Modification of cysteine residues in the ChlI and ChlH subunits of magnesium chelatase results in enzyme inactivation

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The enzyme magnesium protoporphyrin chelatase catalyses the insertion of magnesium into protoporphyrin, the first committed step in chlorophyll biosynthesis. Magnesium chelatase from the cyanobacterium *Synechocystis* PCC6803 has been reconstituted in a highly active state as a result of purifying the constituent proteins from strains of *Escherichia coli* that overproduce the ChlH, ChlI and ChlD subunits. These individual subunits were analysed for their sensitivity to *N*-ethylmaleimide (NEM), in order to assess the roles that cysteine residues play in the partial reactions that comprise the catalytic cycle of Mg^{2+} chelatase, such as the ATPase activity of ChlI, and the formation of ChlI–ChlD–MgATP and ChlH–protoporphyrin complexes. It was shown that NEM binds to ChlI and inhibits the ATPase activity of this subunit, and that prior incubation with MgATP affords protection against inhibition. Quantitative analysis of the effects of NEM binding on ChlI-catalysed ATPase activity showed that three out of four thiols per ChlI molecule are available to react with NEM, but only one cysteine residue per ChlI subunit is essential for ATPase activity. In contrast, the cysteines in ChlD are not essential for Mg^{2+} chelatase activity, and the formation of the ChlI–ChlD–ATP complex can proceed with NEM-treated ChlI. Neither the ATPase activity of ChlI nor NEM-modifiable cysteines are therefore required to form the ChlI–ChlD–MgATP complex. However, this complex cannot catalyse magnesium chelation in the presence of the ChlH subunit, protoporphyrin and Mg^{2+} ions. The simplest explanation for this is that in an intact Mg^{2+} chelatase complex the ATPase activity of ChlI drives the chelation process. NEM binds to ChlH and inhibits the chelation reaction, and this effect can be partially alleviated by pre-incubating ChlH with magnesium and ATP. We conclude that cysteine residues play an important role in the chelation reaction, in respect of the ChlI–MgATP association, ATP hydrolysis and in the interaction of ChlH with MgATP and protoporphyrin IX.

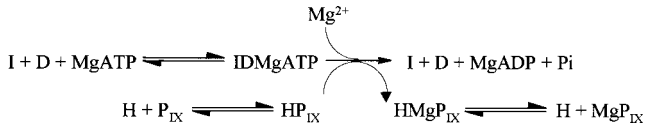
Key words: ATPase, chlorophyll biosynthesis, cysteine modification, Mg^{2+} chelatase, subunit interaction, *Synechocystis*, tetrapyrrole.

INTRODUCTION

Magnesium protoporphyrin IX chelatase catalyses the insertion of Mg^{2+} into protoporphyrin IX, the first step unique to chlorophyll production. The Mg^{2+} chelatase enzyme consists of three subunits, ChlI (38–42 kDa), ChlD (60–74 kDa), and ChlH (140–150 kDa), in (bacterio)chlorophyll *a*-producing prokaryotes [1–3] and also in higher plants [4–7]. The enzyme-catalysed insertion of Mg^{2+} requires hydrolysable ATP in addition to the metal ion and porphyrin substrates.

The Mg^{2+} chelatase enzymes from the cyanobacterium *Synechocystis* PCC6803 (ChlH, ChlI and ChlD) and the purple bacterium *Rhodobacter sphaeroides* (BchH, BchI and BchD) have been reconstituted in a highly active state as a result of purifying the constituent proteins from strains of *Escherichia coli* that overproduce these subunits [8,9]. Furthermore, a physical interaction can be demonstrated between the ChlI and ChlD subunits, which requires the presence of both ATP and Mg^{2+} [9,10]. High rates of ATP hydrolysis are not correlated with the ChlI–ChlD or BchI–BchD complexes, and instead ATP hydrolysis was clearly attributable to the process of Mg^{2+} insertion, since a 7-fold increase in ATP hydrolysis was seen when all three subunits and the three substrates were present [10]. On the basis of this work, a provisional model of the Mg^{2+} chelatase cycle was suggested in which the ChlI and ChlD subunits interact with MgATP and form a complex. It was also proposed that the ChlH subunit with bound protoporphyrin IX reacts with this ChlI–ChlD–MgATP complex, and a short-lived complex consisting of all three subunits and the three substrates is formed. Next, Mg^{2+} is inserted into protoporphyrin IX with concomitant hydrolysis of ATP, resulting in another, probably short-lived, complex consisting of ChlI, ChlD, ChlH, MgADP and Mg^{2+}-protoporphyrin IX. This complex dissociates into ChlI–ChlD–MgADP and ChlH–Mg^{2+}-protoporphyrin, which can be recharged with MgATP and protoporphyrin IX, respectively, to enable them to participate in a new reaction cycle following removal of the reaction products (Scheme 1).

