Catalytic Turnover Triggers Exchange of Subunits of the Magnesium Chelatase AAA+ Motor Unit*

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Background: Magnesium chelatase is an AAA+ protein complex involved in chlorophyll biosynthesis.

Results: An exchange of subunits occurs during the catalytic cycle.

Conclusion: Dissociation of the complex may be part of the reaction mechanism.

Significance: Deciphering of the mechanism of AAA+ protein complexes is crucial for our understanding of the catalytic cycle of a common class of molecular machines.

The ATP-dependent insertion of Mg\(^{2+}\) into protoporphyrin IX is the first committed step in the chlorophyll biosynthetic pathway. The reaction is catalyzed by magnesium chelatase, which consists of three gene products: BchI, BchD, and BchH. The Bchl and BchD subunits belong to the family of AAA+ proteins (ATPases associated with various cellular activities) and form a two-ring complex with six Bchl subunits in one layer and six BchD subunits in the other layer. This BchID complex is a two-layered trimer of dimers with the ATP binding site located at the interface between two neighboring BchI subunits. ATP hydrolysis by the BchID motor unit fuels the insertion of Mg\(^{2+}\) into the porphyrin by the BchI subunit. In the present study, we explored mutations that were originally identified in semidominant barley (Hordeum vulgare L.) mutants. The resulting recombinant Bchl proteins have marginal ATPase activity and cannot contribute to magnesium chelatase activity although they apparently form structurally correct complexes with BchD. Mixing experiments with modified and wild-type Bchl in various combinations showed that an exchange of Bchl subunits in magnesium chelatase occurs during the catalytic cycle, which indicates that dissociation of the complex may be part of the reaction mechanism related to product release. Mixing experiments also showed that more than three functional interfaces in the Bchl ring structure are required for magnesium chelatase activity.

Magnesium chelatase is the multisubunit protein complex that conducts the enzymatic catalysis in the first specific step of chlorophyll and bacteriochlorophyll biosynthesis. In this reaction, which requires ATP hydrolysis, Mg\(^{2+}\) is inserted into protoporphyrin IX to form Mg-protoporphyrin IX. Magnesium chelatase requires the presence of three gene products, BchH, BchD, and BchI (often referred to as the H, D, and I subunits), which have molecular masses of 140, 70, and 40 kDa, respectively (1–4). The H subunit has been shown to bind the porphyrin substrate with high affinity (3–7) and has also been predicted to bind the magnesium ion substrate, by analogy with the homologous CobN, the cobalt-binding subunit of cobalt chelatase (8). Based on these observations, the H subunit has been suggested to be the catalytic component of the enzyme (3–5).

Bchl and BchD belong to the structurally conserved family of AAA+ proteins (ATPases associated with various cellular activities). They form a two-ring structure with six I subunits in one layer and six D subunits in the other layer. Members of this family are known to act as molecular machines that remodel their various target molecules in an ATP-dependent manner (9). In the case of magnesium chelatase, the ID motor unit has the H subunit as a target protein. The I subunit, which contains the characteristic ATP-binding Walker A and Walker B motifs, is the only ATPase-active component of magnesium chelatase (10–14). The D subunit has an AAA+ module at its N terminus with distinct homology to Bchl (10, 15). However, the Walker A and Walker B motifs, which are necessary for ATP hydrolysis, are poorly conserved in the D subunit. Despite this, BchD is still capable of forming oligomeric ring structures, even in the absence of ATP, and has been suggested to function as a platform for the assembly of the ID complex (16). The ID complex has been studied by cryoelectron microscopy (15, 17). The complex has a 3-fold symmetry built up from a trimer of homodimers in each ring. Different structures were observed in the presence of ADP, ATP, or a nonhydrolyzable ATP analog AMPPNP, which probably reflect large conformational changes of the complex during the ATP hydrolytic cycle. The Bchl ring has three intact ATPase sites within the homodimers when it is in complex with BchD. It is not known how ATP hydrolysis between the subunits is coordinated with functional output. Various mechanistic models have been proposed for other AAA+ protein family members, including concerted (18, 19), sequential or semisequential (20–22), probabilistic (22), and partly stochastic models (23). We have previously used barley (Hordeum vulgare L.) mutants to study the cooperativity of...

* This work was supported by the Danish Council for Independent Research and the Carlsberg Foundation.

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‡ The abbreviations used are: AAA+, ATPases associated with various cellular activities; AMPPNP, adenosine 5’-(3’-thio)triphosphate; ATPγS, adenosine 5’-3’-(thio)triphosphate; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.

