The cbbQ Genes, Located Downstream of the Form I and Form II RubisCO Genes, Affect the Activity of Both RubisCOS

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Hydrogenovibrio marinus strain MH-110, an obligately lithoautotrophic hydrogen-oxidizing bacterium, possesses three sets of the genes for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO): namely, two form I type (cbbLS-1 and cbbLS-2) and one form II type (cbbM) enzymes. The cbbQ-m gene was located downstream of cbbM, and showed high similarity to other cbbQ genes and nirQ/norQ genes in denitrifying bacteria. Phylogenetic analysis of CbbQ and NirQ/NorQ indicated that CbbQ-m from H. marinus closely resembled CbbQ from Thiobacillus intermedius and Thiobacillus neapolitanus and less closely resembled NirQ and NorQ. The cbbQ-m gene has been shown to activate the form II RubisCO in E. coli cells, and the cbbQ-t from Hydrogenophilus thermoluteolus could also activate the form II RubisCO. Both cbbQ genes have also been shown to activate the form I RubisCO from H. thermoluteolus in E. coli cells. However, the activation levels of two form I RubisCOs from H. marinus were smaller than that of form I RubisCOs from H. thermoluteolus. Form II RubisCO activated by CbbQ-m (QM) was purified from E. coli cells. The result of the 8-anilino-1-naphthalenesulfonate binding assay and the circular dichroism spectra indicated that QM was conformationally different from Form II RubisCO that was not activated by CbbQ.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC4.1.1.39) is one of two specific enzymes of the Calvin-Benson cycle. The enzyme in plants, algae, cyanobacteria, and most photo- and chemoautotrophic bacteria, is a high molecular weight protein (M, 500,000–560,000) composed of eight large and eight small subunits (8L8S; form I type). The large subunit (M, 50,000–64,000) containing the active site is encoded by the cbbL gene, and the small subunit (M, 10,000–16,000) is encoded by the cbbS gene. The second type of RubisCO, form II type (8L) RubisCO, encoded by the cbbM gene and consisting of only large subunits, has also been described in Rhodospirillum rubrum (8L; 1, 2), Gonyaulax dinoflagellates (8L; 3), and Rhodopseudomonas palustris (8L; 4). Rhodobacter sphaeroides (5), Rhodobacter capsulatus (6), Thiobacillus denitrificans (7), and Thiobacillus intermedius (8) have one form I and one form II type RubisCO.

Hydrogenovibrio marinus strain MH-110 is an obligately lithoautotrophic, halophilic, and aerobic hydrogen-oxidizing bacterium isolated from a marine environment (9, 10). This bacterium fixes CO2 via the Calvin-Benson cycle. In previous papers, we demonstrated that H. marinus possessed two different sets of genes for two form I type (cbbLS-1 and cbbLS-2) and one form II RubisCO gene (11, 12). All of these RubisCOs have been purified from H. marinus cells harboring a set of genes for each, and some properties of the purified enzymes have been investigated (13). The form II RubisCO has been also purified from H. marinus cells (14).

The cbbQ gene that encodes a protein carrying an ATP-binding motif is located downstream of cbbLS in Hydrogenophilus thermoluteolus (15) (formerly known as Pseudomonas hydrogenothermophila (16)), in Chromatium vinosum (15), and in Rhodobacter capsulatus (17). CbbQ is thought to play an important role in the posttranslational regulation of RubisCO, because coexpression of cbbQ with cbbLS in E. coli affects the conformational state and the activity of RubisCO (18). The cbbQ genes are extremely similar to the nirQ genes from Pseudomonas aeruginosa (19) and Pseudomonas stutzeri (20) as well as to the norQ genes from Para-

Abbreviations used: Hv, Hydrogenovibrio; Hp, Hydrogenophilus; cbbQ-m, cbbQ of Hv. marinus; cbbQ-t, cbbQ of Hp. thermoluteolus; cbbLS-1, cbbLS of Hp. thermoluteolus; UM, CbbM that is not activated by CbbQ; QM, CbbM that is activated by CbbQ.

