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Title: ATPase activity associated with the magnesium chelatase H-subunit of the chlorophyll biosynthetic pathway is an artifact

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Abbreviations:

AAA⁺, ATPases Associated with various cellular Activities; ATP[S], adenosine 5'-O-(3-thiotriphosphate); D, magnesium chelatase D-subunit; EM, electron microscopy; I, magnesium chelatase I-subunit, LM, light membrane-bound fraction; MIDAS, metal ion dependent adhesion site; S, soluble; TFA, trifluoroacetic acid

SYNOPSIS

Magnesium chelatase inserts Mg^{2+} into protoporphyrin IX and is the first unique enzyme of the chlorophyll biosynthetic pathway. It is a heterotrimeric enzyme, composed of I- (40 kDa), D- (70 kDa) and H- (140 kDa) subunits. The I- and D- proteins belong to the family of ATPases Associated with various cellular Activities (AAA⁺), but only I hydrolyses ATP to ADP. The D-subunits provide a platform for the assembly of the I-subunits, which results in a two-tiered hexameric ring complex. However, the D-subunits are unstable in the chloroplast unless ATPase active I-subunits are present. The H-subunit binds protoporphyrin and is suggested to be the catalytic subunit. Previous studies have indicated that the H-subunit also has ATPase activity, which is in accordance with an earlier suggested two-stage mechanism of the reaction. In this work we demonstrate that gel filtration chromatography of affinity purified *Rhodobacter capsulatus* H-subunit produced in *Escherichia coli* generates a high and a low molecular weight fraction. Both fractions were dominated by the H-subunit, but the ATPase activity was only found in the high molecular weight fraction and magnesium chelatase activity was only associated with the low molecular weight fraction. We demonstrated that light converted monomeric low molecular weight H-subunit into high molecular weight aggregates. We conclude that ATP utilization by magnesium chelatase is solely connected to the I-subunit and suggest that a contaminating *E. coli* protein, which binds to aggregates of the H-subunit, caused the previously reported ATPase activity of the H-subunit.

INTRODUCTION

Magnesium chelatase (EC 6.6.1.1) catalyses the insertion of Mg^{2+} into protoporphyrin IX, the first committed step in chlorophyll biosynthesis [1]. Early characterizations of magnesium chelatase employed intact chloroplasts to assay enzyme activity [2-4]. It was shown that magnesium chelatase required the porphyrin and metal ion substrates, but it also had a requirement for ATP. Further characterization of magnesium chelatase was hampered by a complete loss of activity after plastid breakage. The first work on magnesium chelatase from broken chloroplasts showed that enzyme activity required both a soluble (S) and a "light" membrane-bound fraction (LM) [5]. It was also shown that the time course of enzyme activity was not linear, but exhibited a lag phase before the enzymatic product, Mg-protoporphyrin IX, was detected [6]. The lag phase could be eliminated if the S and LM extracts were mixed and preincubated in the presence of ATP before protoporphyrin was added. Through a series of experiments where the preincubation and the reaction were performed at different concentrations Walker and Weinstein [7] found that adenosine 5'-O-(3-thiotriphosphate) (ATP[S]) could replace ATP in the activation stage during preincubation, but not in the chelation stage of the reaction. From this observation they concluded that magnesium chelation proceeds via a two-stage ATP dependant mechanism.

It is now known that the magnesium chelatase is composed of three heterogeneous subunits (I, D and H) with average molecular masses of 40-, 70- and 140-kDa, respectively. Today, recombinant proteins of all three subunits are available from *Rhodobacter sphaeroides* [8-10], *Rhodobacter capsulatus* [11], *Chlorobium vibrioforme* [12], *Synechocystis* PCC6803 [13, 14] and *Acidophilium rubrum* [15]. X-ray structural analysis revealed that the *R. capsulatus* I-subunit belongs to the large family of ATPases Associated with various cellular Activities, commonly referred to as AAA^+ proteins [16]. The N-terminal half of the D-subunit shows high sequence similarity to the I-subunit, while the C-terminal half includes a metal ion coordination motif (MIDAS) characteristic of integrin I domains [16]. The N-terminal similarity to the I-subunit strongly suggests that the D-subunit is also an AAA^+ -protein although no ATPase activity has been detected from this subunit [17-19]. AAA^+ proteins form oligomeric ring structures and indeed from negative stained electron microscopy studies it was shown that the I-subunit from *R. capsulatus* [20] and *Synechocystis* PCC6803 [21] form hexameric and heptameric rings, respectively. In addition, a two-

tiered hexameric ring structure of an ID-complex was recently demonstrated (unpublished). Typically AAA⁺-proteins are chaperones that can bind and hydrolyze ATP. During the ATP hydrolytic cycle, AAA⁺ oligomeric ring structures go through a conformational change that transfer mechanical energy to the substrate protein or DNA [22-24]. Today it is well established that ATP hydrolysis can be performed by the magnesium chelatase I-subunit from various organisms [17-19, 25-27].