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the I subunits (24, 25). The mutant lines Xantha-h.clo161, Xan-
th-a.h.clo125 and Xantha-h.clo157 have missense mutations in
the barley gene orthologous to Rhodobacter capsulatus bchI
and cause the single amino acid exchanges L111F, D207N, and
R289K, respectively (25). Recombinant R. capsulatus BchI pro-
teins carrying the amino acid exchanges have a trace of ATPase
activity but cannot contribute to magnesium chelatase activity.
Magnesium chelatase assays were also performed with mix-
tures of wild-type BchI and BchI carrying any of the three
amino acid exchanges. All mixed assays showed a reduced mag-
nesium chelatase activity compared with assays containing only
wild-type BchI and BchI carrying any of the three
amino acid exchanges. The three residues are affected by the semidominant mutations Xantha-h.clo125 (D207N), Xantha-h.clo157 (R289K), and Xantha-h.clo161 (L111F). The long pre-
sensor II helix insertion (blue), which is unique to magnesium chelatase BchI compared with other AAA proteins, determines the unusual position of the C-terminal helical domain (red). Light red and blue have been used in the left
subunit, and darker corresponding colors have been used in the right subunit. The distance between Leu-111 and Asp-207 is ~12 Å, and that between Leu-
111 and Arg-289 is ~16 Å.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Purification of BchI Proteins—
An expression system of R. capsulatus bchI was previously con-
structed in pET3a and not pET15b because an N-terminal His
tag added to R. capsulatus BchI resulted in an inactive protein
(3). To obtain a one-step purification system, we cloned R. cap-
sulatus bchI in the pET23b expression vector (Novagen), result-
ing in a recombinant BchI with a C-terminal His tag. The plas-
mid obtained was verified by DNA sequencing and named pET23bBchI. Plasmid pET23bBchI was used as template in site-directed mutagenesis (the QuikChange method; Strat-
agene) to construct four different BchI proteins with the mod-
ifications L111F, V113G, D207N, and R289K. The resulting plasmids were named pET23bBchI-L111F, pET23bBchI-
V113G, pET23bBchI-D207N, and pET23bBchI-R289K. The following primers were used to introduce the mutations:
L111Fu (5’-CGCGGCTCCTTGCGT(CG)GACGCTTCC-3’),
L111Fl (5’-CGGACACGCGCAaCGGCAGATCAGCAG-3’),
V113Gu (5’-GCTGCGCTCTCGGCCGAGCGC-3’),
V113Gl (5’-CGGGGTCTCCAGCACGCGGCCGA-3’),
D207Nu (5’-GCGCGACACGCGTGAAGCTGC-
GGCCG-3’),
D207Nl (5’-GACGGAAACGTTrCAAGAGCTGCG-
GGCCG-3’),
R289Ku (5’-GCTTCGAGCTGTTgaaGCGGAG-
GCTGACGC-3’),
R289Kl (5’-GTCAGCTCGCCGAAAaCGC-
GTCGGAACGCC-3’).
The three
mutations described, were expressed in Escherichia coli
BL21pLys. The cells were grown at room temperature, and iso-
propyl β-d-thiogalactopyranoside (final concentration 0.5
mM) was added at an A600 of 0.4. The following day, the cells
from 1 liter of culture were harvested by centrifugation at
20,000 × g for 15 min at 4 °C and resuspended in 100 ml of
binding buffer (20 mM imidazole, 20 mM sodium phosphate, pH
7.4, 500 mM NaCl) prepared from stock solutions (His Buffer
Kit; GE Healthcare). The cells were lysed with a cell disrupter
(Constant Cell disruption system, Buch & Holm) and aliquoted
into eight 12.5-ml portions, one of which was used for purific-
ation each time. The protein was loaded onto a HisTrap FF
crude column (GE Healthcare) and washed according to the
manual using a peristaltic pump at 1 ml/min (Ole Dich,
Hvidovre, Denmark). Elution, fractionation, and recording were
performed using an ÄKTA FPLC system (GE Healthcare) with a
20-ml isocratic gradient to 100% elution buffer (500 mM imid-
azole, 20 mM sodium phosphate, pH 7.4, 500 mM NaCl).
Protein purification fractions were desalted on NAP-5 columns
(GE Healthcare) into 50 mM Tricine-NaOH, pH 8.5, 15 mM Mg-
Cl2, 5 mM DTT, and 6% glycerol. Protein concentration was
determined using Bradford reagent (Bio-Rad) according to the
manufacturer’s instructions. SDS-PAGE (NuPage 4–12%;Invi-
rogen) was performed according to the manufacturer’s instruc-
tions, with PageBlue Protein staining solution (Fermentas).

