The CHLI1 Subunit of Arabidopsis thaliana Magnesium Chelatase Is a Target Protein of the Chloroplast Thioredoxin*§

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Insertion of magnesium into protoporphyrin IX by magnesium chelatase is a key step in the chlorophyll biosynthetic pathway, which takes place in plant chloroplasts. ATP hydrolysis by the CHLI subunit of magnesium chelatase is an essential component of this reaction, and the activity of this enzyme is a primary determinant of the rate of magnesium insertion into the chlorophyll molecule (tetrapyrrole ring). Higher plant CHLI contains highly conserved cysteine residues and was recently identified as a candidate protein in a proteomic screen of thioredoxin target proteins (Balmer, Y., Koller, A., del Val, G., Manieri, W., Schurmann, P., and Buchanan, B. B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 370–375).

To study the thioredoxin-dependent regulation of magnesium chelatase, we first investigated the effect of thioredoxin on the ATPase activity of CHLI1, a major isoform of CHLI in Arabidopsis thaliana. The ATPase activity of recombinant CHLI1 was found to be fully inactivated by oxidation and easily recovered by thioredoxin-assisted reduction, suggesting that CHLI1 is a target protein of thioredoxin. Moreover, we identified one crucial disulfide bond located in the C-terminal helical domain of CHLI1 protein, which may regulate the binding of the nucleotide to the N-terminal catalytic domain. The redox state of CHLI was also found to alter in a light-dependent manner in vivo. Moreover, we successfully observed stimulation of the magnesium chelatase activity in isolated chloroplasts by reduction. Our findings strongly suggest that chlorophyll biosynthesis is subject to chloroplast biogenesis regulation networks to coordinate them with the photosynthetic pathways in chloroplasts.

The reactions encompassing higher plant chlorophyll biosynthesis consist of 12 enzymes that work in unison to produce chlorophyll a from 5-aminolevulinic acid and constitute one of the most important pathways for chloroplast biogenesis. Among the chlorophyll biosynthetic enzymes, magnesium chelatase (EC 6.6.1.1) catalyzes the insertion of Mg2+ into protoporphyrin IX, the first dedicated step in chlorophyll biosynthesis. In (bacterio)chlorophyll a-producing prokaryotes (1), chlorophyll a-synthesizing bacteria, and higher plants, the magnesium chelatase complex consists of ~40-, ~70-, and ~140-kDa subunits named I, D, and H respectively (2, 3). The stoichiometry of these subunits within the magnesium chelatase complex as well as its complete three-dimensional structure is yet to be determined. From the biochemical analysis using recombinant subunits of magnesium chelatase from the photosynthetic bacteria Rhodobacter and cyanobacterium Synechocystis sp. PCC6803, it was determined that the largest H subunit binds protoporphyrin IX noncovalently (4), suggesting that the H subunit catalyzes the metal chelation reaction. Although ATP and Mg2+ are required for the interaction between I and D subunits (5, 6), I-D complex formation is independent from the ATP hydrolysis activity of I subunit. In contrast, the ATP hydrolysis process is required for the insertion of Mg2+ into protoporphyrin IX.

Jensen et al. (6) reported that the purified I subunit (CHLI) shows ATP hydrolysis activity. Moreover, three-dimensional structure of Rhodobacter capsulatus I subunit (Protein Data Bank code 1GBP) (7) has recently been determined. Based on extensive homology search studies, CHLI has been grouped among the large family of AAA+ proteins (ATPase associated with various cellular activities), which normally shows hexameric ring structure. The AAA+ protein family is an important family of enzymes that transform chemical energy into biological events and are usually found in a variety of multimeric states in a number of organisms (8). The N-terminal half of the middle size subunit D, CHLD, is homologous to CHLI, whereas the C-terminal half of CHLD includes a metal ion coordination motif (7). A conformational transition of CHLD induced upon binding to CHLH and the I-D complex formation may trigger porphyrin metallation and also relieve the blockade of the ATP-binding site of CHLI by CHLD and induce ATP hydrolysis (7).

Because magnesium chelatase functions at a branch point in tetrapyrrole biosynthesis where insertion of Mg2+ eventually

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9 The online version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

1 This work is dedicated to Prof. Ken-ichiro Takamiya of Tokyo Institute of Technology (Yokohama, Japan) who passed away as a result of a traffic accident in 2005.