Work with extracts from chloroplasts showed that the higher-plant Mg^{2+} chelatase is sensitive to the cysteine-reactive reagent



Scheme 1 Provisional model of magnesium chelation

The ChlI (I) and ChlD (D) subunits interact to form a complex with magnesium-ATP (MgATP). The protein–nucleotide complex is proposed to interact, perhaps transiently, with a ChlH (H)–protoporphyrin IX (P_{IX}) complex. Following metallation, which is driven by the hydrolysis of ATP, product release occurs. It is not known whether there are subsequent changes in subunit associations in the product-release stages of the catalytic cycle.

Abbreviations used: NEM, *N*-ethylmaleimide; DTT, dithiothreitol; MESG, 2-amino-6-mercapto-7-methylpurine riboside; PNPase, purine nucleoside phosphorylase; 2PDS, 2,2′-dipyridyl disulphide; 2-TP, 2-thiopyridone.

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N-ethylmaleimide (NEM), suggesting that cysteine residues are involved in the proper functioning of the enzyme [11,12]. However, these studies were performed with crude chloroplast preparations, and a detailed analysis was difficult to achieve. We have used the overexpressed and highly purified subunits of the *Synechocystis* Mg^{2+} chelatase to analyse the individual subunits for their sensitivity to NEM. NEM binds to ChlI and inhibits the ATPase activity of this subunit, although prior incubation with MgATP affords protection against inhibition. In contrast, the cysteines in ChlD are not essential for Mg^{2+} chelatase activity, and the formation of the ChlI–ChlD–MgATP complex can proceed with NEM-treated ChlI. NEM binds to ChlH and inhibits the chelation reaction, and this effect can be partially alleviated by pre-incubating ChlH with Mg^{2+} and ATP. We conclude that cysteine residues play an important role in the chelation reaction in respect to the ChlI–MgATP association, ATP hydrolysis and the interaction of ChlH with MgATP and protoporphyrin IX.

EXPERIMENTAL

Expression and purification of recombinant ChlI, ChlD and ChlH proteins

Production of recombinant ChlI, ChlD and ChlH proteins was performed as described by Jensen et al. [2], and purification of both histidine-tagged and non-tagged proteins was as described by Jensen and colleagues [8,10]. A ChlI–ChlD complex was formed by mixing purified histidine-tagged ChlD with purified non-tagged ChlI in the presence of 5 mM ATP and 5 mM $MgCl_2$. Subsequently, ChlI–ChlD complexes were purified by passing the mixture over a Ni^{2+}–agarose affinity column equilibrated in buffer containing 5 mM imidazole, 100 mM NaCl, 20 mM Tris/HCl, pH 7.9, 5 mM ATP and 5 mM $MgCl_2$. To remove unbound proteins, the column was washed with 10 column vols. of the same buffer, followed by 6 column vols. of buffer containing 60 mM imidazole, 100 mM NaCl, 20 mM Tris/HCl, pH 7.9, 5 mM $MgCl_2$ and 5 mM ATP. The bound complexes were eluted with buffer containing 500 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9, 5 mM ATP and 5 mM $MgCl_2$. Finally, the complexes were transferred to 50 mM Mops, pH 7.7, 300 mM glycerol, 5 mM $MgCl_2$ and 5 mM ATP using a PD-10 desalting column (Pharmacia, Milton Keynes, U.K.).

Magnesium protoporphyrin chelatase assay

The standard stopped assay was performed in 100 μl of buffer containing 50 mM Mops/NaOH, pH 7.7, 300 mM glycerol, 16 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5 mM ATP, 4 μM protoporphyrin IX and protein amounts, as indicated below in the Figure and Table legends. The assay mixtures were incubated for 30 min at 34 °C, stopped by addition of 900 μl of acetone/water/32 % (v/v) ammonia (80:20:1, by vol), and centrifuged at 15000 ***g*** for 5 min. The aqueous phase was analysed on a SPEX FluoroLog spectrofluorimeter (Jobin Yvon Ltd, Stanmore, Middx., U.K.). The excitation wavelength was set to 420 nm, and the emission spectrum was recorded between 550 and 650 nm. 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 otherwise identical with the stopped assay. Standard curves for both the stopped and the continuous assay were made by diluting known amounts of authentic Mg^{2+}-protoporphyrin IX (Porphyrin Products, Logan, UT, U.S.A.) in assay buffer containing the same concentrations of ATP, $MgCl_2$ and chelatase subunits as the assays. In assays, care was taken to avoid prolonged light exposure by keeping all materials covered in foil.

ATPase assays

The ATPase activity of the ChlI subunit was measured using the EnzChek Phosphate Assay Kit (Molecular Probes Europe BV, Leiden, The Netherlands) as described previously [10]. Briefly, the chelatase components were incubated with 200 μM 2-amino-6-mercapto-7-methylpurine riboside (MESG) and 1 unit/ml purine nucleoside phosphorylase (PNPase) in an assay volume of 1 ml. Phosphate release was then measured continuously as the increase in absorbance at 360 nm using a Shimadzu UV2101PC spectrophotometer with a temperature-controlled cuvette holder set at 34 °C.