Continuous Magnesium Chelatase and ATP Hydrolysis
Assays—Assays were performed in 300-μl volumes. The protein
content was 14 μg of BchH, 3.2 μg of BchD, and 1.4 μg of
BchI. The assays also contained 2 mM ATP, 4 μM deuteropor-
phyrin IX (preincubated with BchH), 15 mM MgCl2, 5 mM DTT,
and 50 mM Tricine-NaOH, pH 8.5. For measurement of ATP
hydrolysis, the reaction also included MESG (2-amino-6-mer-
capto-7-methylpurine riboside) and purine nucleoside phos-
phorylase at concentrations recommended by the manufac-

FIGURE 1. Location of amino acid residues Asp-207 (magenta), Arg-289 (green), and Leu-111 (cyan) in the ATP binding site (with ATP molecule in yellow) at the interface between two neighboring I subunits. The three residues are affected by the semidominant mutations Xantha-h.clo125 (D207N), Xantha-h.clo157 (R289K), and Xantha-h.clo161 (L111F). The long pre-
sensor II helix insertion (blue), which is unique to magnesium chelatase BchI compared with other AAA proteins, determines the unusual position of the C-terminal helical domain (red). Light red and blue have been used in the left
subunit, and darker corresponding colors have been used in the right subunit.
turer (EnzChek Phosphate Assay Kit; Molecular Probes). The two assay systems had no negative effect on each other. All reactions were performed in the dark with a Tecan robot system (Tecan Group) connected to a Magellan M1000 plate reader (Tecan Group) for continuous spectroscopic analysis. Samples were mixed in black 96-well plates with transparent bottoms (BrandTech Scientific). Data points for magnesium chelatase activity were collected every 4th min with top-measured fluorescence (excitation wavelength 408 nm, slit 5 nm, emission wavelength 582 nm, slit 10 nm). ATPase activity was measured simultaneously as an increase in absorbance at 360 nm. The plate reader was adjusted to 22 °C, and the plate was shaken between measurements. The plate was removed from the reader at t = 72 min for the addition of extra BchI subunits. Buffer solution was added to control samples to compensate for dilution effects.

**Electron Microscopy**—Electron microscopy was done as described previously (7). 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 8.5, 15 mM MgCl₂, 2 mM ATP, 1 mM DTT. Excess liquid was removed by brief contact with filter paper. Protein samples were diluted to 0.01 mg/ml with the wash buffer. A drop of the protein solution was applied to the grid for 1 min, and excess sample was removed. The grid was immediately stained with filtered 2% (w/v) uranyl formate for 30 s, and excess stain was removed. Electron microscopy was performed using a 120-kV Philips CM-10 microscope at 55,000× magnification, giving a sampling size corresponding 4.67 Å/pixel.

**Barley Mutants and Quantification of Chlorophyll**—Green plants of segregating barley (*H. vulgare*) (*L*) mutants *Xantha-h.clo161*, *Xantha-h.clo125*, and *Xantha-h.clo157* were crossed with each other in all possible combinations. Yellow seedlings that were confirmed by DNA sequencing. To determine the total amount of chlorophyll, 3 cm of a barley leaf (corresponds to 20–30 mg) was cut, weighed, and ground in liquid nitrogen. One ml of methanol was added, followed by mixing on a vortex mixer. The extract was left for 1 h in the dark and then centrifuged for 5 min to remove debris. Spectra were recorded between 500 and 800 nm (slit width 10 nm) using methanol as blank. The peaks at 646, 663, and 750 nm were recorded. The total amount of chlorophyll (chlorophyll *a* and chlorophyll *b* in μg/ml) was calculated according to the formula (17.76 × (A₆₄₆ – A₇₅₀) + 7.34 × (A₆₆₆ – A₇₃₀)) × Vtotal/Vsample and normalized to the amount of leaf material used (26).

**RESULTS**

**Mutant-derived Bchl and Wild-type BchD Form a Complex**—*R. capsulatus* Bchl proteins with modifications L111F, D207N, and R289K are all affected in the ATP binding site (25). To obtain a Bchl protein with a modification outside the ATP binding site, we changed Val-113 to a glycine by site-directed mutagenesis. Val-113 is homologous to Val-92 in the heat shock protein HslU (27). The residue is located in the pore region of the respective AAA+ complex. In HslU, the V92G modified protein has ~80% ATPase activity and can form hexamers but is deficient in substrate recognition (27).