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results in the production of chlorophyll or the insertion of Fe\(^{2+}\) produces heme, this enzyme is a primary candidate for the regulation of overall tetrapyrrole biosynthesis pathway. In addition, the mutation in CHLH of magnesium chelatase results in a GUN (genome uncoupled) phenotype, which is affected in the retrograde signaling pathway from chloroplasts to control transcription of nuclear-encoded photosynthetic genes. Consequently, magnesium protoporphyrin IX, the product of the magnesium chelatase catalyzed reaction, is thought to be involved in the communication pathway from the chloroplast to the nucleus (9). The regulation of the activity of magnesium chelatase is thus important also for the production of such a signaling molecule.

Transcriptional and post-transcriptional regulation of magnesium chelatase is well documented; CHLH transcript levels are light-responsive and oscillate significantly during the diurnal cycle (10–12), thereby obeying the imperatives of a circadian clock (13). Magnesium chelatase activity is also enhanced by the addition of the GUN4 protein, whose mutation causes a gun phenotype in Arabidopsis (14). The activity of magnesium chelatase is also partially regulated by a cooperative response to Mg\(^{2+}\), because the concentrations of free Mg\(^{2+}\) are thermodynamically linked to [ATP]:[ADP]:[Pi] ratios in chloroplasts (15). In the dark, Mg\(^{2+}\) concentrations in chloroplasts are low, and magnesium chelatase is essentially inactive, whereas the Mg\(^{2+}\) concentrations increase in the light, and the enzymes become active, permitting the flux of the substrate, protoporphyrin IX, into the chlorophyll biosynthetic pathway.

In chloroplasts, the regulation of the activity of a number of enzymes involved in the photosynthetic reactions is coupled to photosynthetic electron transport. The most extensively characterized example of this kind of regulation is the thioredoxin (Trx)\(^3\) system in which a light-induced change in enzyme activity is linked to the redox state of a disulfide bond located on the enzyme molecule. Trx, a ubiquitous 12-kDa protein containing a redox reactive cysteine pair within the active site, is a critical regulatory protein involved in this process. In higher plant chloroplasts, two stromal types of Trx, f-type (Trx-f) and m-type (Trx-m), were originally identified and named according to their first identified target proteins (16). Although Trx-f and Trx-m have different evolutionary origins, they both operate via the chloroplast ferredoxin/Trx system to link photosynthetic electron flow with the activity of a number of enzymes including the Calvin cycle enzymes. Enrichment of the genome data base has revealed that higher plants possess various Trx isoforms in chloroplasts, mitochondria, and cytosol (17–19), although their precise function and localization within these cellular subcompartments are yet not fully understood (20, 21).

Moreover, our knowledge of the potential target proteins of Trx has increased significantly by the recent development of a proteomic screen like two-dimensional gel electrophoresis and Trx affinity chromatography (22–25). Using Trx affinity chromatography technique, CHLI was recently identified as a potential Trx target protein (24).

Magnesium chelatase activity has been shown to be sensitive to thiol group reagents (26–29), suggesting that thiol groups in the molecule are essential for the proper function and optimal activity of this enzyme. In addition, the specific binding of a thiol-modifying reagent N-ethylmaleimide (NEM) to CHLI and the resulting inhibition of the ATPase activity have been reported (30). Because CHLII- catalyzed ATP hydrolysis is required for overall magnesium chelatase activity (6), we sought to investigate whether magnesium chelatase activity is regulated by control of the redox state of the cysteine residues in CHLII by Trx. In the present study, we found that Arabidopsis CHLII, one of CHLI isoform, is efficiently reduced by Trx-f and that CHLII ATPase activity is regulated by the reduction of an internal disulfide bond. In addition, we identified the cysteine residues subject to redox-dependent disulfide bond formation in CHLII molecule.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant CHLI1 Protein—**

