The Novel Genes, cbbQ and cbbO, Located Downstream from the RubisCO Genes of Pseudomonas hydrogenothermophila, Affect the Conformational States and Activity of RubisCO

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The cbbQ and cbbO genes are located downstream from the RubisCO genes (cbbLS) in the thermophilic hydrogen-oxidizing bacterium, Pseudomonas hydrogenothermophila. Recombinant RubisCO enzymes were purified from E. coli cells which were transformed with plasmids expressing cbbLS, cbbLSQ, cbbLSQO, or cbbLSQO. Co-expression of cbbQ and/or cbbO with cbbLS made the maximal rates of carboxylation (Vmax) of the recombinant RubisCOs about two-fold higher than that of the enzyme derived from only cbbLS. The RubisCOs with high Vmax also had a high stability when undergoing ultrasonic treatment. The results of the circular dichroism spectra and the 8-anilino-1-naphthalenesulfonate binding assay indicated that these recombinant RubisCOs were conformationally different to each other.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is one of two unique enzymes of the Calvin-Benson cycle. The enzyme which can be bound in plants, algae, cyanobacteria, and most photo- and chemotrophic bacteria, is composed of eight large and eight small subunits (L8S8). The large subunit (M1, ~56,000), encoded by the cbbL gene, contains the active site, while the precise role of the small subunit (M2, ~15,000), encoded by the cbbS gene, is still unclear (1,2).

Pseudomonas hydrogenothermophila strain TH-1 is an aerobic, thermophilic hydrogen-oxidizing bacterium and is a facultative chemolithotroph able to grow not only autotrophically using hydrogen, oxygen, and carbon dioxide, but also heterotrophically in organic media. The strain is known to assimilate CO2 via the Calvin-Benson cycle (3,4). The genes for the large (cbbL) and small (cbbS) subunits for RubisCO were cloned from P. hydrogenothermophila genomic DNA and sequenced (5). We found an open reading frame (cbbQ) of unknown function downstream from cbbLS. The gene encodes a protein carrying an ATP-binding motif and is highly similar to the nirQ gene from Pseudomonas aeruginosa or Pseudomonas stutzeri (5) or the norQ gene from Paracoccus denitrificans (6) or Rhodobacter sphaeroides (7). The nirQ and norQ genes are located in the gene cluster for denitrification in the genome of P. aeruginosa (8), P. stutzeri (9), Pa. denitrificans (6) or R. sphaeroides (7). In P. stutzeri, the NirQ protein is believed to regulate the respiratory NO reduction and nitrite reduction (9), however, the function of NirQ or NorQ is not investigated thoroughly.

In the case of heterocystous cyanobacteria, Anabaena sp., the gene encoding an ATP-binding protein called Rubisco activase is located downstream from RubisCO genes (10-12). Plants and algae have Rubisco activase and this enzyme is known to be required for posttranslational regulation of the L8S8 form (form I type) RubisCO (13,14). In Anabaena sp., an open reading frame, rbcX, is located between RubisCO genes (rbcL and rbcS) (11, 15). In the results of Anabaena sp. strain CA, rbcX gene is required for maximum activity of RubisCO in E. coli cell extract (16). The protein corresponding to Rubisco activase has not been identified in the photo- and chemotrophic bacteria. The genes, cbbX and cbbY, are located immediately downstream from cbbLS in Alcaligenes eutrophus (17) and R. sphaeroides (18). The cbbX gene encode another ATP-binding protein and the corresponding genes have been found downstream from cbbLS in Xanthobacter flavus (19) or a red alga, Antithamnion sp. (20). In R. sphaeroides, the cbbX deletion mutation can not grow in photoautotrophic condition (18).

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In this report, we describe the ability of the \( \text{cbbQ} \) and \( \text{cbbO} \) genes to change the activity and conformational states of \( P. \) \( \text{hydrogenothermophila} \) \( \text{Rubisco} \) in \( E. \) \( \text{coli} \) cells.

**MATERIALS AND METHODS**

Bacterial strain, vectors and media. \( E. \) \( \text{coli} \) strain \( M109 \) (21) was used as a host for all plasmids used in this paper and was routinely cultivated at \( 37^\circ \text{C} \) in LB medium with \( 100 \mu \text{g/ml ampicillin} \). \( pUC119 \) (21) was used for subcloning and as an expression vector.

