Plant Physiology and Biochemistry 109 (2016) 365–373

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Chlorophyll *a* is a favorable substrate for *Chlamydomonas* Mg-dechelatase encoded by *STAY-GREEN*

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ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form
18 October 2016
Accepted 23 October 2016
Available online 24 October 2016

Keywords:
Chlamydomonas
Mg-dechelatase
Stay green
Substrate specificity

ABSTRACT

Mg removal from chlorophyll by Mg-dechelatase is the first step of chlorophyll degradation. Recent studies showed that in *Arabidopsis*, *Stay Green* (*SGR*) encodes Mg-dechelatase. Though the *Escherichia coli* expression system is advantageous for investigating the properties of Mg-dechelatase, *Arabidopsis* Mg-dechelatase is not successfully expressed in *E. coli*. *Chlamydomonas reinhardtii* SGR (CrSGR) has a long, hydrophilic tail, suggesting that active CrSGR can be expressed in *E. coli*. After the incubation of chlorophyll *a* with CrSGR expressed in *E. coli*, pheophytin *a* accumulated, indicating that active CrSGR was expressed in *E. coli*. Substrate specificity of CrSGR against chlorophyll *b* and an intermediate molecule of the chlorophyll *b* degradation pathway was examined. CrSGR exhibited no activity against chlorophyll *b* and low activity against 7-hydroxymethyl chlorophyll *a*, consistent with the fact that chlorophyll *b* is degraded only after conversion to chlorophyll *a*. CrSGR exhibited low activity against divinyl chlorophyll *a* and chlorophyll *a*′, and no activity against chlorophyllide *a*, protochlorophyll *a*, chlorophyll c_2, and Zn-chlorophyll *a*. These observations indicate that chlorophyll *a* is the most favorable substrate for CrSGR. When CrSGR was expressed in *Arabidopsis* cells, the chlorophyll content decreased, further confirming that SGR has Mg-dechelating activity in chloroplasts.

1. Introduction

Land plants and green algae have antenna complexes that absorb light energy for photosynthesis. The level of these complexes changes in response to light conditions (Schöttler and Toth, 2014). When plants are subjected from low to high light conditions, the antenna complex is degraded to decrease the absorbance of light energy and prevent photodamage. The developmental stage of a plant also affects the level of this chlorophyll protein complex. In senescent leaves, the chlorophyll protein complex, which is one of major nitrogen source, is degraded, and its nitrogen is exported to the growing organs (Hörtensteiner and Feller, 2002). In this process, chlorophyll degradation is thought to trigger chlorophyll protein degradation, because mutants defective in chlorophyll degradation do not degrade chlorophyll proteins (Bachmann et al., 1994; Wu et al., 2016). Understanding the enzymes and the regulation of chlorophyll degradation is therefore essential for gaining insight into the process of chlorophyll protein degradation.

The extraction of a central Mg is the first step of chlorophyll degradation (Hortensteiner, 2006) (Fig. 1), and this reaction is the committed step of chlorophyll degradation. The extraction of Mg from chlorophyll is catalyzed by Mg-dechelatase, and recent studies show that it is encoded by *Stay Green* (*SGR*) (Shimoda et al., 2016). *SGR* was initially identified in stay-green mutant plants (Park et al., 2007; Christ and Hörtensteiner, 2014). In many of the plants examined, the *SGR* mutants preserve chlorophyll during senescence. This is consistent with the fact that SGR is the Mg-dechelatase that catalyzes the first step of chlorophyll degradation. *SGR* homologs are found in green algae and land plants. In land plants, *SGR* homologs can be classified into two subfamilies, the *SGR* subfamily, and *SGR-like* (*SGRL*) subfamily. *Arabidopsis* has two SGR, *Arabidopsis* SGR1 (*AtSGR1*) and *Arabidopsis* SGR2 (*AtSGR2*) and one *Arabidopsis* SGRL (*AtSGRL*). These three *Arabidopsis* SGR homologs remove Mg from chlorophyll *a*, but none of them removes Mg from chlorophyll *b* (Shimoda et al., 2016). This substrate specificity serves to degrade chlorophyll *b* correctly. For degradation, chlorophyll *b* converts to chlorophyll *a* (Hortensteiner, 2006). Therefore, chlorophyll *a* is believed to be the only chlorophyll from which Mg is