The primers 5’-CATATGGTTATGATGCTAGCAATCATGAA-AC-3’ and 5’-CTCGAGGCTGAAAATCTCGGCGAAC-3’ were used to amplify the CHLI1 mature protein coding sequence from an Arabidopsis thaliana cDNA full-length clone obtained from the RAFL clone collection of RIKEN institute of Japan (31, 32). LA Taq polymerase (Takara, Japan) was used according to the manufacturer’s instructions. The PCR settings were the following: 98 °C for 5 min, followed by 30 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 5 min. The resulting PCR fragment was digested with Ndel and Xhol and ligated to the pET24b vector (Novagen), predigested with the same restriction enzymes. Plasmid for overexpression was transformed into Escherichia coli BL21(DE3) (33), and transformed E. coli cells were grown at 37 °C in 100 ml of LB medium containing 100 µg·ml\(^{-1}\) kanamycin until the A\(_{600}\) of the culture reached 0.5. Protein expression was induced by the addition of isopropyl \(\beta\)-d-thiogalactoside to the cultures at a final concentration of 1 mM and further incubated at 20 °C overnight. The cells were harvested and suspended in 10 ml of suspension buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl) and disrupted by sonication. Cell debris was removed by centrifugation, and the supernatant was processed on a nickel-nitrilotriacetic acid-agarose (Novagen) affinity column, according to the manufacturer’s protocol. The recombinant protein was recovered by elution with imidazole buffer (500 mM imidazole, 20 mM Tris-HCl, pH 7.9, 500 mM NaCl). CHLII protein purified from the soluble fraction was dialyzed into the suspension buffer and stored at −80 °C. Although the CHLII protein obtained remained stable at −80 °C, repeated freeze-thaw cycles resulted in an activity loss of up to 45%.

To prepare the mutant CHLII, CHLII\(_{C102S}\), CHLII\(_{C193S}\), CHLII\(_{C364S}\), CHLII\(_{C396S}\), CHLII\(_{C1095S/C1193S}\), and CHLII\(_{C354S/C396S}\) site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of the resultant plasmids were confirmed by DNA sequencing. Mutated proteins were purified as described and stored at −80 °C.

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\(^3\) The abbreviations used are: Trx, thioredoxin; NEM, N-ethylmaleimide; AMS, 4-acetoamido-4'-maleimidyl-stilbene-2,2'-disulfonate; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TMR, tetramethylrhodamine; NTR, NADPH-dependent thioredoxin reductase; MOPS, 4-morpholinopropanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
**Thioredoxin Regulates Magnesium Chelatase Activity**

*In Vitro Reduction of CHLI1 Assisted by Trx—*In vitro reduction of the recombinant CHLI1 protein was carried out essentially as described (23, 34) with some modifications. The recombinant CHLI1 (1 μM) in suspension buffer (50 μl) was incubated for 1 h at 25 °C with 50 μM CuCl₂ or various concentrations of dithiothreitol (DTT) in the presence or absence of recombinant spinach Trx-f (35) or Trx-m (34). For *in vitro* reduction of CHLI1 by Trx, NAPDH, and NADPH-dependent thioredoxin reductase (NTR), NTR from Arabidopsis was prepared (36). To assess the redox state of CHLI1, 4-acetoamide-maleimidyl reagent that specifically modifies cysteine residues, was added to CHLI1 in 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1% (w/v) bovine serum albumin, and various concentrations of DTT. After 20 min of incubation at 25 °C in the dark, the reaction was terminated by successive extraction of porphyrin pigment from chloroplasts with 400 μl of ice-cold acetone and 200 μl of 0.1 N ammonia/acetone (1:9, v/v). Removal of esterified pigments by two consecutive hexane washes, the product magnesium deuteroporphyrin IX was quantified fluorometrically (28), using the excitation wavelength at 404 nm and the emission spectrum between 550 and 650 nm.

*In Vivo Redox State of CHLI1 in Arabidopsis Leaves—*Three-week-old Arabidopsis seedlings grown under continuous light (50 μmol photons m⁻² s⁻¹) were placed in darkness or maintained in the light for 12 h. Rosette leaves were collected from dark- and light-treated seedlings and homogenized in suspension buffer in darkness or light, respectively. Proteins were denatured by the addition of 4% trichloric acid, precipitated by centrifugation, and labeled with AMS as described previously (23). After separation by SDS-PAGE, the proteins were blotted onto nitrocellulose membrane, and CHLI1 protein was detected by immunoblot analysis using antiserum against soybean CHLI protein (41).

