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Expression of genes for subunits of plant-type RuBisCO from *Chromatium* and production of the enzymically active molecule in *Escherichia coli*

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A DNA fragment containing genes for both large (A) and small (B) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from a photosynthetic bacterium *Chromatium vinosum* was ligated with vectors for expressing unfused proteins and introduced into cells of *Escherichia coli*. The expressers of RuBisCO were screened on agar plates using the specific antibody raised against the native enzyme from *Chromatium*. The production of both subunits A and B in the expressers was demonstrated by an immunoblotting experiment. The amount of RuBisCO produced in the *E. coli* cells was as high as 15% of the total soluble protein after induction with isopropyl-β-D-thiogalactoside. The specific activity of enzyme molecules produced in *E. coli* was nearly the same as that of the original *Chromatium* enzyme. On gel filtration high-performance liquid chromatography the two enzymes showed identical elution behavior, strongly indicating their similar quaternary structures.

(Chromatium) *Ribulose-1,5-bisphosphate carboxylase/oxygenase* *Subunit* *Expression* *Assemblage* (Escherichia coli)

1. INTRODUCTION

In recent years, molecular mechanisms governing the biosynthesis of 2 constituent subunits (A and B) of RuBisCO and their functional roles in the enzyme reaction have received a great deal of attention from many investigators [1]. Since genes for the subunits are encoded by chloroplast and nuclear genomes [2], it is frequently argued whether or not a synchronous mechanism operates in the assemblage of 2 subunits to make up the enzymically active holoenzyme in chloroplasts. *Chromatium vinosum*, a photosynthetic purple sulfur bacterium, contains a plant-type RuBisCO molecule consisting of the octamers of each of subunit A (50 kDa) and B (15 kDa) (A_8B_8) [1,3]. It has been demonstrated that genes for both subunits are present in chromosomal DNA [4] and their biosynthesis in the bacterial cells is shown to proceed in a tightly coordinated manner [5]. This communication presents experimental evidence that genes for subunits of *Chromatium* RuBisCO are expressed and the products are assembled to make up the A_8B_8 type molecule of RuBisCO in *Escherichia coli*.

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Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; IPTG, isopropyl-β-D-thiogalactoside; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; RuBP, ribulose 1,5-bisphosphate

2. MATERIALS AND METHODS

The procedure for making plasmids (pCKSs) composed of a DNA fragment containing genes for 2 subunits of RuBisCO from *Chromatium* and

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an expression vector is schematically illustrated in fig.1. Another series of plasmids, pCTPs, were made in basically the same way as that for pCKSs except for the use of *Pvu*II-digest ptac12 [10], for which *Sma*I-treated pKK223-3 (P-L, Pharmacia) was substituted. After transformation of *E. coli* JM105 with approx. 0.1 μg of the plasmids by the $CaCl_2$/RbCl procedure [9], 1800 and 1900 transformants with pCKSs and pCTPs, respectively, were obtained on LB agar plates containing ampicillin. All of the colonies were transferred to a nitrocellulose filter (S & S BA85, 0.45 μm) either by laying and lifting the filter or using toothpicks [9], and incubated on LB agar containing IPTG (1 mM) and ampicillin at 37°C for 6 h.

RuBisCO producers were detected by highly purified specific antibody against *Chromatium* RuBisCO which was prepared by passing the IgG fraction of serum through an affinity column immobilized with *Chromatium* RuBisCO by CNBr-activated Sepharose [11]. The antiserum against the holoenzyme of *Chromatium* RuBisCO was prepared by published methods [12,13]. For preparing the antibody against subunit A, the purified *Chromatium* RuBisCO was dissociated into the constituent subunits in an alkaline solution composed of 50 mM Tris-Cl (pH 9.2), 0.1 mM EDTA, 1 mM dithiothreitol and 10% glycerol; subunit A separated by Sepharose 6B was injected into rabbits. The anti-subunit A serum was also purified by affinity chromatography.

Colonies on the nitrocellulose filter were lysed with chloroform vapor, followed by incubation overnight at room temperature in a solution composed of 50 mM Tris-Cl (pH 7.0), 0.15 M NaCl, 5 mM $MgCl_2$, 3% (w/v) bovine serum albumin, 1 μg/ml DNase and 40 μg/ml lysozyme [14]. Expressers were detected using horseradish peroxidase-conjugated anti-rabbit goat IgG and 4-chloro-1-naphthol according to the instruction manual from Bio-Rad. Four and six colonies of *E. coli* transformed by pCKSs and pCTPs, respectively, showed high signal-to-noise levels.