The availability of recombinant magnesium chelatase systems has afforded detailed *in vitro* investigations showing that the oligomerization of I- and D-subunits is responsible for the observed lag phase. Preincubation of I- and D-subunits with Mg²⁺ and ATP eliminates the lag phase [9, 10, 14, 28]. As earlier found by Walker and Weinstein [7], this initial step does not require hydrolysis of ATP, as ADP and nonhydrolysable ATP analogues (but not AMP) can substitute for ATP [18, 25, 29]. It is clear, however, that the overall magnesium chelatase reaction requires ATP hydrolysis. Therefore, ATPase activity assigned to the H-subunit has been interesting since this subunit also binds the porphyrin substrate and is regarded as the catalytic subunit [9, 11, 14, 30]. The ATPase activity of the H-subunit of *R. sphaeroides* and *C. vibrioforme* was 0.90 and 1.8 nmol min⁻¹ mg⁻¹ of protein, respectively [17, 19]. Otherwise, the H-subunit of *Synechocystis* PCC6803 has been most intensively studied. In one report no ATPase activity could be detected [19], whereas 0.95±0.34 nmol min⁻¹ mg⁻¹ was reported in another study [18]. In addition, the binding of the porphyrin substrate analogue deuteroporphyrin IX to the H-subunit of *Synechocystis*, but not *R. sphaeroides*, was stimulated 4-fold by ATP [30]. In the present study we have investigated the ATPase activity of *R. capsulatus* H-subunit and showed that the ATPase activity could be separated from magnesium chelatase activity. Therefore, we suggest that the H-subunit participates in the magnesium chelatase reaction without hydrolyzing ATP. Instead, ATP utilization by magnesium chelatase is connected to activities of the I-subunit.

EXPERIMENTAL

Production of the magnesium chelatase H-subunit

Plasmid pET15bBchH [11] contains the *R. capsulatus bchH* gene cloned into the pET15b vector (Novagen). The *bchH* gene encodes the magnesium chelatase H-subunit and when expressed from pET15bBchH it obtains a His-tag in its N-terminus.

Plasmid pET15bBchH was transformed into *Escherichia coli* BL21 (DE3) pLys (Novagen) and the His-tagged H-subunit was produced and purified as described by [11] using a Ni²⁺-chelating column. During the expression and purification procedure the cultures, the pellets, lysates and purification fractions were protected from light where possible in order to avoid photo-oxidation.

Gel filtration chromatography

A Sephacryl S-200 HR 16/60 (Amersham Pharmacia Biotech) column was equilibrated with buffer consisting of 50 mM Tricine-NaOH pH 8.0, 15 mM MgCl₂, 6% glycerol and 4 mM dithiothreitol at a flow rate of 1 ml/min. His-tagged H-subunit (400 µl of 10 µg/µl) purified on a Ni²⁺-chelating column was loaded in the dark on the Sephacryl column and 500 µl fractions were collected. When necessary eluted fractions were pooled and concentrated using YM-10 centricon (Amicon). The column was calibrated with the following molecular weight standards: bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (443 kDa).

Magnesium chelatase activity measurements

Recombinant *R. capsulatus* H-protein was thawed quickly and kept on ice. During the entire assay procedure it was kept in darkness in order to prevent photo-oxidation and bleaching of protein and endogenously bound porphyrin. All assays were performed in 100 µl volumes with 25 µg H-subunit, 4.5 µg I-subunit and 10 µg D-subunit. The reaction contained: 50 mM Tricine-NaOH pH 8.0, 15 mM MgCl₂, 1 mM dithiothreitol, 10 mM phosphocreatine, phosphocreatine kinase (2 units per reaction), 4 mM ATP and 1 µM protoporphyrin IX. Protoporphyrin IX was first mixed with a small amount of 1 M NaOH and then dissolved in DMSO. The concentration of protoporphyrin IX was determined in 2.7 M HCl using the ϵ_{408} of 262 mM⁻¹ cm⁻¹. The final concentration of DMSO in assays was not greater than 0.5%. Assays were incubated for a maximum of 90 minutes at 32°C and the reaction was terminated with the addition of 900 µl of acetone : H₂O : 13.4 M ammonia mixture (80:20:1, v/v/v), followed by centrifugation to remove precipitants. For Mg-protoporphyrin IX detection, the supernatants were analyzed on a FluroMax-2 Spectrophotometer (Jobin Yvon Spex, Horiba) with excitation set at 418 nm and emission detected between

550-650 nm. Excitation and emission slit widths were set to 10 nm. A peak at 590 nm indicated the presence of Mg-protoporphyrin IX. A standard curve was produced using authentic Mg-protoporphyrin IX (Porphyrin Products, Logan, UT, U.S.A.) acetone mix.

ATPase activity measurements

ATPase activity was measured by hydrolysis of [8-¹⁴C]ATP (specific activity 2.1 Gbq/mmol, Perkin-Elmer). The reaction contained: 50 mM Tricine-NaOH pH 8.0, 15 mM MgCl₂, 6% glycerol, 1 mM dithiothreitol, 80 μM ATP, 20 μM [8-¹⁴C]ATP and 7 μg protein in a total volume of 10 μl. During incubation at 32°C, 1 μl samples were removed at 2 h and quenched by addition of 1 μl of 2 M formic acid. The ATP and ADP components were then separated by thin layer chromatography (polyethyleneimine-cellulose F, Merck), using 1 M formic acid and 0.7 M LiCl as the mobile phase. A STORM phosphoimager system (Amersham Pharmacia) was used for detection and quantification.