**RESULTS**

*Kinetic Properties of ATPase Activity of Recombinant CHLI1 Protein of Arabidopsis—*A. thaliana possesses two isoforms of CHLI: CHLI1 (At4g18480) and CHLI2 (At5g45930). Macorray analysis of the genes involved in tetrapyrrole biosynthesis indicates that the expression of CHLI1 is higher than CHLI2 under both light and dark growth conditions (11). In addition, whereas T-DNA knock-out mutations in CHLI1 (ch42) result in a severe reduction in chlorophyll accumulation (42), the impact of CHLI2 on chlorophyll synthesis is thought to be limited (43).

To further characterize CHLI1 function, we first carried out a biochemical analysis of recombinant CHLI1. Because CHLI1 is a member of the AAA⁺ ATPase family, we first investigated the ATPase activity of Arabidopsis CHLI1. Magnesium chelatase activity is known to be sensitive to thiol group reagents (26–29); *Synechocystis* magnesium chelatase activity is dependent on DTT concentrations, and the thiol-modifying reagent NEM bound to CHLI inhibits its ATPase activity (30). Therefore, we first determined DTT dependence of the ATPase activity of recombinant CHLI1; whereas ATPase activity was not detected in the absence of DTT, it could be restored with increasing concentrations of DTT (Fig. 1A). Nearly complete activation could be achieved upon incubation of the enzyme with 5 mM DTT. This result was in agreement with the reported DTT-dependent activation of the magnesium chelatase activity of *Synechocystis* (30).

The ATPase activity of CHLI1 was also found to positively correlate with increasing Mg²⁺ concentrations in the assay mixture (Fig. 1B). Consistent with *Synechocystis* CHLI1 ATPase, full activation could be achieved with 10 mM Mg²⁺ (44). Under the optimized assay conditions and by using freshly prepared...
recombinant CHLI1 protein, Arabidopsis CHLI1 ATPase activity showed $V_{\text{max}}$ and $K_m$ ATP values of 55 nmol min$^{-1}$ mg protein$^{-1}$ and 460 µM, respectively.

**Trx Controls the ATPase Activity and Redox State of CHLI1**—It has been shown that CHLI1 is a possible target protein of Trx in spinach chloroplasts (24). Therefore, we first examined whether the reduced form of Trx could affect the ATPase activity of CHLI1. Full oxidation of CHLI1 was achieved by incubation with 50 µM CuCl$_2$ and resulted in the abolishment of the CHLI1 ATPase activity (Fig. 2A). Enzyme activity was restored upon incubation of oxidized protein with 5 mM DTT, whereas 10 µM DTT only partially restored the activity. Recovery of the ATPase activity was enhanced by the addition of Trx in the presence of 10 µM DTT (Fig. 2A). We also note that even in fully oxidized CHLI1 by CuCl$_2$, the ATPase activity was completely restored by the reduction with DTT.

We then examined the reduction of the intramolecular disulfide bonds in CHLI1 molecule in the presence or absence of Trx-f. The redox state of CHLI1 was visualized as a change in mobility in nonreducing SDS-PAGE following labeling of the free thiols by the thiol-modifying reagent, AMS (45), allowing the fully oxidized and reduced CHLI1 to be distinguished (Fig. 2B). CHLI1 was reduced upon incubation with a minimum concentration of 50 µM DTT alone, and reduction of CHLI1 showed two bands, indicating that the reduced form CHLI1 protein contains at least two distinct reduced forms (Fig. 2B, red1 and red2). Reduction by DTT could only be accomplished by Trx-f in the presence of a minimum concentration of 10 µM DTT (red2 band).

Reduction of CHLI1 Protein by NADPH-NTR-Trx System—Because DTT efficiently reduces the disulfide bond on CHLI1, it is not suitable to extensively study the effect of Trx in the...
presence of this reductant (Fig. 2A). We therefore applied an in vitro NADPH-NTR system as an alternative source of reducing equivalent. Chloroplast Trxs, Trx-f and Trx-m, are efficiently reduced by the electrons from NADPH via NTR as well as the cytosolic Trx (36). By using this NADPH-NTR system, we observed a remarkable difference in the activation of CHLI1 when comparing Trx-f and Trx-m, because this system eliminated the possible increase in basal level of ATPase activity (Fig. 3A). We then examined the effect of Trx-f on the redox state of CHLI1 by AMS labeling. CHLI1 protein was immunologically detected by Western blot analysis using anti-soybean CHLI antiserum (41) because the mobility of oxidized form of CHLI1 was almost same as that of NTR. When NADPH, NTR, and Trx-f were incubated with the oxidized enzyme, Trx-f was reduced well, and the reduction of CHLI1 protein was only observed under this combination (Fig. 3B).