It was previously established that Bchl proteins with the modifications L111F, D207N, and R289K are able to interact with wild-type BchI (25). Using immobilized BchD, it was further shown that the modified BchI subunits can associate with BchD (12). In both experiments, the interactions occurred in presence of ATP or ADP but not when AMP was added. To confirm that a structurally comparable ID complex is obtained with modified Bchl proteins, Bchl-L111F was incubated together with wild-type BchD in the presence of ATP and stained with uranyl formate on a copper grid suitable for electron microscopic analysis. Images revealed clear and defined ring-shaped particles with a diameter of ~130 Å (Fig. 2), which is the expected size of an ID complex (15). Thus, Bchl-L111F forms a complex with BchD that is similar to that of wild-type Bchl in combination with BchD. It should therefore be possible to use Bchl-L111F for conclusive analyses concerning the stability and intactness of the magnesium chelatase complex during the catalytic cycle. Because Bchl-D207N and Bchl-R289K had behavior similar to that of Bchl-L111F in our previous experiments (12, 25), and from the ability of HslU-V92G to form hexamers (27), we assume that Bchl with the modifications D207N, R289K, and V113G would also be suitable for these kinds of studies.

**Internal Regulation of ATPase Activity**—ATP hydrolysis plays a central role in the magnesium chelatase reaction. In the present study, we developed a continuous assay where we could simultaneously measure ATP hydrolysis and the formation of
Mg-deuteroporphyrin IX. Magnesium chelatase activity is directly correlated with ATPase activity, and no Mg-deuteroporphyrin IX is formed without ATP hydrolysis (Fig. 3). In contrast, ATP hydrolysis can occur at a low but detectable rate without formation of Mg-deuteroporphyrin IX, but it is considerably higher during magnesium chelation (Fig. 3). Incubation of 3.2 μg (54 pmol) of BchD with 1.4 μg (37 pmol) of BchI and 2 mM ATP allowed ATP hydrolysis at a rate of 10 pmol of P_i/min, which is approximately 6-fold less than with BchI alone (58 pmol of P_i/min) under equal conditions (Fig. 4). Addition of 15 μg (116 pmol) of BchH preincubated with deuteroporphyrin IX to the solution initiated the formation of Mg-deuteroporphyrin IX (1.14 pmol of Mg-deuteroporphyrin IX formed per min) and instantly caused a 20-fold increase in ATP hydrolysis (200 pmol of P_i/min) (Fig. 4A). We conclude that the highest ATPase activity is observed when the enzyme is catalyzing the formation of Mg-porphyrin and the lowest ATPase activity is seen when only BchD is mixed with BchH. Similar observations have previously been reported from studies with Synechocystis magnesiu m chelatase (11).

Combining Inactive and Active BchI Subunits in Vivo—BchI-L111F, BchI-D207N, and BchI-R289K are totally deficient even though the ATPase activity of all four proteins is of similar magnitude. The three modifications L111F, D207N, and R289K probably destabilize the conformational changes that are necessary to maintain the chelatase catalytic cycle.

Magnesium chelatase assays containing a mixture of BchI-V113G and wild-type BchI had the effect of stimulating the activity (1.47 pmol of Mg-deuteroporphyrin IX/min) (Fig. 4E); that is, the activity was higher than in magnesium chelatase assays where BchI-V113G was excluded (1.14 pmol of Mg-deuteroporphyrin IX/min, Fig. 4A). This is the first time that we have observed that an ATPase-deficient BchI subunit can stimulate magnesium chelatase activity in an assay in which it is combined with wild-type BchI subunits. BchI-V113G will be studied further to learn about allosteric modifications in the ID complex during the catalytic cycle of magnesium chelatase.

Mixing Experiments with Inactive BchI Subunits—Structural analyses have shown that the Bchl ring is formed by six subunits in a trimer of dimers (15). The x-ray structure of BchI shows that residue Leu-111 is located on one side of the wedge-shaped BchI structure and that Asp-207 and Arg-289 are located on the opposite side. However, in a dimer they all share the same ATP binding pocket (Fig. 1). In a subunit-mixing experiment involving BchI-L111F and BchI-D207N or a mixture of BchI-L111F and BchI-R289K, it was possible to form ATP binding sites with no modified residues in the interface between BchI subunits (Fig. 5). However, none of the resulting trimers of dimers contained more than three wild-type subunit interfaces (Fig. 5, E and F). We performed the mixing experiments to determine whether BchI complexes with only a few wild-type interfaces could contribute to magnesium chelatase activity. However, BchI-L111F mixed with BchI-D207N or BchI-R289K could not contribute to magnesium chelatase activity in vitro (Fig. 4, F–H).