These expressers were grown in LB liquid medium containing ampicillin until absorbance at 550 nm reached 0.5. IPTG (final 1 mM) was added and the incubation was continued for an additional 2 h at 37°C. Cells were collected by centrifugation, washed with 10 mM Tris-Cl (pH 7.0), 0.1 M NaCl and 1 mM EDTA, and disrupted by Kubota 200M insonator at maximal power for 10 min in the same medium containing 10% glycerol and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation, and RuBP carboxylase activity [13] determined. RuBisCO content (1–100 μg) was measured by ELISA [15] using horseradish peroxidase-conjugated anti-rabbit goat IgG and *o*-phenylenediamine.

3. RESULTS AND DISCUSSION

DNA fragments from *Chromatium* cells were ligated with pUC8 [6], transformed into *E. coli* JM83 or JM103, and screened by ^{32}P-labeled genes for both the subunits of RuBisCO from *Anacystis nidulans* [7,8]. The insertion containing genes for RuBisCO was treated with Bal31 exonuclease and ligated with expression vectors possessing a fused promoter of trp-lacUV5 and an insertion site just after their ribosome-binding region (Shine-Dalgarno sequence), i.e. pKK223-3 (P-L, Pharmacia) or ptac12 [10] (fig.1). The bacterial colonies producing RuBisCO molecules were screened by immunoassay on LB agar employing an affinity column-purified antibody (IgG) raised against *Chromatium* RuBisCO after induction with IPTG. We have found that affinity column chromatography is essential to remove antibodies against cellular proteins of *E. coli* which produce undesirable signals in the immunoblotting experiments. Among 3700 colonies tested which were resistant to ampicillin, only 10 were found to produce high signal-to-noise levels. Despite the possible operation of the Shine-Dalgarno sequence of ptac12, it is likely that the Shine-Dalgarno sequence of pKK223-3 is not functioning because of the distance between the sequence and insertion *Sma*I site. Therefore, a Shine-Dalgarno sequence preceding structural genes for the RuBisCO subunits should be functional for expressing RuBisCO. This notion was supported by nearly the same frequency of positive clones using pKK223-3 and ptac12.

The specific enzyme activities of RuBisCO in the positive colonies were determined by assaying the enzyme activity as well as its content by ELISA [15] (table 1). The highest amount of RuBisCO produced in the cells of *E. coli* was approx. 15% of the total soluble protein after induction by IPTG for 8 h. Among the 10 positive transfor-

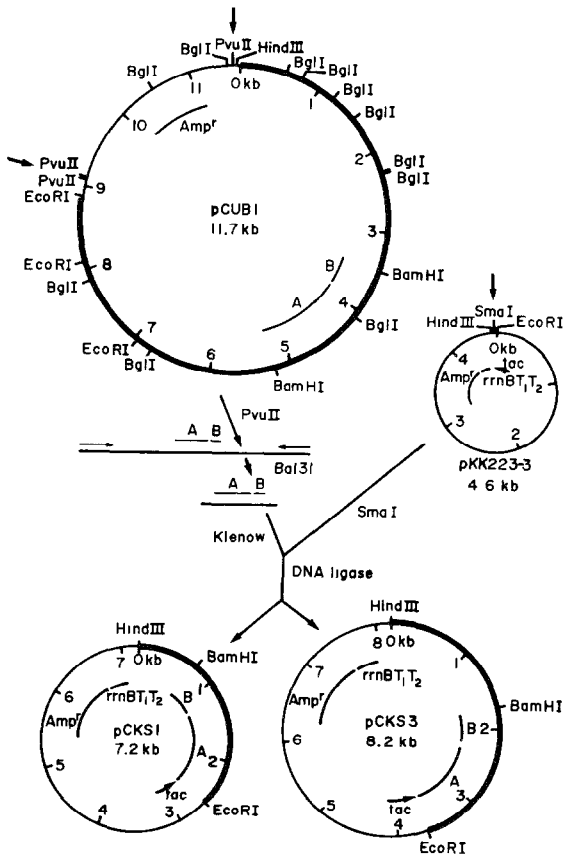

Fig.1. Diagrammatic representation of a strategy for preparing plasmids expressing RuBisCO. DNA fragments derived from *Chromatium* are represented by thick lines in the plasmids. *Chromatium* DNA was digested with *Bgl*II, ligated with the *Bam*HI site of pUC8 [6], and subsequently introduced into *E. coli* JM83 or JM103. RuBisCO genes were screened by DNA probes of genes for RuBisCO from *A. nidulans* which were kindly provided by Dr K. Shinozaki [7,8]. We have designated the plasmid pCUB1. A *Pvu*II fragment of 9.2 kb containing genes for subunits A and B of *Chromatium* RuBisCO was prepared from pCUB1 by agarose gel electrophoresis and electroelution [9], and digested with exonuclease Bal31 [9] for 20 min at 30°C to remove approx. 3 kbp from both ends of the fragment. The resulting about 3 kbp fragments were treated with Klenow fragment of *E. coli* DNA polymerase I for filling the ends [9], and ligated with *Sma*I-digested pKK223-3 (P-L, Pharmacia) by T_4 DNA ligase and T_4 RNA ligase to achieve efficient blunt end ligation [9]. After transforming *E. coli* JM105 with the produced plasmids, expressers of RuBisCO were screened according to the procedure described in section 2. Two of these, pCKS1 and pCKS3 were analyzed by several restriction enzymes for making construction maps.