NEM solution

NEM (Sigma, Poole, Dorset) was dissolved in 96 % (v/v) ethanol ($\approx$ 1 M), dilutions were made in water and the final concentration was determined using a molar absorption coefficient of $\varepsilon_{305} = 620\ M^{-1} \cdot cm^{-1}$ [13].

Titration of ChlI ATPase activity with NEM

The total number of protein thiol groups available was determined by titrating with 2,2′-dipyridyl disulphide (2PDS) and following spectrophotometrically the appearance of 2-thiopyridone (2-TP) product at 343 nm ($\varepsilon_{343} = 8080\ M^{-1} \cdot cm^{-1}$; [14]). 2PDS was supplied by Aldrich (Gillingham, Dorset) as Aldrithiol-2, and recrystallized from petroleum ether (60–80 °C b.p.). Any residual DTT was removed from ChlI by gel filtration (using a G-25 column). Protein was incubated with NEM for 30 min at room temperature, and the reaction was quenched with 10 mM DTT. Control experiments demonstrated that no further ATPase activity was lost on a longer ($\approx$ 3 h) incubation, and it was therefore assumed that the NEM modification had gone to completion. Residual ATPase activity was determined as described above. Data were analysed using the non-linear regression function of Sigmaplot (V. 5.05; SPSS Inc.).

Other methods

SDS/PAGE was performed as described in [15]. Protein concentrations were determined using the Bio-Rad protein assay with BSA as the standard. Protoporphyrin IX (Sigma) was prepared as outlined in Jensen et al. [8].

RESULTS

Stimulation of Mg^{2+} chelatase activity by DTT

It has been shown previously that Mg^{2+} chelatase activity is sensitive to thiol-group reagents [11,12,16,17], suggesting that cysteines are essential for the proper functioning and optimal activity of the enzyme. In the protein preparations and assays used in this paper, 1 mM DTT was usually present, and when extra DTT in the 1–20 mM range was added to the assay no further stimulation of activity was observed (results not shown), indicating that 1 mM DTT was sufficient for the reaction. However, diluting the individual protein subunits in buffer without DTT and using them immediately in assays without DTT resulted in an $\approx$ 90 % reduction in Mg^{2+} chelatase activity. The inhibition of the Mg^{2+} chelatase activity in the absence of DTT was reversible, as seen in the restoration of activity following incubation of the DTT-depleted enzyme with increasing concen-

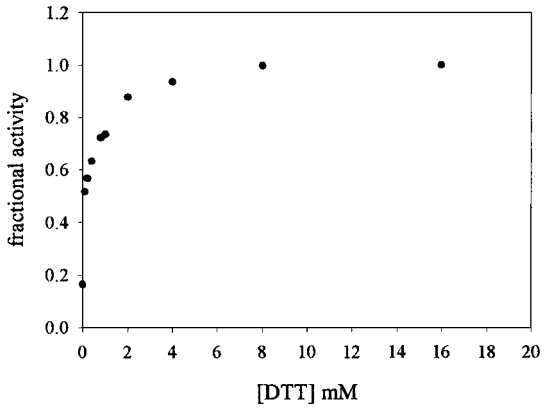

Figure 1 Stimulation of Mg^{2+} chelatase activity by DTT

The ChlI–ChlD complexes prepared without DTT as described in the Experimental section were mixed with ChlH and the three substrates to give a 'master mix'. Aliquots of this master mix were subsequently added to reaction tubes containing increasing concentrations of DTT, resulting in a final DTT concentration as indicated. The assays were incubated for 30 min before being stopped, and the Mg^{2+}-protoporphyrin formed was estimated from the fluorescence emission at 595 nm. In the final assay, the amount of ChlI–ChlD complex was 40 nM and the amount of ChlH was 200 nM. The only source of DTT in the master mix was that added with the ChlH protein, which results in a final concentration of approx. 14 μM DTT.

trations of DTT (Figure 1); almost full activity was regained with 8 mM DTT. This strongly suggests that one or more of the *Synechocystis* Mg^{2+} chelatase subunits contains essential cysteines.

Inhibition of individual Mg^{2+} chelatase subunits by NEM: effects on individual subunits

Performing experiments with NEM requires the omission of DTT from the protein preparations and assay buffers, which otherwise quenches the effect of NEM. Preliminary experiments with varying concentrations of NEM had demonstrated inhibition of the Mg^{2+} chelatase reaction by NEM (results not shown). In order to obtain more precise information, individual subunits were modified by NEM in turn and their consequent behaviours were measured. Subsequently, a mixture containing the other two subunits, the three substrates and 10 mM DTT was added, and the amount of Mg^{2+}-protoporphyrin formed was measured following incubation for 30 min at 34 °C. In the control assay, when the three proteins and substrates were mixed in the presence of 10 mM DTT and then added to tubes containing NEM, it was shown that 3–12 μM NEM was easily prevented from reacting with protein thiols by 10 mM DTT (results not shown).