Mass spectrometry

Protein bands of interest were cut from SDS-polyacrylamide gels and destained in 75 μl 50 mM NH₄HCO₃, 50% ethanol for 60 minutes. The liquid was removed and 50 μl ethanol was added to shrink the gel pieces for 15 minutes. The liquid was removed and the gel pieces were dried in a SpeedVac concentrator (Savant, Farmingdale, NY, USA). The gel pieces were rehydrated in 10 μl digestion buffer consisting of 25 mM NH₄HCO₃ and 12.5 μg/μl of sequencing-grade trypsin (Promega, Madison, WI, USA) on ice for 45 minutes. Digestion buffer without trypsin (10 μl) was added to cover the pieces and to keep them wet during enzyme cleavage. The samples were incubated at 37°C overnight and the supernatant was collected. To further extract the peptides from the gel pieces, 10 mM NH₄HCO₃, 50% (v/v) ethanol, 0.5% (w/v) trifluoroacetic acid (TFA) was added and incubated for 60 minutes at 37°C. The gel pieces were spun down and the supernatant was collected and pooled with the supernatant from the overnight incubation. The samples were dried using a SpeedVac concentrator and were then redissolved in 5 μl 0.1% TFA. A half μl was spotted directly onto a stainless steel MALDI-target plate and the solvent was allowed to completely evaporate. A half μl of a matrix solution containing 5 mg/ml α-cyano-4-hydroxy

cinnamic acid, 50% (v/v) acetonitrile, 0.1% (w/v) TFA and 5 mM $\text{NH}_4\text{H}_2\text{PO}_4$ [31] was added and allowed to dry. MALDI-TOF MS spectra were recorded automatically using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, CA, USA) mass spectrometer in positive reflector mode. The MS spectra were internally calibrated using two trypsin autoproteolysis peptides with the m/z values 842.51 and 2211.097 Da. Protein identification was performed using the GPS Explorer software, with an in-house installed Mascot search engine (Matrix Science, London, UK) [32] searching the Swissprot database. Parameters specified in the search were: taxa, *E. coli* or eubacteria; missed cleavages, 1; peptide mass tolerance, ± 50 ppm. A protein was considered as identified when the level of confidence exceeded 99%.

Electron microscopy

Carbon-coated 400 mesh copper grids (Ax-labs, Vedbæk, Denmark) were first washed for 1 min with a drop of 50 mM Tricine-NaOH, pH 8.0, 15 mM MgCl_2 , 1 mM dithiothreitol. Excess liquid was removed by brief contact with filter paper. Ni^{2+} affinity purified H-subunit was diluted to 0.01 mg/ml with the wash buffer. A drop of the protein solution was applied to the grid and incubated for 1 min in darkness and excess sample was removed. The grid was immediately stained with filtered 2% (w/v) uranyl acetate for 30 sec and excess stain was removed. Electron microscopy (EM) was performed using a 120 kV Philips CM-10 microscope at 55000 \times magnification. Using a single particle reconstruction of *R. capsulatus* H-subunit as a guide (unpublished data), we searched for areas in the micrographs that contained stain excluding particles equal to or greater than the monomeric size.

Other methods

SDS-PAGE (10% w/v acrylamide) was performed according to Fling and Gregerson [33] with the Tris-Tricine buffer system of Schagger and von Jagow [34]. Proteins on SDS-PAGE were visualized by staining with colloidal Coomassie Brilliant Blue G-250 [35]. Amino acid sequence alignments were performed using the ClustalW algorithm [36]. The ScanProsite tool was used to search for ATPase moieties [37].

RESULTS

H-subunits do not contain conserved ATPase moieties.

An abundance of sequence and crystallographic data analysis [38-42] have shown that the best conserved motif for binding of ATP or GTP is the Walker A consensus ([AG]XXXXGK[ST]), which is also known as the P-loop [38, 40]. An alignment of magnesium chelatase I-subunits shows the conserved P-loop motif characteristic of AAA⁺ proteins (G[DH]RGTGKS) (Figure 1). ATP hydrolysis by this AAA⁺ protein is strongly supported by biochemical and structural investigations [16-19, 25-27]. We performed a careful search for the P-loop signature in a representative set of nine magnesium chelatase D- and H-subunits (Figure 1). Concerning the D-subunit, only the *Heliobacillus mobilis* protein contained the full P-loop motif (GRRGTGKT). An incomplete P-loop motif was found in *Nicotiana tabacum*, *Arabidopsis thaliana*, *Hordeum vulgare* and *Synechocystis* PCC 6803 (G[KR]RGTA[KTS]). *R. capsulatus* and *R. sphaeroides* completely lacked the motif and *Chlorobium tepidum* and *Chlorobium vibrioforme* contain only traces of the motif (GKS). The relative position of motif in the I- and D-subunits is conserved within their AAA⁺ domains. It can be speculated that the semi-conserved motif in the D-subunits is due to a gene duplication event followed by divergence over the course of evolution without selective pressure to maintain the P-loop motif in the D-subunit.

Regarding the H-subunit, no traces of a conserved site with a P-loop signature could be found by analysis of amino acid sequence alignments. However, when analyzing individual polypeptides a perfect motif was found in the polypeptide of *R. capsulatus* (AYGRDGKS) (Figure 1). Of the eight other organisms included in the alignment, *R. sphaeroides* contained a sequence most reminiscent of a P-loop (AYGRSGKP). Only the first Ala residue in the motif is conserved in all eight polypeptides. It is likely that this motif is a semi-conserved artifact between two closely related organisms, since not all proteins that contain the P-loop motif are ATP binding e.g. human ferritin light chain and chymotrypsin. This analysis did not strengthen the argument that ATPase activity is a general feature of the H-subunit, although it could explain ATP hydrolysis by the *R. capsulatus* enzyme.