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coccus denitrificans (21), Rhodobacter sphaeroides (22), and Paracoccus halodenitrificans (23). In these bacteria, the nirQ and norQ genes are located in the genome, within the gene cluster for denitrification. The nirQ gene is reportedly necessary for the posttranslational activation of nitric oxide reductase in P. stutzeri (20).

We have previously reported that NirQ from P. aeruginosa can activate RubisCO from H. thermoluteolus (24). In this report, we identified the cbbQ gene downstream of form II RubisCO gene in H. marinus and described the ability of cbbQ to change the activities of the form I and form II RubisCOs in E. coli cells.

**MATERIALS AND METHODS**

Bacterial strain, vectors and media. E. coli strain J M109 (25) was used as a host for all plasmids used in this paper and was routinely cultivated at 37°C in LB medium with 100 μg/ml ampicillin. 2× YT medium (25) was used for cultivation of E. coli for purification of CbbM. pUC119 (Takara Shuzo, Kyoto, Japan) was used for subcloning and as an expression vector. pACYC184 (26) was used for expression of CbbQ.
DNA sequence. DNA sequence of the region downstream of cbbM was determined from both directions according to the method described previously (15). The nucleotide sequence data for cbbQ have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence database under the Accession Number AB025104.

Phylogenetic analysis of CbbQ. The CbbQ amino acid sequences of Hv. marinus were multiply-aligned using CLUSTAL W ver. 1.6 (27). After being multiply-aligned, the multiple sequence alignment was corrected manually when necessary. A phylogenetic tree was constructed from evolutionary distance data by applying the algorithm of the neighbor-joining method (28). To evaluate the robustness of the branches of the inferred tree, the bootstrap resampling method of Felsenstein (29) was used with 1,000 replications.

Accession numbers of the sequences used for phylogenetic analysis of the CbbQ/NirQ/NorQ type proteins are as follows: Bacillus subtilis (YojN), AF026147; Bradyrhizobium japonicum (NorQ), AJ132911; Hp. thermoluteolus (CbbQ), D30764; Pa. denitrificans (NorQ), U28078; Pa. halodenitrificans (NorQ), AB010889; P. aeruginosa (NirQ), D37883; Pseudomonas sp. G-179 (NorQ), AF083948; P. stutzeri (NirQ), Z17423; Rb. capsulatus (CbbQ), L82000; Rb. sphaeroides (NorQ), AF000233; T. intermedius, AF012127; T. neapolitanus, AF046932.

Construction of the plasmid including cbbL, cbbS, cbbM, and cbbQ genes. The vectors used for the expression of cbbLS-1, cbbLS-2, and cbbM were pYAH501, pYAH506, and pYAH507, respectively. pYAH501 was constructed by ligating the SphI-MroI region into the SphI and HinclI sites of pUC119 after the MroI end was blunted by Klenow fragment (Fig. 1A). pYAH506 and pYAH507 were constructed by ligating the NdeI-SacI fragment of pJS305 and pJE225 (13) into the HinclI and SacI sites of pUC119 after the NdeI ends were blunted by Klenow fragment, respectively (Fig. 1B and C). pYAH508 was constructed by ligating the EcoRI-EcoRV region into the EcoRI and ScaI sites of pACYC184 (Fig. 1C). The constructions of pYAH303 (18), pYAH315 (24), and pACYC184Cm (24) were described previously (Fig. 1D). Recombinant DNA manipulations were carried out as described by Sambrook et al. (25).
Preparation of cell extract. E. coli cells harboring plasmids were grown at 37°C in 150 ml of LB-medium with 100 \( \mu \)g/ml ampicillin and 25 \( \mu \)g/ml tetracycline. Cells were suspended in 10 ml of the buffer A (bicine 10 mM, EDTA 0.1 mM, MgCl\(_2\) 6H\(_2\)O 1 mM, 2-mercaptoethanol 1 mM, monoiodoacetic acid 1 mM, pH 7.5) and disrupted twice by passage through a French Pressure Cell at 110 MPa. The cell extract was centrifuged at 10,000 \( \times \) g for 1 h to remove cell debris.

RubisCO assay. The enzyme assay for RubisCO was performed using NaH\(^{14}\)CO\(_3\) as described previously (13, 18). One unit of enzyme activity was defined as the amount catalyzing the fixation of 1 \( \mu \)mol CO\(_2\) per min at 30 or 50°C.

Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Lab., Richmond, CA) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and Western immunoblot analysis. Cell extract was separated by electrophoresis in a 12.5% polyacrylamide gel (PAGE) in the presence of sodium dodecyl sulfate (SDS). The other methods for PAGE and Western immunoblot analysis were described previously (13).

Fluorescence measurement. Fluorescence measurement was performed as described previously (18), with the exception that the buffer A was used, and incubation was carried out 10 min at 30°C. A spectrum of the buffer was subtracted from each protein spectrum.

Measurement of CD spectra. Circular dichroic (CD) spectra of RubisCO proteins were measured on a JASCO automatic recording spectropolarimeter, Model J 720, from 320 to 200 nm.

RESULTS AND DISCUSSION

Sequence Analysis and Identification of the Downstream Region of cbbM

The nucleotide sequence was determined for a 1.4-kb fragment of DNA downstream of cbbM (data not shown). An ORF of 798 nucleotides was located there and was preceded by a reasonable ribosome binding site. A search for amino acid homology with other proteins showed that this protein had high identity to CbbQ, NirQ, and NorQ proteins. The two nucleotide-
binding domain had high similarity among these proteins (Fig. 2).

Phylogenetic Analysis of the CbbQ/NirQ/NorQ Type Proteins

A phylogenetic tree of the CbbQ/NirQ/NorQ type proteins was constructed using the neighbor-joining method (Fig. 3). Phylogenetic analysis indicated that CbbQ from *Hv. marinus* resembled CbbQ from other bacteria more closely than NirQ, NorQ, and YojN. Bootstrap values were indicated and strongly supported that these proteins should be clustered into four major groups. The four groups were classified by the location of the genes encoding the proteins; the first group genes (cbbQ) was located downstream of cbbM, the second group (cbbQ) was located downstream of cbbL, the third group (nirQ) was located downstream of norCB encoding nitric oxide reductase, and the last group (nirQ) was located upstream of nirS encoding nitrite reductase. YojN had lower identity to the other proteins, and had no nucleotide-binding motif. YojN seemed to be different from the CbbQ/NirQ/NorQ type proteins. The sequences of the large subunits of form I RubisCOs are clustered into two major groups, designated as Red-like and Green-like (30, 31). The RubisCOs (including cbbLS-1 from *C. vinosum*) encoded by

The genes located upstream of cbbQ have been located in the form II group and the Green-like group (17, 30, 31). On the other hand, cbbX genes encoding another ATP-binding protein have been located downstream of cbbL genes in *Ralstonia eutropha* (32), Rhodobacter *sphaeroides* (33), and *Xanthobacter flavus* (34), as well as downstream of RubisCO genes in a red alga, *Antithamnion sp.* (35), and in *Porphyra purpurea* (36). In *Rb. sphaeroides*, a cbbX deletion mutation cannot grow photoautotrophically (33). The RubisCOs encoded by the genes located upstream of cbbX have been located in the Red-like group (30). These finding indicate that the RubisCO genes (cbbLS and cbbM) and neighboring genes were acquired by horizontal gene transfers. However, the RubisCOs (cbbLS-1 and cbbLS-2 from *Hv. marinus*, cbbLS-2 from *C. vinosum*, and RubisCO genes from plant, green algae, and cyanobacteria) encoded by the genes not located upstream of cbbQ were located in the Green-like group.