Combining Inactive and Active BchI Subunits in Vivo—To detect possible subtle magnesium chelatase activity, we also performed the mixing experiment in vivo. Heterozygous plants of barley magnesium chelatase mutant Xantha-h.clo161 (carrying modification L111F) were crossed with heterozygous lines of Xantha-h.clo125 (D207N) and Xantha-h.clo157 (R289K). The genotype of the resulting F1 plants was confirmed by DNA sequencing of individual seedlings. The cross Xantha-h.clo161 × Xantha-h.clo125 resulted in segregation of the F1 generation into dark green wild-type plants, light green heterozygous plants with the genotypes WT/Xantha-h.clo161 or WT/Xantha-h.clo125, and yellow plants with the genotype Xantha-h.clo161/Xantha-h.clo125. Correspondingly, the cross Xantha-h.clo161 × Xantha-h.clo157 resulted in segregation of the F1 generation into dark green wild-type plants, light green heterozygous plants with the genotypes WT/Xantha-h.clo161 or WT/Xantha-h.clo157, and yellow plants with the genotype Xantha-h.clo161/Xantha-h.clo157. The chlorophyll content was analyzed in leaves that had been grown for 7 days. The wild type contained 1.6 mg of chlorophyll/g of fresh leaf weight (Fig. 6). The pale green heterozygote leaves of WT/Xantha-h.clo161, WT/Xantha-h.clo125, and WT/Xantha-h.clo157 contained...
0.59, 0.62, and 0.58 mg/g, respectively, which was 36, 38, and 36% of the wild-type level. The amount of chlorophyll in the mutants \textit{Xantha-h.clo161/Xantha-h.clo125} and \textit{Xantha-h.clo161/Xantha-h.clo157} was less than the detection limit of 1 μg/g. Thus, the mutation in \textit{Xantha-h.clo161} could not be functionally complemented by the mutations in \textit{Xantha-h.clo125} or \textit{Xantha-h.clo157}. The result of the \textit{in vivo} experiment is congruent with the \textit{in vitro} data, in that more than three wild-type subunit interfaces in the ID complexes were required for magnesium chelatase activity.

Catalytic Turnover Triggers Subunit Exchange—Dissociation of the proteins making up the ring structure has been suggested as a possible mechanism of AAA+ proteins releasing their product after catalysis (28). Magnesium chelatase is unusual in the sense that Mg-protoporphyrin IX rather than a protein is the ultimate product of the AAA+ activity, although the BchH...
min after addition of wild-type Bchl (Fig. 7B). Assays initiated with Bchl-V113G, which had 18 and 23% of the wild-type ATPase- and chelatase activity, respectively, were stimulated 31 min after addition of wild-type Bchl. This was ~47 min less than the time required to activate the assays initiated with Bchl-L111F, Bchl-D207N, and Bchl-R289K. It should also be noted that the assay started with Bchl-V113G was stimulated by addition of wild-type Bchl 9 min later than the wild-type Bchl assay was inhibited by addition of Bchl-L111F, Bchl-D207N, or Bchl-R289K. Our results demonstrate that there is an exchange of Bchl subunits. Furthermore, the exchange is more pronounced in active assays, which suggests that dissociation and reassembly of the Bchl ring is part of the catalytic cycle.