Light exposure increases the heterogeneity of affinity purified H-subunit

When excited by light, porphyrins generate reactive oxygen species. The magnesium chelatase H-subunit is brownish upon purification due to bound protoporphyrin IX. It has previously been suggested that light induced inactivation of the H-subunit via the bound protoporphyrin could account for the rapid inhibition of bacteriochlorophyll

biosynthesis in *R. capsulatus* during transition from anaerobic photosynthetic growth to aerobic heterotrophic growth [43]. We analyzed the effect of light treatment on the H-subunit *in vitro*. A system for producing *R. capsulatus* H-subunit in *E. coli* has been developed including a one-step affinity purification method [11]. In this study, we produced affinity purified H-subunit in the dark, although some light exposure could not be avoided. Two 40 mg aliquots of this protein were analyzed. One aliquot was kept in the dark and on ice as a control. The other identical sample was also kept on ice, but exposed to ordinary room light of $15 \mu\text{E m}^{-2} \text{s}^{-1}$ from a 58 W fluorescent light tube for 30 minutes. These samples were analyzed by gel filtration and in both cases a peak of high molecular weight was obtained in addition to a peak of lower molecular weight (Figure 2A). Interestingly, light treatment resulted in an increased amount of protein of high molecular weight and a decreased amount of low molecular weight protein. Fractions 9 and 22 corresponded to approximate molecular masses of 400 and 150 kDa, respectively. Fractions 7-12 from the respective analysis were pooled and selected to represent the high molecular weight fraction. Fractions 18-27 were pooled and represented the low molecular weight fraction. In the dark control, the H-subunit dominated both the high and the low molecular weight peak according to SDS-PAGE analyses (Figure 3, lanes 2 and 3). This was also supported by mass spectrometry performed on non-trypsin treated material. The high and low molecular protein fractions gave masses of 132,830 and 132,581 Da, respectively, which was close to the predicted molecular mass of 132,524 Da for the His-tagged *R. capsulatus* H-subunit.

The SDS-PAGE profile of the light treated material was different from that of the dark control. The amount of H-subunit corresponding to the monomeric size was reduced in the high molecular fraction and instead a significant amount of protein was retarded in the stacking gel (Figure 3, lane 4). The observation suggested that the H-subunits form large aggregates upon light exposure. The existence of aggregates could be demonstrated by negative staining electron microscopy (Figure 4). In a parallel study we could easily pick over 5000 single particles that correspond to monomeric H-subunit and a 3D model was produced (unpublished). The H-subunit is approximately 100 Å across. Figure 4 illustrates a region of a micrograph containing a few monomeric H-subunit particles and a large number of particles of up to 400 Å across. In order to further analyze the light dependent formation of aggregates, a low

molecular weight fraction from a dark control was split in two equal samples. One sample was exposed to light as described above, while the other sample was kept in the dark as a control. Upon gel filtration, a new high molecular weight fraction was obtained at the expense of the low molecular weight fraction in the light treated sample, whereas the low molecular weight proteins remained in the dark treated control (Figure 2B). The experiment demonstrated that the protoporphyrin-binding H-subunit is very sensitive to light and that even brief exposure causes novel aggregation of H-subunits.

Separation of magnesium chelatase and ATPase activity

Bioinformatic analyses of the magnesium chelatase subunits call the ATPase activity of the H-subunit in question. However, it is clear that affinity-purified H-subunit is active in magnesium chelatase activity measurements (Figure 5), but also hydrolyzed ATP to ADP (Figure 6, trace 1). The ATPase activity was 0.65 nmol formed ADP per min and mg of protein, which is in the same range of the activity determined for the H-subunit of *R. sphaeroides*, *C. vibrioforme* and *Synechocystis* PCC6803 [17-19]. Further purification of the affinity purified H-subunit by gel filtration chromatography resulted in a high and a low molecular weight fraction (Figure 2A). When assayed for magnesium chelatase activity (Figure 5) and ATPase activity (Figure 6), it was found that the high molecular weight fraction had ATPase activity, but could not contribute to magnesium chelatase activity. In contrast, the low molecular weight peak had no ATPase activity, but contributed to magnesium chelatase activity. Light treatment of the affinity purified H-subunit did not increase the ATPase activity. When protein from the dark treated low molecular weight peak was pooled and reloaded onto the gel filtration column after light or dark treatment, it was evident that light directly induces the formation of the high molecular weight peak (Figure 2B). However, in this case no ATPase activity was detected in the *de novo* high molecular weight peak. Considering these data, it is unlikely that ATP hydrolysis is an intrinsic activity of the H-subunit that is activated by light. Instead, the ATPase activity is more likely to originate from a contaminating *E. coli* protein that is present in the affinity purified preparation of H-subunit. In order to identify such a contaminating ATPase, we separated proteins with SDS-PAGE and employed mass spectrometry of trypsin digested material to identify low abundant proteins, which were seen in the region between 25- and 100-kDa. All bands formed by low abundant proteins on the gel