Table 1 shows the results of such an experiment on ChlI. It is clear that treatment of ChlI with NEM abolishes its ability to reconstitute an active Mg^{2+} chelatase with the other two subunits (compare assays 1 and 2).

The ChlI protein of *Synechocystis* is a highly active ATPase [10]. To test whether the NEM-mediated inhibition of ChlI could be prevented by ATP, experiments were performed in which 0.2 μM ChlI was incubated with 5 mM ATP and 16 mM $MgCl_2$ before being exposed to 3 μM NEM and subsequently tested for Mg^{2+} chelatase activity. The result was complete protection against NEM, as shown by the Mg^{2+} chelatase activity obtained after mixing with the other assay components (Table 1, assays 3 and 4). Full protection of ChlI could also be obtained with 5 mM ATP or 5 mM ADP alone, but not with 5 mM AMP or 11 mM $MgCl_2$ (results not shown). These protection experiments indicate either that cysteine is involved in the binding of ATP and ADP or that these nucleotides facilitate a conformational change or oligomerization in which essential cysteine is protected.

Pre-incubation of 0.4 μM ChlH subunit with 3 μM NEM also abolished the ability of this subunit to reconstitute an active Mg^{2+} chelatase (Table 2, assays 1 and 2). The ChlH subunit has been proposed to bind the protoporphyrin substrate prior to insertion of the Mg^{2+} ion [8]. However, incubation of ChlH with 4 μM protoporphyrin IX gave only a minimal protection of ChlH against NEM inactivation (Table 2, assays 3 and 4), which suggests that protoporphyrin binding does not protect essential cysteines.

It was possible that an interaction between ChlH and the other two subunits could involve or protect essential cysteines in ChlH. In order to test this, 0.4 μM ChlH was pre-incubated with 0.8 μM ChlI, 0.4 μM ChlD and equal concentrations (6 mM) of Mg^{2+} and ATP (MgATP), which ensures formation of a ChlI–ChlD complex [8], before adding NEM to give 12 μM. Minimal protection of ChlH was seen (Table 2, assays 5 and 6), comparable with the protection observed when pre-incubating ChlH with protoporphyrin IX alone (Table 2, assay 3). As ChlI is protected against NEM by incubation with MgATP, and ChlD is almost unaffected by NEM (see below), this observed inhibition is likely to arise from modification of ChlH by NEM. This result suggests that if a ChlI–ChlD–ChlH complex has formed, then any cysteine residues of ChlH in such a complex are accessible to NEM.

To extend this experiment, 0.4 μM ChlH was incubated with 0.8 μM ChlI, 0.4 μM ChlD, 6 mM MgATP and 4 μM protoporphyrin IX before addition of NEM. After this pre-incubation,

Table 1 Effect of NEM on ChlI- and ATP-mediated protection of essential cysteine residues in ChlI

The ChlI subunit (0.2 μM) was pre-incubated for 4 min at 34 °C with 3 μM NEM or 5 mM ATP + 16 mM $MgCl_2$ as indicated (column A). The 'A' incubations were then incubated for another 4 min at 34 °C with additions, as indicated in column B. Subsequently, a mixture was added (column C) to give final concentrations of 0.1 μM ChlI, 0.05 μM ChlD, 0.2 μM ChlH, 5 mM ATP + 16 mM $MgCl_2$, 4 μM protoporphyrin IX (Pix) and 5 mM DTT. The assays were incubated for 30 min at 34 °C and formed Mg^{2+} protoporphyrin was estimated (stopped assay). The average of three repeats is shown ± S.D.

	Treatment			
Assay	A	B	C	Fractional activity
1	ChlI + NEM	ATP + $MgCl_2$	ChlD + ChlH + Pix + DTT	< 0.012
2	ChlI	ATP + $MgCl_2$	ChlD + ChlH + Pix + DTT	0.92 ± 0.06
3	ChlI + ATP + $MgCl_2$	NEM	ChlD + ChlH + Pix + DTT	0.95 ± 0.09
4	ChlI + ATP + $MgCl_2$	No NEM	ChlD + ChlH + Pix + DTT	1 ± 0.09

Table 2 Effect of NEM on ChlH and protection of essential cysteine residues in the ChlH subunit by MgATP and protoporphyrin IX

The ChlH subunit (0.4 μM) was pre-incubated for 4 min at 34 °C as indicated (column A). The mixtures in column A were then incubated for a further 4 min at 34 °C with additions as indicated in column B. The dashes in column B indicate 'no further pre-incubation'. Subsequently, a mixture of the other assay components was added, as indicated in column C, and the assays were incubated for 30 min at 34 °C, as described in the legend to Table 1. In the pre-incubations 1–4, 3 μM NEM was used and in the respective assays, final concentrations were 0.1 μM ChlI, 0.05 μM ChlD, 0.2 μM ChlH, 4 μM protoporphyrin IX (Pix), 5 mM ATP and 16 mM $MgCl_2$. In the pre-incubations 5–10, 12 μM NEM was used and in the respective assays, final concentrations were 0.4 μM ChlI, 0.2 μM ChlD, 0.2 μM ChlH, 4 μM Pix, 5 mM ATP and 16 mM $MgCl_2$. The average of three experiments is shown $\pm$ S.D. MgATP indicates 6 mM ATP and 6 mM $MgCl_2$.

