed is, as in Figure 2(A), between 30 and 40.

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

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. (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. MESG and PNPase were added to the reaction mixture, and the reaction was initiated by adding the mixture into cuvettes prewarmed to 34 °C. Rates of ATP hydrolysis and Mg chelation were estimated from the continuous absorbance readings obtained at 360 and 424 nm respectively; these rates are indicated on the left-hand and right-hand y-axes respectively in units of nmol of P_i/min (○) and nmol of Mg protoporphyrin/min (nmol MgP/min; ●).

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 on to a Ni²⁺-resin that retains the histidine-tagged proteins. If a physical interaction

Figure 4 Effect of different nucleotides on the establishment of the ChII–ChlD complex

The same experimental strategy as outlined in the legend for Figure 3 was employed. The wash and eluted fractions were assayed for Mg chelatase activity by mixing with either ChII protein alone or with ChII + ChlH proteins, and by SDS/PAGE. Shown is an SDS-polyacrylamide gel of the eluted fractions containing the most protein from each of the three experiments corresponding to fraction 22 in Figure 3. Experimental conditions set up in each of the lanes were as follows: lane 1, 5 mM MgATP (i.e. 5 mM ATP + 5 mM MgCl_2); lane 2, 5 mM ATP; lane 3, 10 mM MgCl_2 ; lane 4, 5 mM AMP + 10 mM MgCl_2 ; lane 5, 5 mM AMP-PNP + 10 mM MgCl_2 ; lane 6, 5 mM AMP-PCP + 10 mM MgCl_2 ; lane 7, 5 mM ATP[S] + 10 mM MgCl_2 .

occurs between histidine-ChlD and ChII, this should result in the retention of both subunits on the resin. The wash fractions and eluted fractions were analysed for Mg chelatase activity in assays with saturating amounts of ChII protein (to test for ‘I–D activity’) or the ChII–ChlH protein complex (to test for ‘D–activity’), or by SDS/PAGE (to observe co-purification of the ChII and ChlD proteins). As seen in Figure 3(A), a physical interaction between ChII and ChlD was indeed observed, which was dependent on the presence of ATP and MgCl_2 . The ChII–ChlD interaction is apparent from the Mg chelatase activity obtained when the eluted fractions were assayed with ChII, and also from the gel of the corresponding fractions, which reveals co-purification of non-histidine-tagged ChII and histidine-tagged ChlD. In a separate experiment in which a histidine-tagged version of ChII was mixed with a non-tagged version of ChlD in the presence of ATP and MgCl_2 , a similar result was obtained (results not shown). Substitution of ATP for ADP (but keeping MgCl_2 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 ChII protein, and from an inspection of the corresponding gel (Figure 3B). This suggests that ATP is necessary for establishing the ChII–ChlD interaction, and that ADP only supports the ChII–ChlD interaction to a minor extent.

That the interaction was specific and required both the presence of nucleotide and MgCl_2 was verified by repeating the experiments, but omitting either ATP or MgCl_2 , which resulted in only trace amounts of co-purified ChII (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 ChII–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

Figure 5 Stability of the ChII–ChlD complex

Histidine-tagged ChlD protein was mixed with non-histidine-tagged ChII protein in the presence of 5 mM ATP and 10 mM MgCl_2 to allow complex formation. The mixture was then loaded on to the Ni^{2+} -resin column, which was then washed with buffers BB and WB (see the Materials and methods section) containing 5 mM ATP and 10 mM MgCl_2 to remove unbound protein. Subsequently, the bound complexes were washed in 6 column vols. of WB containing: lane 1, 5 mM ATP + 5 mM MgCl_2 ; lane 2, 5 mM ADP + 10 mM MgCl_2 ; lane 3, 10 mM MgCl_2 ; lane 4, 5 mM AMP + 10 mM MgCl_2 ; lane 5, 5 mM AMP-PNP + 10 mM MgCl_2 ; lane 6, 5 mM AMP-PCP + 10 mM MgCl_2 ; and lane 7, 5 mM ATP[S] + 10 mM MgCl_2 . Finally, the complexes were eluted with imidazole buffer. The Figure shows the SDS/PAGE analysis of the eluted fractions, each one corresponding to fraction 22 in Figure 3.