DNA sequence. DNA sequence of the region downstream from \( \text{cbbLSQ} \) was determined from both directions according to the method described previously (5). The nucleotide sequence data for \( \text{cbbO} \) have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence database under the accession number AB003906.

Construction of the plasmids including \( \text{cbbL}, \text{cbbS}, \text{cbbQ}, \) and \( \text{cbbO} \) genes. The fragments of the plasmids used in this work are designated in Fig. 1. \( pYAH302 \) was constructed by ligating the \( \text{HedAI} - \text{EcoRI} \) region containing the \( \text{cbbLSO} \) genes into the \( \text{HindIII} \) and \( \text{EcoRI} \) sites of \( pUC119 \) after the \( \text{HedAI} \) end was blunted by T4 DNA polymerase. The \( \text{HedAI} - \text{Smal} \)-fragment carrying the \( \text{cbbLSQ} \) genes was cut from \( pYAH302 \) by \( \text{SphI} \) and \( \text{Smal} \) and ligated into the respective sites of \( \text{pUC119} \), yielding \( pYAH303 \), \( pYAH322 \) was constructed by inserting the fragment carrying the \( \text{cbbLSQ} \) genes, which was cut out by \( \text{SphI} \) and \( \text{PstI} \), into the respective sites of \( pUC119 \), and deleting 200-bp from \( \text{PstI} \) site by \( \text{Exonuclease III} \). The fragment from \( pYAH323 \) carrying the \( \text{cbbSO} \) genes was obtained by removing the 400-bp \( \text{BsiWI} - \text{PpuMI} \)-fragment, in which two \( \text{ATP} \)-binding sites of \( \text{CbbQ} \) were encoded, from the fragment of \( pYAH302 \). The fragment was removed by digesting \( pYAH302 \) with \( \text{BsiWI} \) and \( \text{PpuMI} \), blunting the cohesive ends with the \( \text{Klenow} \) enzyme, and recircularizing. Recombinant DNA manipulations were carried out as described by Sambrook et al. (21).

Rubisco assay. The enzyme assay for Rubisco was performed by using \( \text{NaH}^{14} \text{CO}_3 \) as described previously (22), except that the activity was measured at \( 50^\circ \text{C} \) and the BEMND buffer (bicarbonate \( 50 \text{mM}, \text{EDTA} \) \( 0.1 \text{mM}, \text{MgCl}_2 \) \( 6H_2O \) \( 10 \text{mM}, \text{dithiothreitol} \) \( 1 \text{mM}, \text{NaHCO}_3 \) \( 25 \text{mM}, \text{pH} \) \( 7.8 \)) was used as a reaction buffer. One unit of enzyme activity was defined as the amount catalyzing the fixation of \( 1 \mu \text{mol CO}_2 \) per min at \( 50^\circ \text{C} \).

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

Purification of Rubisco enzymes from recombinant \( E. \) \( \text{coli} \) cells. \( E. \) \( \text{coli} \) cells harboring plasmid were grown at \( 37^\circ \text{C} \) in \( 600 \text{ml of LB-medium with} \) \( 50 \mu \text{g/ml ampicillin} \). Cells were suspended in 20 ml of the BEMND buffer (bicarbonate \( 50 \text{mM}, \text{EDTA} \) \( 0.1 \text{mM}, \text{dithiothreitol} \) \( 1 \text{mM}, \text{pH} \) \( 7.8 \)) and disrupted twice by passage through a French Pressure Cell at \( 100 \text{Mpa} \). The cell extract was centrifuged at \( 10,000 \times g \) for \( 1 \text{h} \) to remove cell debris. The supernatant was incubated at \( 60^\circ \text{C} \) for \( 30 \text{min} \) and then centrifuged at \( 200,000 \times g \) for \( 1 \text{h} \) to remove the denatured proteins. The Rubisco protein purified from \( P. \) \( \text{hydrogenothermophila} \) cells was stable under \( 60^\circ \text{C} \) (23). The supernatants were subsequently fractionated by fast protein liquid chromatography (FPLC; Pharmacia) equipped with a Superdex 200 column (Pharmacia). Fractions were collected, and those fractions corresponding to peaks of absorbance at \( 280 \text{nm} \) were collected for further purification. The protein solutions were subsequently fractionated by FPLC with a Mono Q column (5 mm \( \times \) \( 5 \text{cm} \), Pharmacia). The enzymes were eluted with a linear gradient containing \( 0.05 \text{mM NaCl} \) with the BEMND buffer, and the active fractions were pooled and desalted on a PD-10 column (Pharmacia). The cell extract and the purity of each preparation were monitored by both nondenaturing polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE.