Table 1

Specific enzyme activities of RuBisCO produced in *E. coli*

Preparation	Specific carboxylase activity (μmol CO_2/min per mg protein)
Chromatium	1.0
E. coli pCKS1	1.2
E. coli pCKS2	1.0
E. coli pCKS3	1.2
E. coli pCKS4	1.0
E. coli pCTP1	1.0
E. coli pCTP2	1.1
E. coli pCTP3	1.0
E. coli pCTP4	0.9
E. coli pCTP5	0.8
E. coli pCTP6	1.2
E. coli pCUB1	undetectable
E. coli pKK223-3	undetectable

E. coli cells were grown in 2 ml LB medium containing ampicillin, subsequently incubated with IPTG, and subjected to the preparation of extracts as described in section 2. Specific enzyme activities of RuBisCO were determined using extracts from the different positive colonies of *E. coli* JM105 transformed by the pCKS and pCTP series. RuBisCO content was determined by ELISA [15]

mants, the specific activities were nearly the same as that of the original RuBisCO in *Chromatium*. RuBisCO molecules were not synthesized as directed by the original promoter of RuBisCO genes on pCUB1 in *E. coli* (table 1).

The immunoblotting analysis showed the presence of the 2 constituent subunits (A and B) in *E. coli* cells transformed by pCKS1 (fig.2) as well as in the other expressers (not shown). The possibility of the presence of a degraded product of subunit A at the position of subunit B is excluded, and the presence of subunit B in the cellular extract of *E. coli* cells can be substantiated by detecting a band using the anti-native RuBisCO IgG but not anti-subunit A IgG (fig.2). It was observed that the ratio of subunit A to subunit B was nearly equal in the cellular extracts from *E. coli* and *Chromatium*, indicating that an equimolar amount of the 2 constituent subunits is present in the cells of *E. coli* (fig.2).

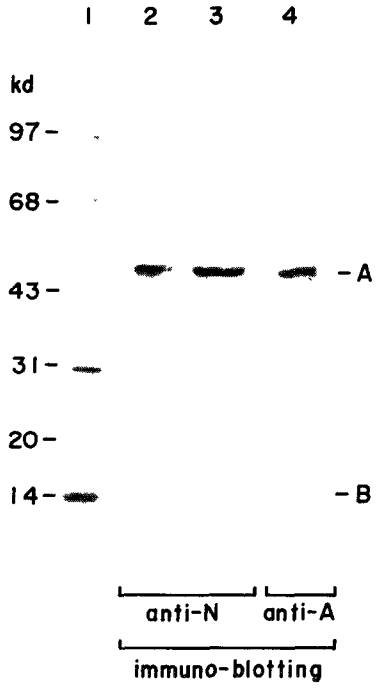

Fig.2. Immunoblotting analysis of RuBisCO synthesized by *E. coli* cells transformed by pCKS1. 1 μg purified *Chromatium* RuBisCO (lane 2) and 10 μg soluble protein from the *E. coli* extract (lanes 3 and 4) were first subjected to SDS-polyacrylamide gel electrophoresis (7.5–15% linear gradient) [16], and electrophoretically transferred to nitrocellulose filter (S&S PH79, 0.1 μm) [17]. Subunits of RuBisCO were detected by the specific antibody raised against the whole enzyme (lanes 2 and 3) or its purified subunit A (lane 4). Bands were detected using horseradish peroxidase-conjugated anti-rabbit goat IgG and 4-chloro-1-naphthol according to the instruction manual from Bio-Rad with washing buffers containing dry milk [18]. Molecular mass markers (in kDa) in lane 1 are 2 μg each of phosphorylase *b* (97), bovine serum albumin (68), ovalbumin (43), carbonic anhydrase (31), soybean trypsin inhibitor (20) and lysozyme (14), which are stained with Coomassie brilliant blue R-250.