analogues tested, only ATP[S] supports a ChII–ChlD interaction. AMP-PNP facilitates co-purification of a small amount of the non-histidine-tagged ChII protein, and in the presence of AMP-PCP no detectable ChII 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 ChII and 40 μg of ChII, and it was found that all possessed activity (results not shown). We therefore conclude that ChII and ChlD form a complex in the presence of ATP, and that Mg^{2+} 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 MgCl_2 , and in WB with 5 mM ATP and 10 mM MgCl_2). The column was subsequently washed again with different combinations of nucleotide and MgCl_2 , as indicated in Figure 5, before elution with imidazole buffer. Significant co-purification of the ChII and ChlD subunits was only observed when the wash contained MgATP , ADP + MgCl_2 or ATP[S] + MgCl_2 (Figure 5, lanes 1, 2 and 7 respectively). Washing with MgCl_2 alone did not maintain the complex to any significant degree, nor did washing with AMP + MgCl_2 . Washing with the two non-hydrolysable ATP analogues, AMP-PNP and AMP-PCP, 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 ChII–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.

Figure 6 Inhibition of Mg chelatase activity by different nucleotides

Amounts of ChII, ChID and ChIH (0.4 μ g, 0.36 μ g and 2.7 μ g respectively) were incubated with 0.9 mM ATP (twice the measured K_m for ATP), 11 mM $MgCl_2$ and 8 μ M protoporphyrin for 20 min (stopped assay) with various concentrations of ADP, ATP[S], AMP, AMP-PNP or AMP-PCP, as indicated. A constant excess of approx. 10 mM free $MgCl_2$ was ensured by adding equimolar concentrations of $MgCl_2$ with these nucleotides. The Mg chelatase activity in the absence of any inhibitory nucleotide added was taken as being 100%.

Having established the effects of ADP, ATP[S], AMP-PNP, AMP-PCP and AMP on promoting I-D complex formation, it was important to correlate these data with Mg chelatase activity in the presence of ATP (Figure 6). It is clear that inhibition of Mg chelatase activity by ADP, ATP[S] and AMP-PNP does indeed correlate with the observed effect of these nucleotides on either the formation or the maintenance of the I-D interaction, i.e. the better the nucleotide is in forming the ChII-ChID complex, the more inhibition of Mg chelatase it exerts. AMP-PCP and AMP are poor inhibitors of the *Synechocystis* Mg chelatase (Figure 6), and also do not support I-D complex formation. This indirectly suggests that the ATPase activity of the ChII subunit in the Mg chelatase complex is involved in metal chelation, since a hydrolysable nucleotide is required in the active site of the ChII subunit in order for Mg^{2+} chelation to occur.

To test whether high rates of continuous ATP hydrolysis are required to maintain the ChII-ChID complex, purified histidine-tagged ChID was mixed with purified non-histidine-tagged ChII in the presence of ATP and $MgCl_2$ to allow complex formation. The ChII-ChID complex was purified on a Ni^{2+} -resin, as described above, and eluted with imidazole in the presence of ATP and $MgCl_2$. After removal of imidazole and NaCl by quick desalting on a PD-10 column, the complex was assayed for ATPase and Mg chelatase activity. As seen in Table 2, the ChII-ChID complex (ID) has only half the ATPase activity of the reconstituted ChII and ChID (I + D). It is likely that the higher level of ATP hydrolysis seen when ChII and ChID are reconstituted in a 2:1 ratio is caused by free ChII that has not formed a complex with ChID, since it has already been shown that free ChII can hydrolyse ATP. SDS/PAGE analysis of the ChII-ChID complex formed in this experiment suggested that ChII and ChID associate in a ratio of 1:1 in the complex (results not shown). In order to address this, the rate of ATP hydrolysis was monitored under chelation conditions, i.e. in the presence of protoporphyrin and ChIH. It is interesting to note that, under these conditions, the rate of ATP hydrolysis by reconstituted

Table 2 ATPase and Mg chelatase activities of the ChII-ChID complex

The indicated amounts of the ChII-ChID complex (ID) or the individual subunits were incubated with 16 mM $MgCl_2$, 5 mM ATP and 2 μ M protoporphyrin IX (PPIX). The rates were obtained using the continuous assay measuring at A_{424} (Mg chelatase) or at A_{360} (ATPase). The values given are means $\pm$ S.D. and n is the number of independent repeats.