Effect of cbbQ Expression on Form II RubisCO Activity in the *E. coli* Cell

We abbreviate the cbbQ of *H. thermoluteolus* as cbbQ-t and the cbbQ of *Hv. marinus* as cbbQ-m, pUC vector was used for the expressions of cbbLS and cbbM, and pACYC vector was used for the expression of cbbQ. pACYC184Cm (24) was used as a negative control of pYAH315 and pYAH508. The replication origin of pACYC184 is compatible with that of the pUC vectors (26); therefore the plasmids derived from pUC vector are able to co-exist with pACYC derivatives in *E. coli* cells. The cell extract of *E. coli* JM109 harboring both pYAH507 and pACYC184Cm showed RubisCO activity of 0.091 unit/mg protein (Table 1). This activity was about 1.6-fold and 1.5-fold greater when pYAH315 or pYAH508 was used than when pACYC184Cm was
used, respectively. The activity was not detected when the cell was transformed only with pYAH315 (24) or pYAH508 (Table 1). These results clearly indicated that both CbbQ-t and CbbQ-m activated the form II RubisCO from Hv. marinus. SDS-PAGE of the cell extracts and immunological detection of RubisCO expressed in E. coli showed that the expression level of the enzyme protein was not affected by the existence of the cbbQ-t or cbbQ-m gene (Fig. 4). Both CbbQs are thought to mediate the posttranslational activation of form II RubisCO at an almost equal level. The activity was about two-fold greater when cbbLS genes from Hp. thermoluteolus were coexpressed with cbbQ-t or nirQ than when only cbbLS genes were expressed (24). The activation level of form II RubisCO by both CbbQ was smaller than that of form I RubisCO (CbbLS-t) by CbbQ-t. CbbQ-t has 68 and 53% identity to CbbQ-m and NirQ from P. aeruginosa. The proteins possessing up to 50% identity seem to be able to substitute for CbbQ-t functionally. Because CbbQ could activate form II RubisCO that does not possess a small subunit, CbbQ seemed to react to large subunits of RubisCO.

Effect of cbbQ Expression on Form I RubisCO Activity in the E. coli Cell

We also investigated whether cbbQ-t and cbbQ-m could activate form I RubisCOs in E. coli cells (Fig. 5). Three kinds of form I RubisCO genes, cbbLS1 and cbbLS-2 from Hv. marinus and cbbLS from Hp. thermoluteolus, were used in this study. We abbreviate cbbLS from Hp. thermoluteolus as cbbLS-t. We determined that no cbbQ gene was located in the downstream regions of cbbLS-1 and cbbLS-2 (data not shown). The activity in cell extracts of E. coli harboring cbbLS-t with cbbQ-t or cbbQ-m was two-fold greater than that in extracts harboring only cbbLS-t. Both CbbQs seemed to activate CbbLS-t posttranslationally. However, the activation level of CbbLS-1 or CbbLS-2 by cbbQ was smaller than that of CbbLS-t. CbbL-t has 79.0, 76.8 and 29.5% identity to CbbL-1, CbbL-2, and CbbM, respectively. CbbQ seemed to activate the RubisCOs that are encoded by the gene(s) located upstream of cbbQ genes. The activation by CbbQ had a specificity to RubisCO.

Purification of Form II RubisCO Activated by CbbQ-m

The recombinant form II RubisCO was purified so that detailed properties could be examined. Here we refer to the RubisCOs produced in the E. coli cells harboring cbbM-cbbQ and cbbM as QM and UM (unaffected CbbM), respectively. The purification procedure of UM was described previously (13). QM and UM were indistinguishable from each other in the denaturing gel (Fig. 6). QM was inactivated during Q-Sepharose chromatography. The activity of purified QM was quite low (data not shown). The inactivation of UM during Q-Sepharose chromatography had been also observed previously (13). The cbbO gene is located
downstream of cbbLSQ from *H. pylori* and affects the activation and stabilization of Rubisco (18). Another gene may be required for activating CbbM.

8-Anilino-1-naphthalenesulfonate (ANS) has been widely used as a fluorescent probe for the identification of conformational changes in proteins including Rubisco proteins (37). Addition of Rubisco to a solution of ANS caused a large enhancement of the fluorescence intensity of the dye and a blue shift of the emission maximum from 510 to 472 nm (Fig. 7A). UM enhanced ANS fluorescence, as compared to QM, suggesting that the conformation of UM is different from that of QM. The structural integrity of the two Rubisco enzymes was further examined using circular dichroism (CD) (Fig. 7B). CD provides information regarding the relative amounts of helix, sheet, and random-coil structures present in a protein (38), and it has been used to measure the conformational change of Rubisco (39). There were differences observed in the CD spectra of the UM and QM, indicating that the UM and QM differed in protein conformation. CbbQ seemed to mediate the conformational change in CbbM.

REFERENCES