**DISCUSSION**

In light of our present knowledge of the structure and function of magnesium chelatase, the enzyme should be understood as an AAA + system with a dynamic ATPase motor unit (the ID complex) and an AAA +-specific substrate (the H subunit). In general terms, the energetically unfavorable reaction of insertion of Mg2+ into protoporphyrin IX by the H subunit is fueled by ATP hydrolysis performed by the ID complex. The process also involves considerable conformational changes within the ID complex (15) as well as within the H subunit (7). The present study supports earlier observations that the I subunit has ATP hydrolytic activity that exceeds the ATP hydrolytic activity of subunit is the target of the BchID complex. Nevertheless, it is essential to understand the stability of the AAA + complex during the catalytic cycles to understand the reaction mechanism of magnesium chelatase. Because ATPase-deficient Bchl subunits form a complex with wild-type Bchl and interfere with the activity, they could be used to monitor the stability of the magnesium chelatase AAA + complex. Therefore, we added ATPase-deficient Bchl subunits and wild-type Bchl at different stages to magnesium chelatase reaction assays that were already running. All assays contained fixed amounts of wild-type BchD and wild-type BchH subunits. The assays also contained either wild-type Bchl, Bchl-L111F, Bchl-D207N, Bchl-R289K, or Bchl-V113G in the same stoichiometric amounts. The assays were started and continuously monitored for Mg-deuteroporphyrin IX production (Fig. 7) and ATP hydrolysis (data not shown). After 72 min, additional wild-type or modified Bchl proteins were added, and the reactions were monitored further for 178 min. The reactions were run at 22 °C, which permitted a slow reaction rate, giving time for addition of Bchl subunits. Reactions initiated with wild-type Bchl were slowed down by the addition of modified Bchl subunits. Bchl-L111F, Bchl-D207N, and Bchl-R289K showed an inhibitory effect 22–23 min after addition (Fig. 7A). In the opposite situations, in which the assays were initiated with Bchl-L111F, Bchl-D207N, or Bchl-R289K, the stimulatory effect was first observed 74–78 min after addition of wild-type Bchl (Fig. 7B). Assays initiated with Bchl-V113G, which had 18 and 23% of the wild-type ATPase- and chelatase activity, respectively, were stimulated 31 min after addition of wild-type Bchl. This was ~47 min less than the time required to activate the assays initiated with Bchl-L111F, Bchl-D207N, and Bchl-R289K. It should also be noted that the assay started with Bchl-V113G was stimulated by addition of wild-type Bchl 9 min later than the wild-type Bchl assay was inhibited by addition of Bchl-L111F, Bchl-D207N, or Bchl-R289K. Our results demonstrate that there is an exchange of Bchl subunits. Furthermore, the exchange is more pronounced in active assays, which suggests that dissociation and reassembly of the Bchl ring is part of the catalytic cycle.
Subunit Exchange in an AAA+ Motor Unit

![Graph A](image1)

**Graph A**

**Graph B**

The ID complex (11). Addition of H subunit triggers ATP hydrolysis and enables enzymatic production of Mg-porphyrin. It is likely that BchD causes an allosteric inhibition of the intrinsic ATPase activity of BchI, which is released when BchH binds to the complex and the reaction starts. Thus, ATP hydrolysis is controlled internally throughout the catalytic pathway. The ID complex is unusual because it consists of a trimer of dimers instead of a hexamer, as found in most other AAA+ proteins. In experiments involving mixing of inactive BchI subunits, we found that a mixture of BchI-L111F and BchI-D207N or a mixture of BchI-L111F and BchI-R289K could not contribute to magnesium chelatase activity, although 92% of the resulting complexes contained at least one BchI-BchI interface with no modified amino acid residue. The mixing experiment showed that an exchange of BchI subunits occurs in the magnesium chelatase complex. Furthermore, the exchange is faster if the reaction has a high turnover. The second observation was strengthened by the partially active BchI-V113G subunit responding faster than BchI-L111F, BchI-D207N, or BchI-R289K to an addition of wild-type BchI. Although the dissociation of magnesium chelatase complexes involving BchI-L111F, BchI-D207N, and BchI-R289K is slow, it still occurs because enzymatic activity could be seen 74–78 min after addition of wild-type BchI subunits. This time possibly reflects a basic instability of the complex. It also remains to be investigated whether the 5–11% residual ATPase activity seen in BchI-L111F, BchI-D207N, and BchI-R289K is important for the dissociation. Singh et al. (29) reported that dissociation of ClpA and ClpAP is faster in the presence of Mg-porphyrin, which again suggests that dissociation in AAA+ complexes is connected to catalytic turnover. The frequency of dissociation might vary considerably between different AAA+ protein complexes. In the case of ClpA and ClpAP, it was estimated that the dissociation occurs in <1 in 100 cycles (29). In contrast, hexamers of ClpB, the closest relative of ClpA in *E. coli*, show fast dissociation as an intrinsic part of the product release mechanism (28). It should be noted that the ring consisting of six magnesium chelatase D subunits is very stable, and no exchange of D subunits could be detected in an ongoing magnesium chelatase reaction, which indicates that the D ring does not dissociate (16). Instead, the D ring was suggested to function as a platform for the reassembly of the ID double ring structure after dissociation of I subunits (16). The formation of the ID complex might be the so-called complexes, which may reflect the diversity of the AAA+ protein family. AAA+ complexes of ClpAP proteins remained associated during hundreds of rounds of ATP hydrolysis (29). In contrast, the AAA+ chaperone ClpB exchanged subunits fast on a time scale from seconds to minutes (28).
activation step seen as a lag phase at the start of a magnesium chelatase assay (30). From kinetic analysis, it has been suggested that the activation step does not occur in every catalytic cycle (31). The present study clearly demonstrates that catalytic turnover of the magnesium chelatase results in dissociation of I subunits, which may be the mechanism of product release, i.e. the release of the H subunit. We suggest that a transient conformation is formed during catalysis, from which dissociation occurs. The requirement of a complete activation step after product release remains to be investigated.