were identified as fragments of the H-subunit. The fragments were often internal regions of the H-polypeptide rather than C-terminal truncations with a retained His-tag. Therefore, it is likely that fragments were formed after the elution from the affinity column and suggest the presence of proteases. Concerning the contaminating ATPase we conclude that it is present at a very low level in our preparation. We considered the possibility that the H-subunit fragments in the mass spectrometry analysis could mask the contaminating ATPase. Therefore, we performed careful analyses of residual peaks not identified as fragments of the H-subunit. Although it was difficult to obtain good sequence coverage we did identify two peptides from a band of approximately 70-kDa in the high molecular weight fraction that corresponded to regions within the ClpA protein from *E. coli* (accession number P0ABH9). The observed mass peaks were of 1513.82 and 2722.27 Da and match with the internal ClpA polypeptides HPHAVLLLDEIEK (theoretical mass 1513.830 Da) and NEVTGANVLVAIFSEQESQAAYLLR (2722.40 Da). ClpA catalyzes protein unfolding and limited protein remodeling. Together with its proteolytic partner ClpP, it participates in ATP-dependent intracellular protein degradation [44]. Since ClpA is a highly active ATPase and binds to hydrophobic surface patches not normally exposed in native proteins, it is a possible candidate to be the contaminating ATPase in our high molecular weight fractions.

DISCUSSION

Here we present data that strongly question previous reports that the magnesium chelatase H-subunit possesses intrinsic ATPase activity. Instead we suggest that the ATPase activity is attributed to a low abundant contaminating *E. coli* protein that persists during affinity purification of the H-subunit. The one-step affinity purification method employing nickel immobilized on a column to purify His-tagged H-proteins has been used in all studies where ATPase activity of H has been analyzed [17-19, 30]. We suggest that the contaminating ATPase could be a native *E. coli* chaperone involved in refolding or degradation of aggregated protein. A contaminating chaperone, *E. coli* GroEL, has previously been seen during expression and purification of the *R. sphaeroides* D-subunit [9]. However, in contrast to our study the contaminating GroEL-protein dominated the fractions that could contribute to magnesium chelatase activity and the recombinant D-subunit could not be detected.

To us it is clear that the two-stage model for the magnesium chelatase reaction as originally suggested by Walker and Weinstein [7] is better explained without hydrolysis of ATP by the H-subunit. Instead, both the activation step and the catalytic step relate to the use of ATP by the I-subunit. The activation step, first observed by Walker and Weinstein, is now referred to as an oligomerization step involving the I- and the D-subunits. In this process, a hexameric D-ring provides a platform for the stepwise assembly of the I-subunits into a double hexameric ring (Figure 7) (unpublished). This hypothesis is based on EM structural analysis showing that D-subunits can form oligomeric ring structures in the absence of nucleotide and that a two-tiered hexameric ID-complex is formed in the presence of ATP (unpublished). Since this ID-structure has only been characterized at low resolution and it is yet to be determined whether different nucleotides such as ADP and ATP- γ -S result in alternate structures, we cannot rule out the possibility that hydrolyzed ATP is present within the structure. The ability of I-subunit to bind to D-subunit in the absence of ATP hydrolysis has been shown in independent studies. In one experiment, the I-subunit of *Synechocystis* was treated with *N*-ethylmaleimide, which covalently modifies cysteine residues. This treatment resulted in an inactive I-subunit both in respect to ATP hydrolysis and magnesium chelatase activity. However, in the presence of ATP, ADP or a nonhydrolysable ATP analogue the modified I-subunit could still associate with the D-subunit [18]. In another study semidominant barley mutants, deficient in the gene encoding the I-subunit, were characterized at the DNA level and the mutations were introduced into the recombinant system for expression of *R. capsulatus* I-subunit [26]. These modified I-subunits were unable to hydrolyze ATP and were devoid of magnesium chelatase activity. They could still form oligomeric complexes with themselves, with wild type I-protein and with D-protein if ATP or ADP was present [25, 26]. However, the function of the I-subunit as a chaperone of the D-subunit was apparently lost since no D-subunit could be detected in chloroplast extracts of barley I-mutants. This begs the question of when ATP is hydrolyzed in the catalytic cycle, which has so far been narrowed down to the second stage of the magnesium chelatase reaction suggested by Walker and Weinstein [7]. It has been proposed that the D-subunit blocks the ATPase activity of the I-subunit until the ID-complex interacts with a H-protoporphyrin complex [16]. The transient interaction with the H-subunit releases the inhibition triggering ATP hydrolysis as well as insertion of Mg²⁺ into