![FIG. 1.](image.png)
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FIG. 2. Nondenaturing PAGE (A, B) and denaturing PAGE (C) of cell extract of E. coli (A) and purified Rubisco enzymes (B, C). (A) Cell extract of E. coli harboring lane 1; pYAH302, lane 2; pYAH303, lane 3; pYAH322, lane 4; pYAH323. 50 µg protein was electrophoresed in each lane. (B) lane 1; QO-Rubisco, lane 2; unaffected-Rubisco, lane 3; Q-Rubisco, lane 4; O-Rubisco. 5 µg protein was electrophoresed in each lane. (C) lane 1; QO-Rubisco, lane 2; nonactivated-Rubisco, lane 3; Q-Rubisco, lane 4; O-Rubisco, lane 5; marker proteins, phosphorylase (M. W. 97,400), bovine serum albumin (66,200), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). 1 µg protein was electrophoresed in lane 1-4.

pYAH323 was used to transform E. coli compared to when pYAH303, which carries only cbbLS, was used.

Purification of Rubisco Proteins

The recombinant Rubisco enzymes were purified so that more detailed properties could be examined. Here we refer to the RubiscoS produced in the E. coli cells harboring pYAH302, pYAH303, pYAH322, and pYAH323 as QO-, unaffected-, Q-, O-Rubisco, respectively. In gel filtration step, the Rubisco enzymes showed slightly different retention times. However, the results of nondenaturing PAGE indicate that the $L_{8S_8}$ hexadecameric structure of the Rubisco enzymes was maintained in all of these recombinant RubiscoS, even after the purification procedures. These results are also supported by the SDS PAGE, in which the Rubisco enzymes were indistinguishable from one another (Fig. 2). The analyses of PAGE clarity indicated that the gene products of cbbQ and cbbO were not bound to the purified RubiscoS.

Kinetic Properties of Rubisco Enzymes

The kinetic properties of the purified RubiscoS were determined (Table 1). A significant difference was found in the maximal rates of carboxylation; namely, the $V_{max}$ values of QO-, Q-, and O-RubiscoS were about two-fold higher than that of the unaffected-Rubisco. The values of the Michaelis constants for ribulose 1,5-bisphosphate (RuBP) and for the magnesium ion were quite varied, while those for carbon dioxide were almost uniform. For every concentrations of RuBP and the magnesium ion, the specific activities of the QO-, Q-, and O-RubiscoS were higher than that of unaffected-Rubisco (data not shown).

Stability of the RubiscoS Undergoing Ultrasonic Treatment

To examine the differences in stability of the Rubisco enzymes, the activity of purified Rubisco en-

<table>
<thead>
<tr>
<th>Rubisco</th>
<th>$V_{max}$ ($\mu$mol/min/mg)$^a$</th>
<th>$K_{CO_2}$ ($\mu$M)</th>
<th>$K_{RuBP}$ ($\mu$M)</th>
<th>$K_{Mg}$ ($\mu$gM)</th>
</tr>
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<tbody>
<tr>
<td>QO-R</td>
<td>6.5 ± 0.7</td>
<td>88.3 ± 8.6</td>
<td>353 ± 31</td>
<td>596 ± 66.8</td>
</tr>
<tr>
<td>unaffected-R</td>
<td>3.3 ± 0.2</td>
<td>76.8 ± 0.95</td>
<td>163 ± 29</td>
<td>296 ± 22.8</td>
</tr>
<tr>
<td>Q-R</td>
<td>6.7 ± 1.4</td>
<td>89.9 ± 9.7</td>
<td>465 ± 77</td>
<td>749 ± 128</td>
</tr>
<tr>
<td>O-R</td>
<td>7.8 ± 1.8</td>
<td>84.4 ± 8.6</td>
<td>573 ± 87</td>
<td>961 ± 143</td>
</tr>
</tbody>
</table>

$^a$ $V_{max}$ and $K_{m}$ values were obtained with Lineweaver-Burk plots. $V_{max}$ values were obtained the average of the values for CO$_2$, RuBP, and Mg.