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http://dx.doi.org/10.1016/j.plaphy.2016.10.020
0981-9428/

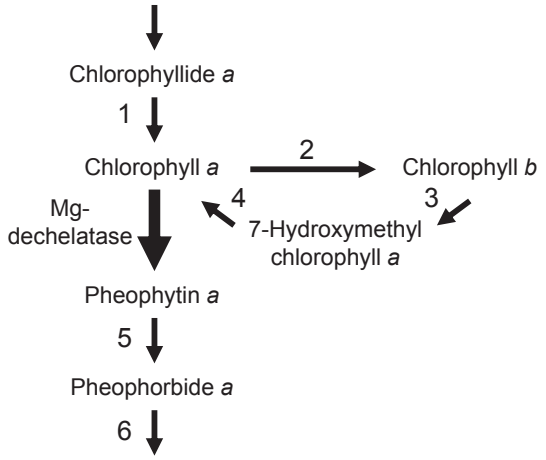

Fig. 1. Chlorophyll metabolic pathway.
A part of the chlorophyll biosynthesis and degradation pathway is shown. Mg-dechelatase catalyzes the conversion of chlorophyll *a* to pheophytin *a*. 1, chlorophyll synthase, 2, chlorophyllide *a* oxygenase, 3, chlorophyll *b* reductase, 4, 7-hydroxymethyl chlorophyll *a* reductase, 5, pheophytinase, and 6, pheophorbide *a* oxygenase.

removed in the chlorophyll degradation process. AtSGR1 and AtSGR2 do not remove Mg from chlorophyllide *a*, whereas, AtSGRL does (Shimoda et al., 2016). The molecular mechanism that causes this difference in substrate specificity is not yet understood.

Mg-dechelatase is an important enzyme for understanding the regulation of chlorophyll degradation, although its enzymatic character remains unclear. *Arabidopsis* SGR was investigated using the recombinant protein expressed by the wheat germ cell-free protein expression system (Shimoda et al., 2016). This system is not as useful as the conventional *Escherichia coli* expression system, because its protein yield is much lower. Low protein yields cause difficulties when examining the enzymatic properties under various conditions and against various substrates with the consistency lot of the recombinant protein. Among the photosynthetic eukaryotes, *Arabidopsis* and *Chlamydomonas* are model organisms. *Chlamydomonas reinhardtii* SGR (CrSGR) has a long hydrophilic tail. The presence of this long tail may allow it to form an active enzyme in *E. coli*. In this study, expression of the active CrSGR in *E. coli* was succeeded and the enzymatic properties of CrSGR were investigated.

2. Materials and methods

2.1. CrSGR preparation

Chlamydomonas reinhardtii (arg7/*cw15*) was obtained from Chlamydomonas Genetic Center. CrSGR (Cre12.g487500) lacking its transit peptide was amplified by PCR using the primers sets (CrSGR; forward 5′-AAGGAGATATACATATGGCCTCCAGGCGACAACCT-3′, CrSGR reverse1; 5′-TCGTCATCGTCTTTGTAGTCGGAGGCCGAGGAGCGCGCTA-3′). The FLAG-tag DNA sequence was added to the 3′ end of CrSGR by PCR using the primes sets (CrSGR; forward 5′-AAGGAGATATACATATGGCCTCCAGGCGACAACCT-3′, CrSGR reverse2; 5′-GGTGGTGGTGCTCGACCTTGTCGTCATCGTCTTT-3′). cDNA was cloned into pET-30a (+) (Novagen) using the *Nde*I and *Xho*I sites with an In-Fusion cloning system (Clontech). The expression plasmid was introduced into *E. coli* Rosetta (DE3). CrSGR was expressed at 37 °C for 3 h with 0.4 mM isopropyl-β-*o*-thiogalactopyranoside. Two hundred mL of the culture was harvested by centrifugation at 5000 × *g* for 5 min and then resuspended in 10 mL of BugBuster Protein Extraction Reagents (Novagen) containing 10 μL of benzonase (Novagen). The culture lysate or the soluble fraction of the culture lysate, obtained by centrifugation at 22,000 × *g* for 5 min, were mixed with the same volume of the sample buffer (25 mM Tris-HCl, pH 6.8, 4% [w/v] SDS, 10% [w/v] sucrose, 5% [v/v] 2-mercaptoethanol, and trace amounts of bromophenol blue) and were denatured by heating at 95 °C for 3 min. The culture lysate and the soluble fraction were subjected to SDS-PAGE. After electrophoresis, proteins were stained with Coomassie Brilliant Blue (CBB), or transferred to the polyvinylidene difluoride membrane for immunoblotting analysis. Antibodies against FLAG-tag (Sigma-Aldrich) detected FLAG-tagged CrSGR.

