Cytoplasmic Localization of the Single Glutamine Synthetase in a Unicellular Red Alga, *Cyanidioschyzon merolae* 10D

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Glutamine synthetase (GS) is a key enzyme for nitrogen assimilation. Although GS contains multiple molecular species found in plastid, mitochondria and cytoplasm in green plants and algae, genome analysis of a red alga, *Cyanidioschyzon merolae*, revealed a single nuclear gene for GS (CmGS). In this study, we experimentally determined the CmGS localization in the cytoplasmic compartment.

Key words: *Cyanidioschyzon merolae*; glutamine synthetase; localization; nitrogen assimilation; red alga

Nitrogen assimilation is an essential process for every cell, and ammonium and nitrate are the usual nitrogen resources in natural habitats for autotrophic organisms like plants. Since nitrate is successively reduced by nitrate reductase (NR) and nitrite reductase (NiR) to ammonium, GS, which synthesizes glutamine from glutamate and ammonium, is a common key enzyme for nitrogen assimilation. Once assimilated as an amide moiety of glutamine, nitrogen is transferred to 2-oxoglutarate (2-OG) to make glutamate by glutamate synthase (GOGAT), and further utilized to make various organic nitrogen compounds. Glutamate dehydrogenase could also serve as the nitrogen assimilating enzyme, but the significance of this enzyme for plant nitrogen assimilation is still a matter of controversy.

In higher plants, multiple nuclear genes encode for type II (eukaryotic type) GS.1,2) GS isoforms localized in cytoplasm are called GS1, and are considered to be required for the primary assimilation of ammonium. The chloroplast isoform is called GS2, and it is usually encoded by a single nuclear gene. The major role of GS2 is to reassimilate the ammonium released by photorespiration in leaves. Interestingly, recent analysis revealed that GS2 is dual targeted into chloroplast and mitochondria in *Arabidopsis*,3) implying complex nitrogen traffic within higher plant cells. In the green alga *Chlamydomonas reinhardtii*, one and two nuclear genes respectively for GS1 and GS2 were identified.4) *Cyanidioschyzon merolae* is a unicellular red alga living in acidic hot springs. Recently the nucleotide sequence of three genomes, present in nucleus, mitochondrion, and chloroplast, was completely determined.5–7) Based on the extremely simple cell structure and the minimally-redundant small genome, this alga is perhaps a very primitive eukaryote suitable for the basic analysis of various aspects of eukaryotic cells.

Using this novel model plant cell, we have begun to characterize the nitrogen assimilation system. In contrast to other plants, a single nuclear gene, CMI233C (http://merolae.biol.s.u-tokyo.ac.jp), was predicted to encode type II GS (CmGS) in *C. merolae*. Although a BLAST search showed slightly higher similarity to GS2s than to GS1s of higher plants, the absence of the amino terminal plastid-targeting sequence suggested the cytoplasmic localization of CmGS (data not shown), but there are few analyses of intracellular protein localization in red algae, and thus it was unclear whether protein localization was determined similarly to green plants. Because the localization of CmGS is essential information to understand the nitrogen traffic in this alga, we examined the localization of CmGS experimentally in this study.

First, after disruption of *C. merolae* cells and fractionation of cellular compartments by density gradient centrifugation, each fraction was examined for CmGS localization by immunoblot analysis. Since the CmGS protein shows very high similarity (>60% identity) to higher plant GS proteins, we used an antiserum against GS1 of rice to detect CmGS. As shown in Fig. 1, immunoblot analysis with *Cyanidioschyzon* lysates detected a single reacted protein around the predicted size of CmGS (365 amino acids, 40.8 kDa). Together with the results of localization of marker proteins for cytoplasm (α-tubulin), chloroplast (large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, RbcL) and mitochondrion (EF-Tu), the CmGS protein was shown to co-fractionate only with α-tubulin (Fig. 1), indicating that CmGS localizes uniquely in the cytoplasm.

Note

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follows: Ammonium imported by ammonium trans-
transporters is assimilated into glutamine by cytoplasmic CmGS, as in higher plants. GOGAT is encoded solely by the chloroplast genome (CMV060C), and thus the assimilated product (glutamine) is imported into the chloroplast to constitute the GS-GOGAT cycle. Since GS is absent in the chloroplast and mitochondrion, the cytoplasmic CmGS must reassimilate ammonium produced by photorespiration. In Cyanidiales, RubisCO enzymes are known to have strong affinity for CO₂ and low oxygenase activity.₉ This probably indicates the low photorespiration rate in these organisms, and might correspond to the dispensability of organelle GS. Localization analysis of CmGS is the first step in understanding nitrogen traffic in this organism, and the simple characteristics of C. merolae should facilitate further analysis. Basic information on this simple alga should be also useful in a consideration of the complicated nitrogen traffic in higher plants.

C. merolae were grown in MA medium⁹ in a glass vessel under continuous white light (50 μmole photon m⁻² s⁻¹) at 42 °C, and bubbled with 2% CO₂.

For fractionation, cells grown to mid-log phase (OD₇₅₀ 0.5–0.7, 500 ml) were collected by centrifugation, washed twice in 35 ml of buffer A (20 mM Heps–KOH, pH 7.6, 5 mM KCl, 5 mM Na–EDTA, pH 8.0, 1.2 mM spermidine, 5 mM MgCl₂, and 180 mM sucrose), and resuspended in 20 ml of the same buffer. Cells were lysed in a French pressure cell (Thermo Electron Corporation, Waltham, MA, USA) at 2,000 p.s.i. After 100 μg/ml DNase I was added, the lysate was incubated on ice for 1 h and filtered with two layers of 10 μm nylon mesh and two layers of Miracloth. After centrifugation for 15 min at 3,000 g, the pellet was suspended in 2 ml of buffer B (20 mM Heps–KOH, pH 7.6, 5 mM KCl, 5 mM Na–EDTA, pH 8.0, 1.2 mM spermidine, 5 mM MgCl₂, and 300 mM sucrose). Four hundred μl of the suspension was layered on 10–80% Percoll linear gradient in buffer B (5 ml) prepared with a Gradient Mate (BioComp, Fredericton, NB, Canada). After centrifugation for 1 h at 28,000 g, eight fractions (650 μl each) were manually separated and centrifuged for 10 min at 17,400 g. The pellet was resuspended in 1 ml of buffer B, and the centrifugation and the resuspension steps were repeated three times to wash the pellets. After the final centrifugation, the pellets were suspended in 250 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1 × Complete Mini protease inhibitor, EDTA-free, Roche Diagnostics, Tokyo, Japan). After 150 mg of glass beads (φ106 μm, SIGMA-ALDRICH, St. Louis, MO, USA) was added and vortexed for 5 min, debris was removed by centrifugation. Immunoblot analysis was performed as described previously, and detection was with an ECL Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer’s instruction.

Immunostaining with an anti-GS antibody and DAPI staining were performed as described previously, with
a few modifications. Cells from a log phase culture were placed in a fixation buffer (1% paraformaldehyde and 10% dimethylsulfoxide in methanol, pre-cooled at −80 °C) for 5 min at −20 °C. Antibodies were diluted at 1:1,000 for rabbit anti-rice GS as the first antibody, and at 1:1,000 for Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) as the second antibody. All the blocking and labeling steps were performed at 30 °C. Cells were examined using an Olympus BX51 microscope. Images were captured with an Olympus DP70 digital camera and processed with Adobe Photoshop software.

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