Analysis of Cysteine Mutants of CHLI1—Arabidopsis CHLI1 contains four cysteine residues, three of which are completely conserved in chlorophyll-α-producing organisms, including cyanobacteria. To determine the cysteine residues involved in CHLI1 redox regulation, we created four mutant proteins by substitution of each of the four cysteines with a serine residue. The cysteine mutants, CHLI1C102S and CHLI1C193S, retained substantial redox response of ATPase activities (Fig. 4). In contrast, the ATPase activities of CHLI1C354S and CHLI1C396S were not affected by reduction and oxidation, although the ATPase activities of these mutants were somehow lower than that of wild type, suggesting that these mutant proteins have already been activated even under the oxidized conditions. These results suggest that two cysteine residues Cys354 and Cys396 form the internal disulfide bond that is involved in the redox control of the ATPase activity of CHLI1 protein. This conclusion was further confirmed by a cysteine mutant CHLI1C102S/C193S, which retained substantial redox sensitivity as well as CHLI1C102S and CHLI1C193S.

Analysis of the Cysteine Residues Involved in the Redox Responses of CHLI1—To identify the cysteine residues involved in disulfide formation that are responsible for redox regulation of CHLI1, CHLI1 was digested by lysyl endopeptidase, and the resultant peptide fragments were separated by reversed phase HPLC after incubation under nonreduced or reduced conditions (Fig. 5). We identified a single redox-responsive peptide fragment in the digested CHLI1ox (LO1). Under reduced conditions (Fig. 5B), the peak corresponding to LO1 disappeared and was replaced by three new peaks (LR1, LR2, and LR3). These redox-specific peptides were analyzed by N-terminal peptide sequencing. The LO1 peptide contained Cys396 (Table 1). Although the LO1 contained other peptide(s), we could not determine their sequence(s) because of limited sensitivity of the analysis. The peptide containing Cys396 was recovered as...
LR2 in the reduced fragments. LR1 containing Cys\textsuperscript{396} and LR3 containing Cys\textsuperscript{102} were only obtained from the reduced fragments (Table 1). We could not detect additional peaks corresponding to cysteine containing fragments under reduced and oxidized conditions, possibly because of incomplete digestion of the protein or insufficient separation of the peptide fragments. Consequently, these results suggest that Cys\textsuperscript{102} and Cys\textsuperscript{193} may also form intramolecular or intermolecular disulfide bond under oxidized conditions.

To confirm whether all cysteine residues on CHLI1 are involved in the formation of disulfide bonds under oxidized conditions, we quantified the number of free sulfhydryl groups in the oxidized or reduced CHLI1 using DTNB. CHLI1\textsubscript{red} contained 3.9 mol of thiol/mol of CHLI1, and CHLI1\textsubscript{ox} contained 0.0 mol of thiol/mol of CHLI1, indicating that all four cysteine residues form two disulfide bonds under oxidized conditions but are fully reduced by reduction. We further examined the free thiol groups on CHLI1 by TMR-maleimide labeling. As shown in Fig. 6A, a fluorescence band corresponding to CHLI1 was clearly detected in the reduced form (lane WT), whereas no fluorescence was detected in the oxidized from. Because the oxidized form of CHLI1 was detected as a monomer on nonreducing SDS-PAGE, it is likely that the four cysteine residues form two intramolecular disulfide bonds under oxidized conditions. We therefore concluded that two disulfide bonds, Cys\textsuperscript{102}–Cys\textsuperscript{193} and Cys\textsuperscript{354}–Cys\textsuperscript{396}, are formed in the oxidized CHLI1.
To corroborate this finding, we constructed the cysteine mutants CHLI1C354S/C396S and CHLI1C102S/C193S and analyzed the redox state of these mutant proteins. Purified recombinant CHLI1C354S/C396S was relatively unstable and always showed a number of low molecular weight bands, most likely degradation products. When mutant proteins were labeled with TMR-maleimide, a fluorescence band with less intensity was detected in the reduced form of CHLI1C102S/C193S, which reflects the loss of cysteines (Fig. 6A). In CHLI1C354S/C396S, weak fluorescence bands were detected in the major protein band and the degradation products. In the oxidized form, these mutants were detected as monomers, but no fluorescence was observed in the protein band. These results also indicated the formation of intramolecular disulfide bonds, Cys102–Cys193 and Cys354–Cys396, in the CHLI1 protein.