Q-R, unaffected-R, Q-R, and O-R indicate recombinant RubiscoS purified from E. coli harboring pYAH302, pYAH303, pYAH322, and pYAH323, respectively.
TABLE 2

<table>
<thead>
<tr>
<th>RubisCO</th>
<th>Specific activity (U/mg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Time (second)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>QO-R</td>
<td>5.52 (100)</td>
</tr>
<tr>
<td>unaffected-R</td>
<td>3.40 (100)</td>
</tr>
<tr>
<td>Q-R</td>
<td>5.12 (100)</td>
</tr>
<tr>
<td>O-R</td>
<td>5.16 (100)</td>
</tr>
</tbody>
</table>

*Time for ultrasonic treatment. RubisCO activity was measured after treatment.

Values in parentheses are percentage of each activity of RubisCO enzymes not treated by ultrasonication. Q-R, unaffected-R, Q-R, and O-R indicate recombinant RubisCOs purified from E. coli harboring pYAH302, pYAH303, pYAH322, and pYAH323, respectively.

Stability of RubisCO Activity Undergoing Ultrasonic Treatment

Analyses of kinetic parameters and the conformational properties of the four purified RubisCOs confirm the existence of both functional and conformational difference in the proteins. When the RubisCO genes (cbbLS) of P. hydrogenothermophila were expressed with both cbbQ and cbbO, in the E. coli cell, the recombinant enzyme had both high activity and stability. The recombinant RubisCO that was expressed without those genes was unstable and had low activity. Expression of cbbLS with cbbQ or cbbO caused a partial activation and stabilization of the recombinant enzyme. These results indicate that both CbbQ and CbbO mediate the posttranslational activation of RubisCO of P. hydrogenothermophila in the E. coli cell. In a cyanobacterium, Anabaena sp. strain CA, the rbcX gene product, which is located between rbcL and rbcS, is necessary for the maximum activity of recombinant RubisCO (16). However, cbbQ or cbbO has no identity with rbcX. In plants, algae, and some cyanobacteria, RubisCO activase is well-known as an activator for L$_{5}$S$_{2}$-form RubisCO (13,14). RubisCO activase regulates the RubisCO activity according to the concentrations of ATP and CO$_{2}$ (27,28). The protein corresponding to RubisCO activase has not been identified in the photo- and chemosynthetic bacteria, whereas the posttranslational regulation of RubisCO activity by the level of carbon sources (29-31) or the nucleotides (32)

DISCUSSION

Conformational Differences of RubisCO Enzymes

Unaffected-RubisCO showed smearable bands, as compared to the others in non-denaturing PAGE, suggesting that the conformation of unaffected-RubisCO is different from the other three recombinant RubisCOs (Fig. 2).

8-anilino-1-naphthalenesulfonate (ANS) has been widely used as a fluorescent probe for the identification of conformational changes and hydrophobic regions of proteins, including the RubisCO protein (24). Addition of RubisCO to a solution of ANS results in a large enhancement of the fluorescence intensity of the dye and a blue shift of the emission maximum from 510 to 472 nm (Fig. 3). Unaffected-RubisCO strongly enhance the ANS fluorescence, as compared to the other RubisCOs, suggesting that the conformation of unaffected-RubisCO is different from that of the other RubisCOs. The structural integrity of the four RubisCO enzymes was further examined using circular dichroism (CD) (Fig. 4). CD provides information regarding the relative amounts of helix, sheet, and random-coil structures present in a protein (25), and it has been used to measure the conformational change of RubisCO (26). There were differences was observed in the CD spectra of the four RubisCOs, indicating that the four RubisCOs differed in protein conformation.

FIG. 3. Fluorescence emission spectra of ANS in 0.2 mg of RubisCO proteins. Q-R, unaffected-R, Q-R, and O-R indicate recombinant RubisCOs purified from E. coli harboring pYAH302, pYAH303, pYAH322, and pYAH323, respectively.
FIG. 4. CD spectra of Rubisco proteins. Proteins were diluted in the BED buffer, pH 7.8, to 0.025 mg/ml and scanned from 280 to 200 nm. Q-R, unaffected-R, Q-R, and O-R indicate recombinant Rubisco purified from E. coli harboring pYAH302, pYAH303, pYAH322, and pYAH323, respectively.

has been observed in R. sphaeroides. In this article, we suggested that at least two proteins, CbbQ and CbbO, are involved in the posttranslational activation of Rubisco in P. hydrogenothermophila.

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