protoporphyrin. However, in the view of an absence of D-subunit in barley I-mutants it seems that binding of I to D is not enough to maintain D in the cell, but is dependant on the ATPase activity of the I-subunit. Thus, ATP hydrolysis should be important for a step after the formation of the ID-complex, but before interaction with the H-subunit (Figure 7). It should be noted that the D-subunit is stably maintained in barley mutants with deletions of the entire gene encoding the H-subunit [45]. It has been shown for several AAA⁺ proteins that a significant conformational change occurs during the ATP hydrolytic cycle [23, 46-48]. In some cases ATP hydrolysis is required for substrate recognition and in others it is not. If ATP hydrolysis occurs after the holoenzyme is formed then a conformational change can be expected to provide the mechanical force that probably remodels the H-subunit and drives the magnesium chelatase reaction. Alternatively, if ATP hydrolysis occurs prior to the formation of the holoenzyme then a conformational change in the I-hexamer would be expected to expose essential amino acid residues in the ID-complex that are only then recognized by the H-subunit resulting in magnesium chelation. This kind of mechanism is found in the phage shock protein F, which is an AAA⁺ protein that regulates transcription in response to stress [49]. This chaperone binds and hydrolyses NTP in order to adopt an alternate conformation so as to expose inaccessible motifs required to interact with its target α^{54} and form the holoenzyme. Future structural studies of the magnesium chelatase involving various ATP analogues will aid in clarifying the role and timing of ATP hydrolysis in the first step of chlorophyll biosynthesis.

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FIGURE LEGENDS

Figure 1

Alignments showing the best candidates for a P-loop motif ([AG]XXXXGK[ST]) in the three polypeptides of magnesium chelatase. The P-loop motif is conserved in the I-subunit sequences. The motif is also found in the individual polypeptides of *Heliobacillus mobilis* D-subunit and the *Rhodobacter capsulatus* H-subunit. The analysis suggests that it is unlikely that ATPase activity is a general feature of the D- and H-subunits. Only 15 amino acid residues are shown from an alignment of the entire polypeptides. Sources: *R. capsulatus* [Genbank accession number Z11165], *Rhodobacter sphaeroides* [AF017642, AJ010302], *Chlorobium tepidum* [AY005135], *Chlorobium vibrioforme* [Z83933], *Nicotiana tabacum* [AF014052, AF014053, Y10022], *Arabidopsis thaliana* [AF083555, X91411, Z68495], *Hordeum vulgare* [AY039003, DQ125942, DQ529207], *Synechocystis* PCC 6803 [13], *H. mobilis* [AF080002].

Figure 2

Gel filtration of affinity purified H-subunit. Elution of protein was followed by absorbance at 280 nm. The column was calibrated with apoferritin (443 kDa, peak at fraction 9), β -amylase (200 kDa, fraction 18), alcohol dehydrogenase (150 kDa, fraction 22) and bovine serum albumin (66 kDa, fraction 29). **A.** Affinity purified H-subunit (40 mg) was exposed to light before loading on a Sephacryl S-200 HR 16/60 column (dashed line). An equal sample, kept in darkness, was used as a control (solid line). The first (fractions 7-12) and second (fractions 18-27) peak of each analysis were selected to represent the high and low molecular weight fraction, respectively, and used in further analysis. **B.** Pooled and concentrated fractions from low molecular weight H-subunit obtained by gel filtration was exposed to light and a second round of gel filtration was performed (dashed line). An identical sample was kept in the dark as a control (solid line). The experiment shows that light causes *de novo* formation of a high molecular weight fraction.

Figure 3

Coomassie stained SDS-polyacrylamide gel of gel filtration fractions. Fractions from a gel filtration experiment with light treated and dark treated His-tagged *R. capsulatus* H-subunit were analyzed on a SDS-PAGE. Ten μg of protein was loaded. Lane 1, size marker; lane 2, pooled fractions 7-12 of the dark treated sample in Figure 2A; lane 3, pooled fractions 18-27 of the dark treated sample; lane 4, pooled fractions 7-12 of the light exposed sample; lane 5, pooled fractions 18-27 of the light exposed sample. The solid and dashed arrows indicate His-tagged H-subunit and high molecular weight aggregates retarded in the stacking gel, respectively.

Figure 4

Negative staining electron micrograph of BchH. The micrograph shows a section containing both aggregated BchH particles (examples marked with white boxes) and monomeric sized BchH particles (black boxes). A 50 nm scale bar is included.

Figure 5

Magnesium chelatase activity of gel filtration fractions of light and dark exposed His-tagged H-subunit. The amount of formed Mg-protoporphyrin IX was analyzed with spectrofluorometry using an excitation wavelength of 418 nm. Mg-protoporphyrin IX forms a peak at 590 nm. Light and dark treated H-subunit from pooled fractions 7-12 and 18-27 in Figure 2A were used in the analysis.

Figure 6

ATPase activity of gel filtration fractions as analyzed with thin-layer chromatography and visualized with autoradiography. Light and dark exposed His-tagged H-subunit fractionated with gel filtration were tested for their ability to hydrolyze ATP into ADP. After 2 h the assay were stopped and the nucleotides were separated with thin-layer chromatography. Lane 1, affinity purified His-tagged H-subunit (activity $0.65 \text{ nmol formed ADP min}^{-1} \text{ mg}^{-1}$ of protein); lane 2, pooled fractions 7-12 of the dark treated sample in Figure 2A ($0.61 \text{ nmol min}^{-1} \text{ mg}^{-1}$); lane 3, pooled fractions 18-27 of the dark treated sample ($<0.05 \text{ nmol min}^{-1} \text{ mg}^{-1}$); lane 4, pooled fractions 7-12 of the light exposed sample ($0.30 \text{ nmol min}^{-1} \text{ mg}^{-1}$); lane 5, pooled fractions 18-27 of the light exposed sample ($<0.05 \text{ nmol min}^{-1} \text{ mg}^{-1}$); lane 6, negative control with no protein ($<0.05 \text{ nmol min}^{-1} \text{ mg}^{-1}$).