We then examined the disulfide bond reduction of the mutants by the reduced form of Trx. As shown in Fig. 6B, CHLI1C102S/C193S and CHLI1C354S/C396S were efficiently reduced by the addition of DTT. In both mutant proteins, reduction was obtained upon incubation with a minimum concentration of 50 μM DTT alone, and reduction by DTT could only be accomplished when Trx-f was added with a minimum of 10 μM DTT. The reduction profile of CHLI1C102S/C193S and CHLI1C354S/C396S was similar to wild type protein, with the exception that both mutant proteins showed a single reduced form, indicating that both contain only one disulfide bond. These results imply that both Cys102–Cys193 and Cys354–Cys396 disulfide bonds are redox-sensitive (Fig. 6C), but only Cys354–Cys396 is involved in redox regulation of the ATPase activity of CHLI1.

Stimulation of Magnesium Chelatase Activity by DTT in Isolated Chloroplasts of A. thaliana—As stated above, magnesium chelatase activity is known to be sensitive to thiol modifiers (26–29). However, there is no report to date on the redox regulation of the magnesium chelatase activity in chloroplasts. To determine whether magnesium chelatase is reduced and activated in vivo, through activation of ATPase activity, we examined whether magnesium chelatase activity in isolated intact chloroplasts of A. thaliana could be altered by exogenous addition of DTT. In this experiment, freshly isolated intact chloroplasts were incubated in the dark to prevent porphyrin-mediated phototoxic damage. As shown in Table 2, an increase of magnesium chelatase activity was found to correlate with increasing DTT concentrations in the assay mixture. This result was in accordance with the previous report on the stimulation of the reconstituted magnesium chelatase activity of Synechocystis by DTT (30). We thus suggest that higher plant magnesium chelatase activity is also regulated by redox state in chloroplasts, via the redox regulation of its ATPase activity.

In Vivo Redox State of CHLI Protein in Leaves of A. thaliana—Finally, we examined whether the redox state of CHLI protein is affected in vivo by a change in photosynthetic conditions or not. Total proteins extracted from rosette leaves of 12 h of dark-treated or continuously light-treated Arabidopsis were labeled by AMS, and CHLI protein was detected by immunoblot analysis. Whereas a single band was observed in light-treated samples, three bands were detected in dark-treated samples (Fig. 7), strongly suggesting that the redox state of CHLI is regulated in vivo by the change of the redox environment in the chloroplasts probably via the Trx system.

**DISCUSSION**

Redox Regulation of CHLI1 Activity by Trx—Proteomic analyses of Trx target proteins in plants have allowed the identification of a large number of candidate target proteins. However, only a small number of the identified candidates have been subject to detailed biochemical analysis to corroborate the nature of their interaction with Trx. In this study we investigated the redox regulation of CHLI1, the I subunit of magnesium chelatase. Because magnesium chelatase is a key enzyme that controls the overall rate of chlorophyll biosynthesis in chloroplasts, we surmised that it may constitute an enzyme of significant physiological importance among the Trx target candidate proteins identified by proteomic screening (24). We first expressed the recombinant CHLI1 protein and examined whether the ATPase activity of CHLI1 is regulated by redox conditions. Although the ATPase activity of the I subunits of the photosynthetic bacterium Rhodobacter sphaeroides and cyanobacterium Synechocystis sp. PCC6803 has already been shown (6), we found that Arabidopsis CHLI1 also possesses the ATPase activity. The *V*ₘₐₓ and *K*ₘₐₚₚ values for ATP of our CHLI1 were almost comparable to those of CHLI from Synechocystis PCC6803; 57.5 μmol min⁻¹ mg protein⁻¹ and 230 μM, respectively (44). We then demonstrated that both the DTT-Trx system and NADPH-NTR-Trx system are capable of stimulating the ATPase activity of CHLI1 by reduction of disulfide bond in CHLI1 (Figs. 2 and 3).