REFERENCES

1. Gibson, L. C., Willows, R. D., Kannangara, C. G., von Wettstein, D., and Hunter, C. N. (1995) Magnesium-protoporphyrin chelatase of *Rhodobacter sphaeroides*: reconstitution of activity by combining the products of the bchH, -I, and -D genes expressed in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1941–1944

2. Walker, C. J., and Weinstein, J. D. (1991) *In vitro* assay of the chlorophyll biosynthetic enzyme Mg-chelatase: resolution of the activity into soluble and membrane-bound fractions. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5789–5793

3. Willows, R. D., and Beale, S. I. (1998) Heterologous expression of the *Rhodobacter capsulatus* bchL, -D, and -H genes that encode magnesium chelatase subunits and characterization of the reconstituted enzyme. *J. Biol. Chem.* **273**, 34206–34213

4. Willows, R. D., Gibson, L. C., Kannangara, C. G., Hunter, C. N., and von Wettstein, D. (1996) Three separate proteins constitute the magnesium chelatase of *Rhodobacter sphaeroides*. *Eur. J. Biochem.* **235**, 438–443

5. Jensen, P. E., Gibson, L. C., Henningen, K. W., and Hunter, C. N. (1996) Expression of the chll, chld, and chlh genes from the cyanobacterium *Synechocystis* PCC6803 in *Escherichia coli* and demonstration that the three cognate proteins are required for magnesium-protoporphyrin chelatase activity. *J. Biol. Chem.* **271**, 16662–16667

6. Karger, G. A., Reid, J. D., and Hunter, C. N. (2001) Characterization of the binding of deuteroporphyrin IX to the magnesium chelatase H subunit and spectroscopic properties of the complex. *Biochemistry* **40**, 9291–9299

7. Sirijovski, N., Lundqvist, J., Rosénbäck, M., Elmlund, H., Al-Karadaghi, S., Willows, R. D., and Hansson, M. (2008) Substrate-binding model of the chlorophyll biosynthetic magnesium chelatase BchH subunit. *J. Biol. Chem.* **283**, 11652–11660

8. Debussche, L., Couder, M., Thibaut, D., Cameron, B., Crouzet, J., and Blanche, F. (1992) Assay, purification, and characterization of cobaltochelatase, a unique complex enzyme catalyzing cobalt insertion in hydroxyenobyrinic acid a-c-diamide during coenzyme B12 biosynthesis in *Pseudomonas denitrificans*. *J. Bacteriol.* **174**, 7445–7451

9. Vale, R. D. (2000) AAA proteins: lords of the ring. *J. Cell Biol.* **150**, F13–19

10. Fodje, M. N., Hansson, A., Hansson, M., Olsen, J. G., Gough, S., Willows, R. D., and Al-Karadaghi, S. (2001) Interplay between an AAA module and an integrin I domain may regulate the function of magnesium chelatase. *J. Mol. Biol.* **311**, 111–122

11. Jensen, P. E., Gibson, L. C., and Hunter, C. N. (1999) ATPase activity associated with the magnesium-protoporphyrin IX chelatase enzyme of *Synechocystis* PCC6803: evidence for ATP hydrolysis during Mg2+ insertion, and the MgATP-dependent interaction of the ChII and ChID subunits. *Biochem. J.* **339**, 127–134

12. Lake, V., Olsson, U., Willows, R. D., and Hansson, M. (2004) ATPase activity of magnesium chelatase subunit I is required to maintain subunit D *in vivo*. *Eur. J. Biochem.* **271**, 2182–2188