Figure 7

Model suggesting progressive subunit assembly of magnesium chelatase in relation to ATP hydrolysis. ① Six D-subunits form a stable hexameric ring in an ATP independent process. ② Using the hexameric D-ring structure as a platform, six I-subunits are assembled stepwise into a hexameric ring. The formation of the two-tiered complex is nucleotide dependant. ③ Two alternative models are shown, which differ in respect to the timing of ATP hydrolysis. In one model the catalytic H-subunit binds to an ID-complex where the ATP hydrolytic capacity of the I-subunit is blocked by the D-subunit. ④ The transient interaction with the H-subunit triggers ATP hydrolysis and insertion of Mg^{2+} into protoporphyrin. ⑤ In the alternative model, ATP hydrolysis by the I-subunits, converts the D-subunits into a stable form and possibly exposes key residues required for the recognition of H-subunits. ⑥ Without the ATP dependent activity of the I-subunits the D-subunits are degraded. ⑦ The catalytic H-subunit interacts with the activated ID-complex and ⑧ Mg^{2+} is inserted into protoporphyrin without ATP hydrolysis at this step. ⑨ After catalysis the IDH-complex disassociates to the level of the D-hexamer, which remains for a new round of catalysis. At present, it is unknown whether the H-subunit docks to the I- or D-side of the complex, although docking to the I-subunits is shown in the figure.

Figure 1

I-subunits

N.tob	MIMGDRGTGKSTTVR	132
H.vul	MIMGDRGTGKSTTVR	36
A.tha	MIMGDRGTGKSTTVR	129
Syn	MIMGDRGTGKSTTIR	58
H.mob	MIMGDRGTGKSTTVR	63
R.sph	LVEGDRGTGKSTAVR	47
R.cap	LVEGDRGTGKSTAVR	63
C.tep	LVMGHRGTGKSTTVR	91
C.vib	LVMGHRGTGKSTTVR	45

D-subunits

N.tob	AICGKRGTAKTLMAR	122
H.vul	AISGKRGTAKTVMAR	122
A.tha	AISGRRGTAKTVMAR	12
Syn	VIAGRRGTAKSVMAR	54
H.mob	AIAGRRGTGKTVLAR	48
R.sph	WLRARASALRDRITG	41
R.cap	WLRSRAGPIRLAFTD	38
C.tep	VIPSTVGSGLKTLAR	44
C.vib	VIPSTVGSGLKTLAR	44

H-subunits

N.tob	KSEAFDCDAPGVGMT	1192
H.vul	KSEAFDSDAPGVGML	1191
A.tha	KSEAFDSDAPGAGMA	1191
Syn	KSEAFNSDNPGM-MD	1142
H.mob	KGFAYGKQVRGEVAD	1103
R.cap	KSEAYGRDGKSTKQV	1005
R.sph	KSEAYGRSGKPVQNA	1005
C.tep	KSEAFSPEGDWRESP	1079
C.vib	NSSAYGCGRKGKQ	1088