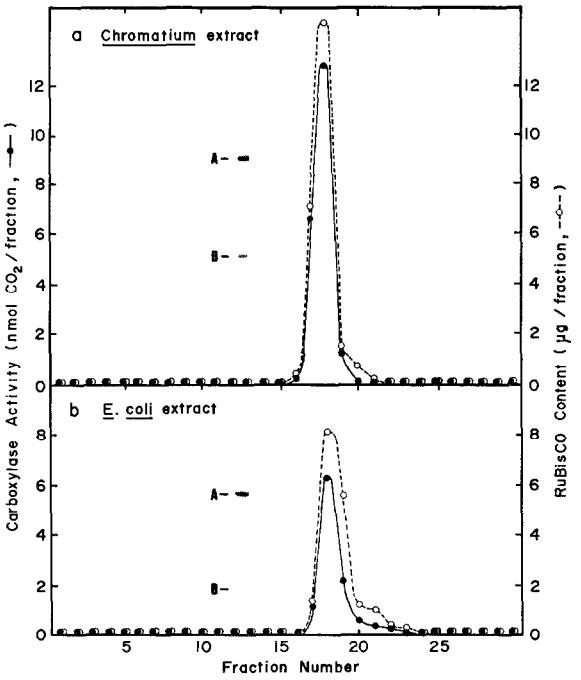

Fig.3. Analysis of RuBisCO produced by *E. coli* cells transformed by pCKS1 by gel filtration HPLC. The 26 μg purified *Chromatium* RuBisCO (a) and approx. 300 μg fresh soluble protein prepared from *E. coli* (b) were subjected to gel filtration HPLC using Superose 6 column (Pharmacia) equilibrated with 50 mM Na phosphate (pH 6.9), 1 mM EDTA, and 0.3 M NaCl. Elution was performed at a flow rate of 0.3 ml/min, and fractionated at 0.9 ml. Immediately after elution, both RuBP carboxylase activity and RuBisCO content were determined. The latter was done by ELISA using the antibody against whole *Chromatium* RuBisCO. The photographs superimposed in the figure represent the immunoblotting analysis of the main active fractions separated by the HPLC.

For the purpose of conclusively defining the quaternary structure of *E. coli* RuBisCO, an aliquot of the extracts from *E. coli* cells transformed by pCKS1 was subjected to gel filtration HPLC. As presented in fig.3, extracts from both *E. coli* and *Chromatium* showed identical elution profiles with respect to maximal RuBisCO activity as well as the enzyme content determined by ELISA. The estimated mass of the molecules at the peak fraction employing several molecular mass standards was 520 kDa in both cases, strongly indicating the A_8B_8 form of RuBisCO. To confirm this assumption, sucrose density gradient centrifugation was carried out. RuBisCO produced in *E. coli* cells co-migrated exactly with the 18 S native enzyme molecule from *Chromatium* (not shown). Therefore, we can reasonably conclude that the quaternary structure of RuBisCO synthesized in *E. coli* is identical with the native *Chromatium* enzyme.

For the biosynthesis and assembly of RuBisCO molecule in chloroplasts, at least 3 kinds of genetic information are reported to be required: (i) processing of the transit sequence of subunit B [2], (ii) processing of the amino terminal of subunit A [19], and (iii) a protein involved in the assembly of subunits A and B making up the symmetric structure of holoenzyme (A_8B_8) [2]. These notions together with the fact that *Chromatium*, a prokaryotic microorganism, produces a plant-type RuBisCO prompt us to further pursue the detailed mechanisms involved in posttranslational events. We have already reported the in vitro homologous reconstitution of enzymically active RuBisCO from the separated subunits of *Chromatium* RuBisCO [20]. Furthermore, since it has been reported that no transit sequence of subunit B exists in DNA sequences from cyanobacteria [8,21], it is conceivable that specific proteins may not be required in the step of subunit assemblage of *Chromatium* RuBisCO.

Several investigators have succeeded in expressing genes only for subunit A of maize RuBisCO [22,23] and *Rhodospirillum rubrum* RuBisCO [24,25] in *E. coli* cells using either the expression vectors or minicells of *E. coli*. The expression of genes for both subunits A and B in *E. coli* cells has been reported by Gatenby et al. [26] using the cyanobacterium *A. nidulans*: they achieved the synthesis of enzymically active RuBisCO. In this investigation, we present the experimental evidence for: (i) expression of genes for both subunits A and B of the plant-type RuBisCO derived from photosynthetic bacterium in *E. coli* using expression vectors, and (ii) assembly of 2 constituent subunits to make up the enzymically active A_8B_8 structure. We have succeeded in producing unfused peptides of subunits A and B in the bacterial cells in the highest yield ever reported. The presently employed experimental system may provide a further clue to clarification of the molecular mechanisms underlying the interaction of the 2 subunits and the regulation operating therein.

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