	Treatment			
Assay	A	B	C	Fractional activity
1	ChlH + NEM	–	ChlI + ChlD + ATP + $MgCl_2$ + Pix + DTT	< 0.008
2	ChlH	–	ChlI + ChlD + ATP + $MgCl_2$ + Pix + DTT	0.88 ± 0.03
3	ChlH + Pix	NEM	ChlI + ChlD + ATP + $MgCl_2$ + DTT	0.03 ± 0.01
4	ChlH + Pix	No NEM	ChlI + ChlD + ATP + $MgCl_2$ + DTT	1 ± 0.1
5	ChlH + ChlI + ChlD + MgATP	NEM	$MgCl_2$ + Pix + DTT	0.05 ± 0.02
6	ChlH + ChlI + ChlD + MgATP	No NEM	$MgCl_2$ + Pix + DTT	1 ± 0.1
7	ChlH + ChlI + ChlD + MgATP + Pix	–	DTT	0
8	ChlH + ChlI + ChlD + MgATP + Pix	NEM	$MgCl_2$ + DTT	0.22 ± 0.03
9	ChlH + MgATP + Pix	NEM	ChlI + ChlD + ATP + $MgCl_2$ + DTT	0.3 ± 0.043
10	ChlH + ChlI + ChlD + MgATP + Pix	No NEM	$MgCl_2$ + DTT	1 ± 0.15

22 % (Table 2, assay 8) of the activity in the control experiment (Table 2, assay 10) was obtained. Thus pre-incubation with protoporphyrin in addition to the other two subunits and MgATP affords some protection of ChlH against NEM. Mg^{2+} chelation is prevented during the pre-incubation because there is no excess of free Mg^{2+} (Table 2, assay 7). A similar level of protection against NEM is also seen when ChlH is pre-incubated with 4 μM protoporphyrin IX and 6 mM MgATP (Table 2, assay 9). Thus the observed protection does not involve a ChlI–ChlD complex, but is most likely a result of the presence of both protoporphyrin IX and MgATP. It therefore appears that substrate binding to ChlH provides partial protection for one or more essential cysteines. We have previously shown that pre-incubation of ChlH with protoporphyrin IX, $MgCl_2$ and ATP increased the rate of Mg^{2+} chelation in continuous assays, indicating that ChlH interacts with both protoporphyrin IX and MgATP before interacting with a ChlI–ChlD–MgATP complex [8]. However, significant ATPase activity was not observed with the ChlH subunit of *Synechocystis* [10].

Surprisingly, the ChlD subunit was almost unaffected by pre-incubation with NEM. Using the same experimental conditions as outlined for the ChlI and ChlH subunits, 0.1 μM ChlD was pre-incubated without or with 4 and 12 μM NEM and subsequently mixed with the two other subunits to assay for Mg^{2+} chelatase activity. When 4 and 12 μM NEM were added, 91 % and 85 % of the activity in the NEM free control were obtained respectively. This indicates that the cysteines in ChlD are not essential for Mg^{2+} chelatase activity. Although ChlI is clearly susceptible to thiol modification and ChlD is unaffected by pre-incubation with NEM, the ChlI–ChlD–MgATP complex [9,10] was not tested. It is possible that binding of substrates, and of ChlI, could lead to exposure of a previously buried cysteine residue in ChlD, as observed with other enzymes, such as the tetrapyrrole biosynthetic enzyme porphobilinogen deaminase [18].

Inhibition of the ATPase activity associated with ChlI by NEM

Recently, it has been shown that ChlI on its own possesses high ATPase activity [10]. Having established that ChlI, after incubation with NEM, was unable to support Mg^{2+} chelatase activity, it was of interest to see whether this inactivation by NEM was due to inactivation of the ATPase activity associated with ChlI. Experiments were performed in which 1 μM ChlI was incubated without or with 3 μM NEM for 4 min at 34 °C. Subsequently, these ChlI preparations were mixed and diluted 10-fold with a mixture containing $MgCl_2$, ATP and DTT (to quench NEM), and 200 μM MESG and 1 unit/ml PNPase to measure ATPase activity. Phosphate release was followed at 360 nm via this linked assay at 34 °C for 30 min. Incubation of ChlI without NEM gave an ATPase activity of 0.37 ± 0.032 $\mu M \cdot min^{-1}$, whereas pre-incubation of ChlI with NEM gave an activity of 0.072 ± 0.042 $\mu M \cdot min^{-1}$. The latter value is close to the detection limit of the ATPase assay, and it is clear that NEM inactivates the ATPase activity associated with the ChlI subunit. It is likely that this inactivation of the ChlI-associated ATPase activity accounts for the inhibitory effects of NEM on ChlI with respect to Mg^{2+} chelatase activity in Table 1.