Subunit	Amount of subunit per assay (μ g)	Rate (nmol/min)		n
		ATPase	Mg chelatase	
ID	7.6	0.090 ± 0.0025	—	3
I + D	4 + 3.6	0.195 ± 0.0195	—	3
ID + H + PPIX	7.6 + 27	1.50 ± 0.16	0.0394 ± 0.0040	2
I + D + H + PPIX	4 + 3.6 + 27	1.762 ± 0.07	0.0412 ± 0.0061	2

ChII and ChID still exceeds the rate measured for the ChII-ChID complex by a similar amount; this emphasizes the importance of using a pre-formed I-D complex.

DISCUSSION

ATP plays at least two roles in the chelation of Mg^{2+} into protoporphyrin IX catalysed by the ChII, ChID and ChIH subunits of Mg chelatase from *Synechocystis*. First, ATP, or rather MgATP, is an absolute requirement for the ChII-ChID interaction, which is a process that precedes Mg^{2+} insertion. Secondly, ATP is hydrolysed during the insertion of Mg^{2+} into protoporphyrin IX.

The dual role of ATP in Mg^{2+} chelation was first proposed by Walker and Weinstein [9]. Working with pea chloroplast fractions, these authors found that the activation step required ATP or the slowly hydrolysable ATP[S], and that the insertion step had a strict requirement for ATP. Here we demonstrate for the first time that a physical interaction takes place between the ChII and ChID subunits in the presence of ATP or ATP[S]. We have previously reported that ChII and ChID are involved in a MgATP-dependent activation step in order to overcome a lag period in product formation when combined with ChIH and protoporphyrin [11]. We suggest that this so-called activation step more correctly should be called an oligomerization step. The fact that the slowly hydrolysable ATP analogue ATP[S], and, to a lesser extent, ADP and the non-hydrolysable analogue AMP-PNP, can substitute for ATP suggests that ATP hydrolysis is not required to maintain the I-D interaction. This is also supported by the fact that the I-D complex, when formed in the presence of ATP, is stable when washed with ADP. Furthermore, only a very small amount of ATPase activity was measured with the ChII-ChID complex, compared with ATPase activity obtained with ChII alone. Presumably, ATP and ATP[S] fulfil the same structural requirement in the ChII-ChID complex.

In a recent report by Hansson and Kannangara [12] it was concluded that the ATPase activities associated with the *R. sphaeroides* Mg chelatase subunits were unlikely to be directly coupled to Mg^{2+} chelation, since the ATPase activity was not affected by addition of deuteroporphyrin to assays containing the BchI, BchD and BchH subunits. This is in direct contradiction with the findings presented here, in which the ATPase activity displayed by the *Synechocystis* Mg chelatase correlates closely with Mg chelatase activity. This could reflect differences in the Mg chelatases from these two species, although the methods employed for detection of ATP hydrolysis provide a more plausible explanation. In the present work, we have used a

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 ChII subunit hydrolyses ATP at a rate of 147.5 nmol/min per mg of protein, and when combined with the ChID 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 ChII alone to 0.23 nmol/min with the same amount of ChII, but with ChID 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 BchI 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/GX₄GKS/T and H/R/KX₃OXOODE 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 ChII 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 ChII–ChID interaction suggests that they bind to the active site of the ChII subunit, and thereby probably inhibit the ATPase activity associated with this subunit. Thus the ATPase activity exhibited by the ChII subunit is normally harnessed to the Mg²⁺ insertion, since it is expected that ChII and ChID 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 protoporphyrin 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 protoporphyrin 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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