2.2. Preparation of chlorophyll derivatives

Pheophytin derivatives were prepared by mixing pigments with 0.1% (v/v) of 1N HCl in acetone. Divinyl chlorophyll *a* was extracted from an slr1923-deficient *Synechocystis* mutant (Ito et al., 2008). 7-Hydroxymethyl chlorophyll *a* was obtained by the reduction of chlorophyll *b* with 1 mM $NaBH_4$ in methanol (Holt, 1959). Chlorophyllide *a* was prepared from chlorophyll *a* through hydrolysis with recombinant chlorophyllase (Tsuchiya et al., 1999). Protochlorophyll *a* was synthesized by the chemical oxidation of chlorophyll *a* with 0.5 mM 2,3-dichloro-5,6-dicyanobenzoquinone in diethyl ether (Shedbalkar et al., 1991). Zn-chlorophyll *a* was prepared from pheophytin *a* solubilized in dichloromethane, by mixing 1/10 volume of zinc acetate saturated methanol (Kunieda and Tamiaki, 2009). Chlorophyll *a*′ was prepared from chlorophyll *a* in triethylamine (Watanabe et al., 1987). Chlorophyll c_2 was prepared from dinoflagellates *Amphidiniella sedentaria* (Horiguchi, 1995) (a kind gift from Prof. T. Horiguchi, Hokkaido University). Chlorophyll derivatives used for the enzymatic analysis were purified, using thin-layer chromatography plates silica gel 60 (MERCK) developed with petroleum ether: acetone (7:3 v/v). The molar extinction coefficient of divinyl chlorophyll in 80% acetone at 664 nm is 69.29 (Shedbalkar and Rebeiz, 1992), 7-hydroxymethyl chlorophyll *a* in diethyl ether at 655.5 nm is 61.1 (Ito et al., 1996), protochlorophyll *a* in 80% acetone at 432 nm is 241 (Kahn, 1983), chlorophyll c_2 in 90% acetone with 1% pyridine at 443.8 nm is 227 (Jeffrey, 1972), Zn-chlorophyll *a* in diethyl ether at 653 nm is 90 (Jones et al., 1968).

2.3. Enzyme assay

A culture lysate expressing CrSGR (10 μL) was suspended in 40 μL of a reaction buffer (20 mM sodium phosphate, pH 7.5, 100 mM NaCl, 0.1% Triton X-100). The pigments were solubilized in acetone, and less than 1.5 μL of the acetone solution was added to the reaction buffer. The final concentration of the pigments used for the reaction was 2.5–10.0 μM. The reaction mixtures were incubated for 30 min at 25 °C and reactions were stopped by the addition of 200 μL of acetone. The pigments were analyzed by HPLC using a C8 column (Waters Symmetry C8, Waters) with a gradient from eluent A (methanol:acetonitrile:aquaeous pyridine solution [0.25 M pyridine] [50:25:25 v:v:v]) to eluent B (methanol:acetonitrile:acetone [20:60:20 v:v:v]) at a flow rate of 1 mL min^{-1} at 40 °C (Shimoda et al., 2012). When the optimal pH and the kinetics parameters were determined, the column was developed with eluent B. The elution profiles were monitored by measuring their fluorescence (RF-20A, Shimadzu). Pigment quantification was performed using the areas of the peaks. Chlorophyll *a*′ was analyzed on a normal-phase HPLC system. The column (Senshupak Silica 2141-N, Senshu Science) was developed with a solvent (hexane: toluene: methanol = 100:4:0.8 [v/v]), at a flow rate of 1 mL min^{-1} at 25 °C (Nakamura et al., 2001).