Quantification of cysteine residues in ChlI essential for ATPase activity

The method of Tsou [19,20] was used to count the number of cysteine residues in ChlI essential for ATPase activity. The total number of thiol groups available was determined using 2PDS as a thiol-specific modification reagent with a chromophoric product (2-TP; ε_{343} 8080 $M^{-1} \cdot cm^{-1}$; [14]). ChlI protein was allowed to react to completion with sub-stoichiometric ratios of NEM to protein thiol, and the residual ATPase activity was determined. The results of such a titration are shown in Figure 2. The relationship between a (the fraction of activity remaining), m (the fraction of thiol groups modified), p (the fraction of groups modified when no activity remains) and α (the number of essential groups) is given by eqn. (1):

$$a = \left[1 - \frac{m}{p} \right]^{\alpha} \quad (1)$$

Analysis of these data (Figure 2) gives $\alpha = 1.072 \pm 0.144$ and $p = 0.739 \pm 0.055$ (means $\pm$ S.E.M.). This suggests that one cysteine residue per ChlI subunit is essential for ATPase activity, and that approx. 74 % of the total thiol, as determined by titration with 2PDS, is accessible to NEM. As the predicted amino acid sequence of ChlI discloses the presence of four cysteine residues

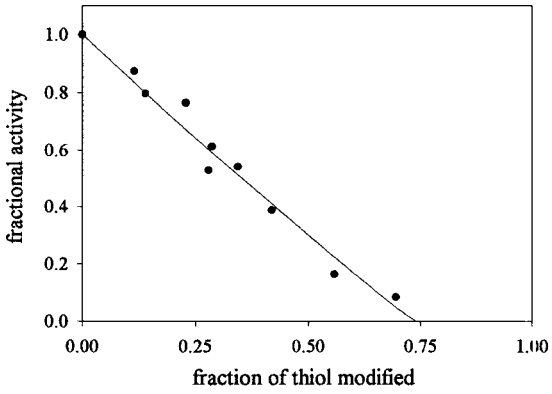

Figure 2 Tsou plot of the loss of ChlI ATPase activity upon thiol modification by NEM

The points are experimental and the line is theoretical for eqn. (1) with the characterizing parameters, p 0.739 (0.055) and α 1.072 (0.144); standard errors in parentheses. Assays were performed at 34 °C in 50 mM Mops/NaOH/0.3 M glycerol, containing 2.1 μM ChlI, 5 mM ATP, 10 mM $MgCl_2$, 5 mM DTT, 200 μM MESG and 1 unit/ml PNPase.

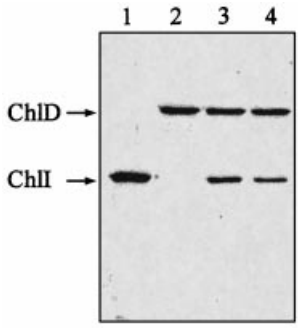

Figure 3 Interaction between ChlD and NEM-inactivated ChlI

Non-histidine-tagged ChlI protein (15 μM) was pre-incubated with or without NEM before being incubated with 6 μM histidine-tagged ChlD protein in the presence of 5 mM ATP and 5 mM $MgCl_2$ to allow the formation of complex. The mixture was then loaded on to the Ni^{2+}-resin column, which was washed with buffers containing 5 mM ATP and 5 mM $MgCl_2$ to remove unbound protein, before the bound complexes were eluted with imidazole buffer (see the Experimental section). The Figure shows the Coomassie-Blue-stained SDS/polyacrylamide gel of the purified proteins used in the experiment and the eluted complexes. Lane 1, non-histidine-tagged ChlI; lane 2, histidine-tagged ChlD; lane 3, eluted complex formed between untreated ChlI and histidine-tagged ChlD; lane 4, eluted complex formed between NEM-treated ChlI and histidine-tagged ChlD.

per chain, this implies that three out of four thiols per ChlI molecule are available to react with NEM.

Effects of NEM on the formation of the ChlI–ChlD complex

Non-tagged ChlI was incubated either with or without NEM as described above, quenched with 5 mM DTT, and then mixed with histidine-tagged ChlD (final concentrations of ChlI and ChlD of 15 μM and 6 μM respectively) to allow complex formation in the presence of 6 mM MgATP. The mixture was then passed over a Ni^{2+}–agarose affinity column. As observed on the SDS gel in Figure 3, the NEM-treated ChlI is still able to form a complex with ChlD, although with slightly decreased yield of the non-tagged ChlI subunit (lane 4) when compared with the control (lane 3). After incubation with NEM, aliquots of ChlI were removed and tested for ATPase activity and, as seen