13. Reid, J. D., and Hunter, C. N. (2004) Magnesium-dependent ATPase activity and cooperativity of magnesium chelatase from *Synechocystis* sp. PCC6803. *J. Biol. Chem.* **279**, 26893–26899

14. Sirijovski, N., Olsson, U., Lundqvist, J., Al-Karadaghi, S., Willows, R. D., and Hansson, M. (2006) ATPase activity associated with the magnesium chelatase H-subunit of the chlorophyll biosynthetic pathway is an artifact. *Biochem. J.* **400**, 477–484

15. Lundqvist, J., Elmlund, H., Wulf, R., Berglund, L., Elm Lund, D., Emanuelsson, C., Hebert, H., Willows, R. D., Hansson, M., Lindahl, M., and Al-Karadaghi, S. (2010) ATP-induced conformational dynamics in the AAA+ motor unit of magnesium chelatase. *Structure* **18**, 354–365

16. Axelsson, E., Lundqvist, J., Savicki, A., Nilsson, S., Schröder, I., Al-Karadaghi, S., Willows, R. D., and Hansson, M. (2006) Recessiveness and dominance in barley mutants deficient in Mg-chelatase subunit D, an AAA protein involved in chlorophyll biosynthesis. *Plant Cell* **18**, 3606–3616

17. Elm Lund, H., Lundqvist, J., Al-Karadaghi, S., Hansson, M., Hebert, H., and Lindahl, M. (2008) A new cryo-EM single-particle *ab initio* reconstruction method visualizes secondary structure elements in an ATP-fueled AAA+ motor. *J. Mol. Biol.* **375**, 934–947

18. Dai, G., Zhao, R., Li, D., Finkielstein, C. V., and Chen, X. S. (2004) Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. *Cell* **119**, 47–60

19. Lenzen, C. U., Steinmann, D., Whiteheart, S. W., and Weis, W. I. (1998) Crystal structure of the hexamerization domain of N-ethylmaleimide-sensitive fusion protein. *Cell* **94**, 525–536

20. Enemark, E. J., and Joshua-Tor, L. (2006) Mechanism of DNA translocation in a replicative hexameric helicase. *Nature* **442**, 270–275

21. Moreau, M. J., McGeoch, A. T., Lowe, A. R., Izhaki, L. S., and Bell, S. D. (2007) ATPase site architecture and helicase mechanism of an archaeal MCM. *Mol. Cell* **28**, 304–314

22. Hoskins, J. R., Doyle, S. M., and Wickner, S. (2009) Coupling ATP utilization to protein remodeling by ClpB, a hexameric AAA+ protein. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 22233–22238

23. Martin, A., Baker, T. A., and Sauer, R. T. (2005) Rebuilt AAA+ motors reveal operating principles for ATP-fuelled machines. *Nature* **437**, 1115–1120

24. Hansson, A., Kannangara, C. G., von Wettstein, D., and Hansson, M. (1999) Molecular basis for semidominance of missense mutations in the XANTHA-H (42-kDa) subunit of magnesium chelatase. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1744–1749

25. Hansson, A., Willows, R. D., Roberts, T. H., and Hansson, M. (2002) Three semidominant barley mutants with single amino acid substitutions in the smallest magnesium chelatase subunit form defective AAA+ hexamers. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 13944–13949

26. Porra, R. J. (2002) The chequeried history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. *Photosynth. Res.* **73**, 149–156

27. Song, H. K., Hartmann, C., Ramachandran, R., Bochtler, M., Behrendt, R., Moroder, L., and Huber, R. (2000) Mutational studies on HsILU and its docking mode with HsIV. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 14103–14108

28. Haslberger, T., Weibeizin, J., Zahn, R., Lee, S., Tsai, F. T., Buka, B., and Mogk, A. (2007) M domains couple the ClpB threading motor with the Dnak chaperone activity. *Mol. Cell* **25**, 247–260

29. Singh, S. K., Guo, F., and Maurizi, M. R. (1999) ClpA and ClpP remain associated during multiple rounds of ATP-dependent protein degradation by ClpAP protease. *Biochemistry* **38**, 14906–14915

30. Walker, C. J., and Weinstein, J. D. (1994) The magnesium-insertion step of chlorophyll biosynthesis is a two-stage reaction. *Biochem. J.* **299**, 277–284

31. Viney, J., Davison, P. A., Hunter, C. N., and Reid, J. D. (2007) Direct measurement of metal-ion chelation in the active site of the AAA+ ATPase magnesium chelatase. *Biochemistry* **46**, 12788–12794