In this study, we examined the effects of Trx-f and Trx-m on the activation of the ATPase activity of the recombinant CHLI1. For this experiment, we used spinach Trx proteins overexpressed in E. coli. Trx-f and Trx-m are both stromal pro-

**TABLE 2**

| DTT concentration | Magnesium chelatase activity (relative to control) |
|-------------------|-----------------------------------------------|
| 0 mM              | 1.00 ± 0.04                                   |
| 0.01 mM           | 1.14 ± 0.01                                   |
| 0.1 mM            | 1.62 ± 0.01                                   |
| 1 mM              | 2.02 ± 0.03                                   |

**FIGURE 7. In vivo redox state of CHLI1 protein in leaves of A. thaliana.** Arabidopsis seedlings were grown for 3 weeks under continuous light and then placed under light or dark conditions for 12 h. The soluble proteins were extracted from leaves and reacted with AMS, and the CHLI1 protein was detected by immunoblot analysis.
Thioredoxin Regulates Magnesium Chelatase Activity

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...target enzymes. The magnesium chelatase I subunit of R. capsulatus, BCHI, contains four cysteine residues, three of which are conserved in A. thaliana (supplemental Fig. S1). Showing an amino acid identity of 49%, R. capsulatus and A. thaliana I subunits show significant sequence homology. Because the crystal structure of BCHI has been reported (7), the distances between Cα-Cα in possible cysteine pairs of CHLI in A. thaliana can be estimated based on the coordinates of the three-dimensional structure of BCHI of R. capsulatus (Protein Data Bank code 1G8P). Here, we identified two disulfide bonds in the CHLI1 protein, and the bond between Cys354 and Cys396 was found to be critically involved in the redox regulation of ATPase activity of CHLI1. According to the structure, the two amino acid residues corresponding to Cys354 and Cys396 are located in close proximity to one another within the C-terminal domain, the distance between Cα-Cα in these two cysteines being estimated as 10.44 Å. In addition, we have predicted the three-dimensional structure of Arabidopsis CHLI1 by homology modeling Swiss Model (swissmodel.expasy.org/), in which the distance between S-S in Cys354–Cys396 was estimated to be 7.78 Å. The observed distance may be too far for disulfide bond formation; thus a large conformational change may be required to allow these two cysteines to be brought into closer proximity. In contrast, Cys102 and Cys193 are located in the N-terminal domain on both sides of Walker motifs, with the distance between Cα-Cα in Cys102–Cys193 estimated at 28.13 Å, based on the crystal structure of BCHI. Hence the formation of a disulfide bond between Cys102 and Cys193 is inconceivable. As mentioned above, in the homology model, these two cysteines were located on both sides of Walker motifs, in which the distance between sulfur atoms of Cys102 and Cys193 was estimated to be 26.5 Å, and it is difficult to conceive of how disulfide bond formation may not affect the ATPase activity of CHLI1. Because BCHI/CHLI forms the hexameric ring structure in the presence of Mg2+ and ATP, the conformation of CHLI1 in the absence of ATP in this study might be different from the determined structure. Further studies are necessary to determine whether redox regulation is applicable to ring structure formation of CHLI1 and complex formation of magnesium chelatase.

Among the four cysteine residues of the I subunits of magnesium chelatase, Cys354 is conserved in all organisms, whereas Cys396 is only present in chlorophyll a-producing organisms, including Synechocystis. Thus, it is probable that the redox-dependent regulation of the ATPase activity of the I subunit is limited to chlorophyll a-producing organisms. When mapped onto the three-dimensional structure of BCHLI, these cysteine residues are located in close proximity within the C-terminal helical domain. It is reported that the spatial orientation of the C-terminal domain to the N-terminal domain in BCHLI is different from those in all known AAA+ protein structures (7); the approximate position of the C-terminal domain is thought to be behind the nucleotide-binding site with extensive contacts with the N-terminal domain. Therefore, it is probable that the conformational change of the C-terminal helical domain of CHLI, caused by the formation or the reduction of the disulfide bond, directly affects the nucleotide binding at the N-terminal catalytic domain of CHLI, which contains the nucleotide binding Walker motif and the so-called second region of homology or sensor-I motif.