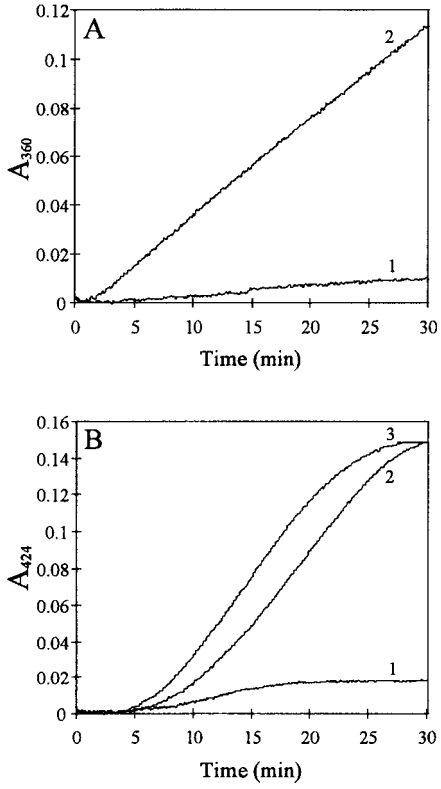

Figure 4 Continuous ATPase and Mg^{2+}-chelatase assays with the NEM-treated and untreated ChlI subunit

The NEM-treated and untreated ChlI used in Figure 3 were tested for activity using the continuous ATPase assay. The obtained traces are displayed in (**A**): trace 1, NEM-treated ChlI; trace 2, untreated ChlI. In both assays, the final concentration of ChlI was 0.1 μM. Similarly, the eluted ChlI–ChlD complexes shown in Figure 3 were tested for their ability to reconstitute an active Mg^{2+} chelatase with added ChlH. The continuous formation of Mg^{2+}-protoporphyrin is shown in (**B**): trace 1, ChlI–ChlD complex formed with NEM-treated ChlI; trace 2, ChlI–ChlD complex formed with untreated ChlI; and trace 3, ChlI–ChlD complex formed with NEM-treated ChlI, but with untreated ChlI (10 nM) added to demonstrate that ChlD is fully active after interaction with NEM-treated ChlI. In the final assay, the concentration of ChlI–ChlD complex ($\approx$ 200 kDa; [9]) was 4 nM; [ChlH] was 20 nM.

from the traces from the continuous assays in Figure 4(A), the NEM-treated ChlI possessed no significant ATPase activity, whereas the untreated ChlI displayed high activity. The rate of ATP hydrolysis for untreated ChlI was 0.45 $\mu M \cdot min^{-1}$, but for NEM-treated ChlI the rate was below the detection limit of the ATPase assay. Thus the ATPase activity of ChlI is not required for ChlI–ChlD complex formation.

The eluted ChlI–ChlD complexes were also tested for their ability to reconstitute an active Mg^{2+} chelatase when mixed with ChlH and the three substrates. As seen in Figure 4(B), observing the formation of Mg^{2+}-protoporphyrin, the ChlI–ChlD complex formed with NEM-treated ChlI only gave a trace amount of activity, corresponding to 0.004 $\mu M \cdot min^{-1}$ (trace 1), whereas the complex formed with the untreated ChlI gave almost 10-fold higher activity, corresponding to 0.038 $\mu M \cdot min^{-1}$ (trace 2). To verify that ChlD in the complex formed with NEM-treated ChlI was still able to form a fully active complex with ChlI, an assay was performed in which extra non-treated ChlI was added (trace 3). From this it is evident that ChlD is unaffected by interaction with NEM-treated ChlI, since the same rate of Mg^{2+} chelation is obtained. The shorter lag period before Mg^{2+}-protoporphyrin starts to accumulate in trace 3 compared with trace 2 was

observed in several repetitions of the experiment, and can probably be explained by the slightly higher protein concentration in the assay with extra amounts of ChlI added.

DISCUSSION

Modification of cysteine residues clearly leads to inactivation of Mg^{2+} chelatase in respect of the association of ChlI–MgATP, ATP hydrolysis and the interaction of ChlH with MgATP and protoporphyrin IX. Quantitative analysis of the effects of NEM binding on ChlI-catalysed ATPase activity shows that three out of four thiols per ChlI molecule are available to react with NEM, but only one cysteine residue per ChlI subunit is essential for ATPase activity.

At present the identity of these cysteines is not known with certainty. Inspection of alignments of deduced primary protein sequences may provide some idea of the extent of conservation of cysteines. As such residues are conserved, it is less likely that structural changes would be tolerated at such positions. Four cysteine residues are present in the ChlI subunit from *Synechocystis* (Cys-121, Cys-244, Cys-282 and Cys-324; numbering on the basis of the *Synechocystis* ChlI sequence). In alignments of 16 ChlI/BchI sequences from bacteria, algae and higher plants (results not shown), Cys-121 is conserved in all sequences, except for that of BchI from *Rhodobacter capsulatus* (accession no. Z11165) and *Acidiphilium rubrum* (accession no. BAA76531). Cys-244 is unique to the *Synechocystis* ChlI sequence. In contrast, Cys-282 is conserved in all 16 ChlI sequences and Cys-324 is conserved in all except the barley, *R. capsulatus* and *A. rubrum* ChlI sequences. Thus there is evidence for absolute conservation of one of the four cysteines, and limited conservation of three of the four cysteines in ChlI from *Synechocystis*.