Figure 2

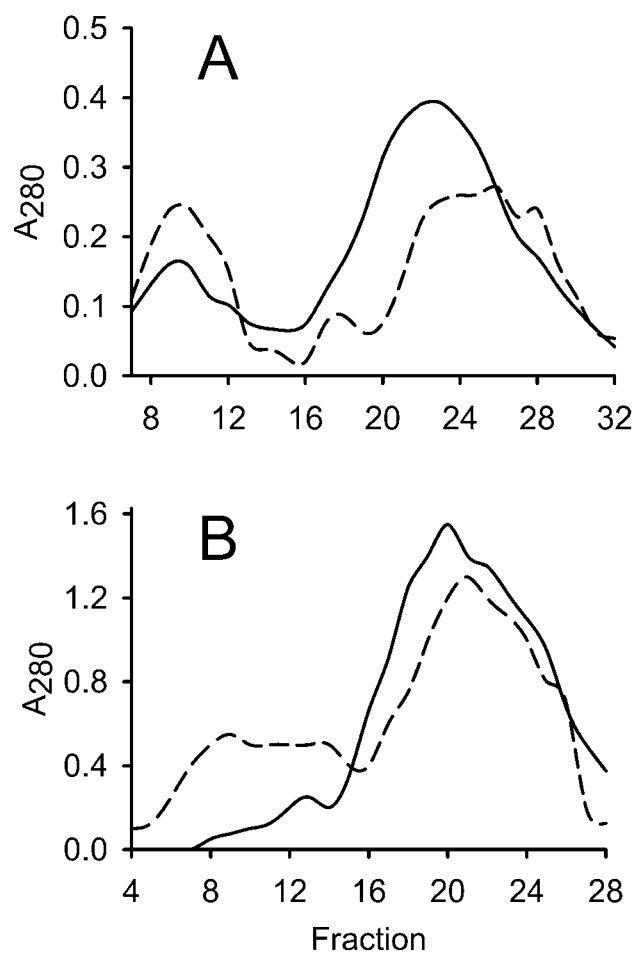


Figure 3

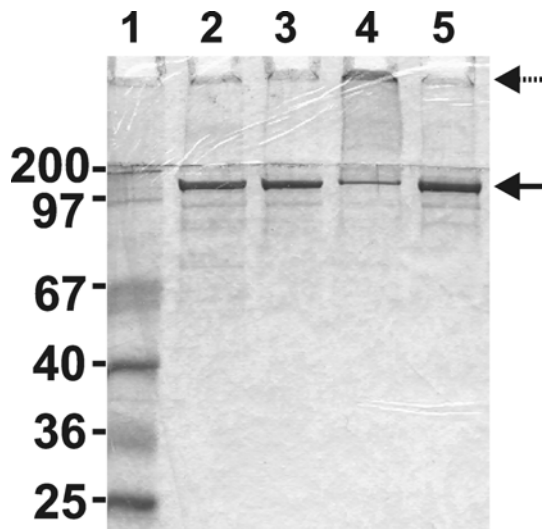


Figure 4

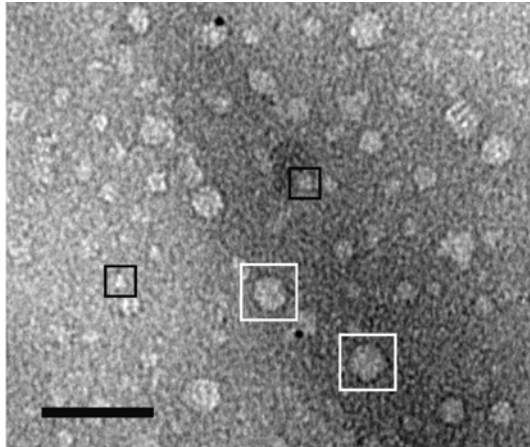


Figure 5

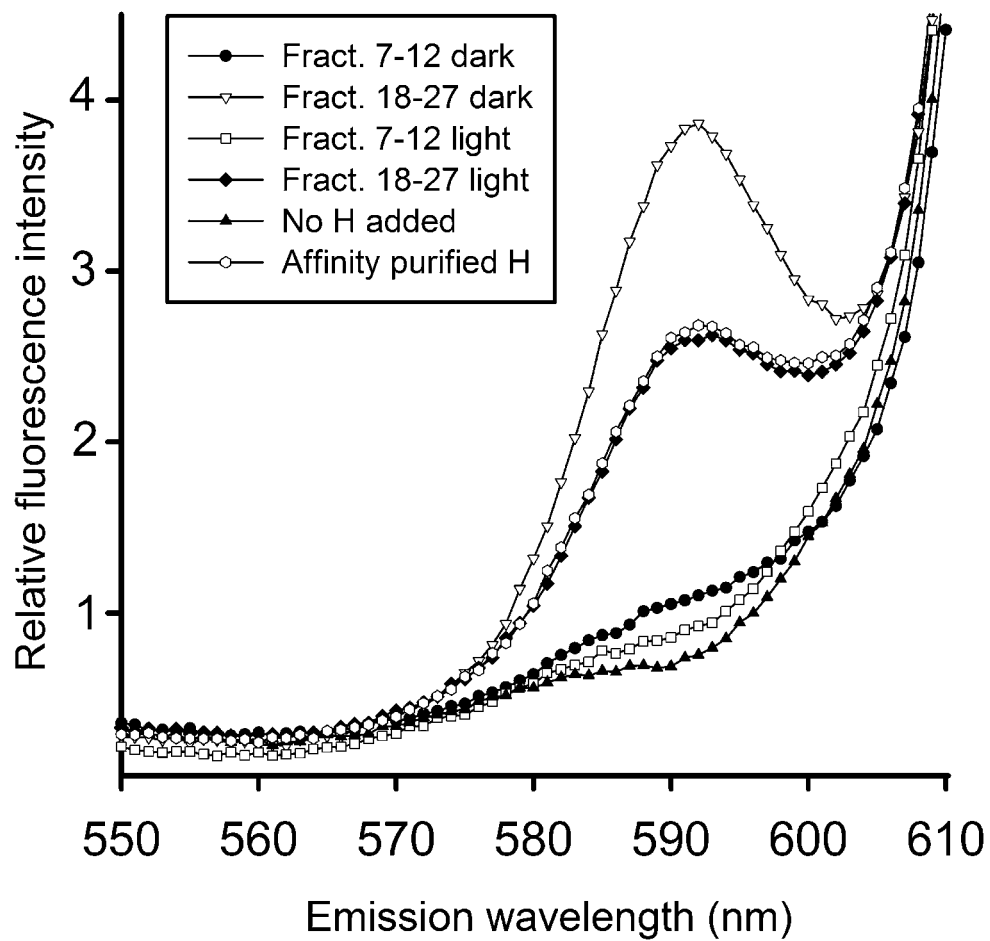


Figure 6

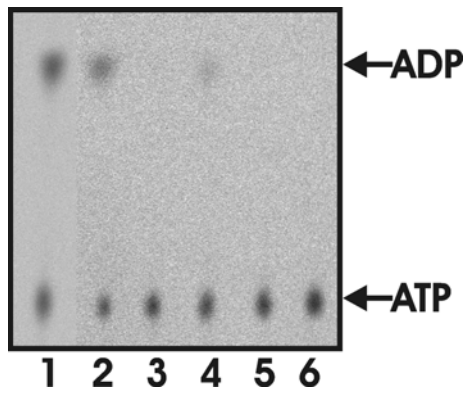


Figure 7