Redox State of CHLI Is Regulated by Light in Vivo—To better understand the regulation of chlorophyll biosynthesis, it is important to know whether the redox state of CHLI is actually regulated in chloroplasts and whether reduction of CHLI is involved in activation of the overall magnesium chelatase activity by light in vivo. In chloroplasts, ferredoxin is reduced by the photosynthetic electron transport system; reduced ferredoxin then reduces the oxidized form of Trx in a reaction catalyzed by ferredoxin-Trx reductase. In the present study, we showed that the redox state of chloroplasts regulate the magnesium chelatase activity in chloroplasts. Furthermore, the redox state of CHLI in chloroplasts was controlled by light, although the method applied in this study could not distinguish endogenous CHLI1 and CHLI2 isoforms (Fig. 7). Under continuous light conditions, the reduced form of CHLI accumulated in chloroplasts, whereas the oxidized form of CHLI could be observed in the dark. These findings indicate that the redox state of CHLI is regulated by light probably through reduction via chloroplast ferredoxin-Trx reductase-Trx system.

In barley, magnesium chelatase activity is induced by light in vivo (2). The activity induction profile is largely consistent with the profile of the expression of CHLI (Xan-h) and CHLH (Xan-f) genes. Under circadian conditions, however, magnesium chelatase activity shows no oscillation, whereas the expression of CHLH (Xan-f) shows rhythmic variation. In contrast, the level of CHLI protein of soybean remains fairly constant in chloroplasts under similar conditions (12). Thus, magnesium chelatase activity must be primarily controlled by the expression of CHLI and CHLH, a somewhat long range regulatory system; additional post-transcriptional/translational regulation independent from rhythmic regulation is likely to also be involved in the control of the magnesium chelatase in vivo as a short range regulation system. Reid and Hunter (15) reported that the activity of magnesium chelatase is regulated in part by a cooperative response to Mg2+. Here, we reported that the
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redox-dependent control of magnesium chelatase constitutes an additional, important regulation mechanism for this enzyme. Indeed, we successfully observed the activation of magnesium chelatase activity in the intact chloroplasts under the reducing conditions, although we could not measure the activation in the light because of the porphyrin-mediated phototoxicative damage. This redox regulation may be advantageous to coordinate the active status of chloroplast caused by photosynthetic electron transport to the network of metabolic pathways including chlorophyll biosynthesis in chloroplasts.

It has been shown that cyanobacterial magnesium chelatase is sensitive to thiol-modifying reagents, and cysteine residues in photosynthetic electron transport to the network of metabolic enzymes are not fully understood. Screening have been found (36); however, their roles in the redox regulation of Trx target proteins in chloroplasts is sensitive to thiol-modifying reagents, and cysteine residues in photosynthetic electron transport to the network of metabolic enzymes are not fully understood. Screening have been found (36); however, their roles in the redox regulation of Trx target proteins in chloroplasts. Further studies on the reconstitution of whole magnesium chelatase complex were identified by this screen. Further work is required to determine whether Trx-dependent regulation of magnesium chelatase is a common control mechanism of photosynthetic organisms.

Concluding Remarks—The reduction/oxidation cascade via Trx in chloroplasts is an important regulation network for the metabolic pathways of chloroplasts. So far a number of metabolic enzymes are suggested to be involved in this network. In the present study, we have further characterized the redox regulation of the ATPase activity of CHLI. Although we cannot conclude yet that magnesium chelatase activity is solely regulated by a change in the redox state of CHLI, this assumption is highly probable given that the ATPase activity of CHLI is one of the primary determinants of the magnesium chelatase activity. Our finding shows that chlorophyll biosynthesis in higher plants is regulated by Trx, the key protein of the redox regulation network in chloroplasts. Further studies on the reconstitution of whole magnesium chelatase with recombinant subunits are required to determine the redox regulation of overall magnesium chelatase activity, together with in vivo functional analysis of cysteine mutants of CHLI by introduction of mutant genes into wild type and mutant CHLI1 (ch42). This should allow a more thorough understanding of the importance of the redox regulation of the ATPase activity for the chlorophyll biosynthesis pathway.

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