It was observed that protection against NEM inhibition of ChlI could be obtained by incubation with MgATP, ATP or ADP alone, but not with AMP or $MgCl_2$. These protection experiments indicate that cysteine(s) are involved in binding of ATP/ADP, or that these nucleotides facilitate a conformational change or oligomerization in which an essential cysteine(s) is protected. In contrast, the formation of the ChlI–ChlD–MgATP complex does not involve NEM-modifiable cysteines. There are several ways in which modification of an amino acid may cause loss of activity. A catalytic-site group (e.g. thiolate anion) may be lost, preventing enzyme turnover. Alternatively, a binding-site residue may be modified, and subsequently this may sterically prevent substrate binding. It is also possible that the modified residue is remote from the active site, but that modification perturbs the enzyme structure enough to prevent catalysis. It has been shown that formation of a ChlID complex requires ATP binding [10]. As NEM-modified ChlI can still form this complex, it is likely still to bind ATP. If NEM-modified ChlI can still bind ATP, it is unlikely that the modified residue is directly involved in substrate binding. We therefore suspect that this residue is not in the active site. Identification of the role of this cysteine will be aided by further analysis using site-directed mutants.

The NEM protection data shown in Table 2 show that, together, MgATP and protoporphyrin provide 30% protection against the effects of NEM on ChlH, which is not as evident as the protection effects seen with ChlI in Table 1. If insufficient MgATP or protoporphyrin IX is present to saturate the putative binding sites on ChlH, it is expected that only partial protection would be observed. Previous work had suggested a role for the Mg^{2+} chelatase ChlH subunit in binding protoporphyrin [1]. However, there had been no indications of a role for MgATP in this process. There is some evidence that the *R. sphaeroides* and the *Chlorobium vibrioforme* ChlH subunits are ATPases [21,22], but porphyrin was shown not to enhance ATPase activity. This highlights the need to quantify the binding of protoporphyrin to ChlH, and also to investigate whether MgATP plays a role in this process, apart from a role in driving the catalysis of magnesium insertion.

There are many possible candidates for NEM-modifiable cysteine residues. In the *Synechocystis* ChlH sequence, 11 cysteine residues are found (Cys-272, -306, -372, -638, -722, -730, -874, -896, -901, -1037 and -1278). Cys-272, -306, -372, -874, -901 and -1278 are unique to the *Synechocystis* ChlH sequence. More interestingly, Cys-722, -896 and -1037 are conserved among *Synechocystis* and all the higher-plant ChlH sequences. Cys-730 is also found in the *C. vibrioforme* ChlH sequence (accession no. Z83933). A compelling candidate would be Cys-638: in an alignment of nine complete ChlH/BchH sequences (five bacterial and four higher-plant sequences), this residue is conserved in all sequences. This cysteine is also conserved in CobN, the porphyrin-binding cobalt-chelatase subunit of *Pseudomonas denitrificans* (accession no. P29929), and in the putative cobalt-chelatase and nickel-chelatase homologues from *Methanococcus jannaschii* (accession nos. U67585 and U67534). It is difficult to assign a role to modified cysteines in the ChlH subunit, since the precise function of this subunit, beyond porphyrin binding, has not been established.

As demonstrated above, NEM abolishes the ATPase activity associated with ChlI. However, the role of this ATPase activity in the Mg^{2+}-chelatase reaction cycle is not clear. It has been proposed that ATPase activity associated with BchI–BchD in *R. sphaeroides* is required to form and maintain an I–D complex [21]. In a more recent report we have shown that the ChlI–ChlD interaction takes place with the slowly hydrolysable ATP analogue adenosine 5′-[γ-thio]triphosphate and, to a minor extent, also with ADP and the non-hydrolysable ATP analogue adenosine 5′-[β,γ-imido]triphosphate, and therefore the formation of complex probably does not require high rates of ATP hydrolysis [10]. The present work demonstrates that, although NEM inhibits the ATPase activity of ChlI, it is still possible to assemble a ChlI–ChlD complex, which provides further evidence that complex formation probably does not require high rates of ATP hydrolysis. However, the proposed catalytic cycle for Mg^{2+} chelatase [8,10] requires a possibly transient association of a ChlI–ChlD–MgATP complex with the ChlH subunit. The fact that NEM treatment of the ChlI subunit abolishes Mg^{2+} chelatase activity suggests that it is the ATPase activity of ChlI that is harnessed in some way to drive either the association of ChlI–ChlD–MgATP complex with ChlH and/or the subsequent insertion of the Mg^{2+} ion into protoporphyrin.

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