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Expression and assembly of active cyanobacterial ribulose-1,5-bisphosphate carboxylase/oxygenase in *Escherichia coli* containing stoichiometric amounts of large and small subunits

(hexadecameric *Escherichia coli* enzyme/small subunit role/high specific activity)

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ABSTRACT **The genes for the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase from the cyanobacterium *Anacystis nidulans* were subcloned into plasmid pUC9. After induction, both genes were expressed in *Escherichia coli* and the subunits were assembled into an active holoenzyme. The enzyme was purified from *E. coli* to high specific activity and was found to contain equimolar amounts of large and small subunits. The assembly of the hexadecameric ribulose bisphosphate carboxylase/oxygenase in *E. coli* should provide the basis for studies on the mechanism of assembly and the role of small subunits in catalysis.**

Ribulose-1,5-bisphosphate (Rbu-P_2) carboxylase/oxygenase is a bifunctional enzyme that catalyzes both the carboxylation and oxygenation of Rbu-P_2. This important enzyme catalyzes the key and primary reaction of the competing pathways of photosynthetic CO_2 fixation and photorespiration. Considerable research over the past several years has elucidated many of the details relative to activation and catalysis (1) as well as the contribution of both nuclear and plasmid genetic information in the coding of the two dissimilar subunits, large and small, which comprise the hexadecameric protein in eukaryotes (2). Rbu-P_2 carboxylase/oxygenase from most prokaryotic organisms is also hexadecameric and is comprised of the familiar eight large (catalytic) and eight small subunit arrangement (L_8S_8) (3). Of particular interest are the cyanobacteria (blue-green algae), organisms that possess a plant-type photosynthetic machinery, yet are typically prokaryotic in structure (4). Recent studies have shown that cyanobacteria contain an L_8S_8 Rbu-P_2 carboxylase/oxygenase (5–8) that is unusual in the fact that small subunits may be stripped from the holoenzyme by mild acid treatment (8–10). Moreover, the genes encoding both the large and small subunits of Rbu-P_2 carboxylase/oxygenase from the cyanobacteria *Anacystis* and *Anabaena* were found to be linked and cotranscribed (11, 12).

In this investigation we have taken advantage of the linkage of the large and small subunit genes of the *Anacystis* enzyme and introduced these genes into the expression vector pUC9 to obtain high levels of fully assembled and active enzyme in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were grown on tryptone/yeast extract (TYE) medium (1% tryptone/0.5% yeast extract/0.5% NaCl) containing appropriate antibiotics. Cultures used for purification of Rbu-P_2 carboxylase/oxygenase were grown in M9 medium (16) containing 0.1% vitamin-free Casamino acids. Antibiotic concentrations were ampicillin (Ap), 50 μg/ml; carbenicillin (Cb), 200 μg/ml; tetracycline (Tc), 12.5 μg/ml; and streptomycin (Sm), 25 μg/ml.

Restriction Enzyme Digestion and Agarose Gel Electrophoresis. All restriction enzymes were used in the buffer systems described previously (16). Agarose gel electrophoresis of DNA samples was performed in 0.8% agarose gels in Peacock's buffer (17).

Isolation of Plasmid DNA. Plasmid DNA for routine restriction enzyme analysis was isolated from *E. coli* by the rapid boiling method (18).

Subcloning of the *Anacystis* Rbu-P_2 Carboxylase/Oxygenase Fragment. The *A. nidulans* 6301 Rbu-P_2 carboxylase/oxygenase DNA fragment containing large subunit and small subunit sequences was subcloned from pANP1155 into pUC9. Plasmid pANP1155 was isolated by the rapid boiling method (18). Following *Pst* I digestion of the pANP1155 DNA and pUC9 DNA, ligation was performed (19). The pUC9-derived plasmid was transformed into *E. coli* JM105 with selection on TYE agar at 200 μg of Cb per ml and 40 μg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (XGal) per ml (19). Plasmid DNAs from white colonies were then examined by *Pst* I digestion for confirmation of insertion; this was followed by *Eco*RI digestion for orientation determination.

Preparation of *E. coli* Plasmid-Containing Extracts for Enzyme Assay. Typically, early- to midlogarithmic-phase cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and allowed to express Rbu-P_2 carboxylase/oxygenase in the M9 medium for 12–15 hr prior to harvesting. Cultures of 500 ml were grown in 2-liter Erlenmeyer flasks at 37°C with shaking at 145 rpm. For large amounts of cell material, 4-liter flasks containing 1 liter of medium were employed. The inoculum for all cultures was 3 ml of an overnight culture grown in TYE medium with 200 μg of Cb per ml. Late-logarithmic early stationary-phase cultures of *E. coli* plasmid-containing strains were harvested by centrifugation, washed in TEM buffer (25 mM Tris·HCl, pH 8.0/1 mM EDTA/10 mM 2-mercaptoethanol), and repelleted in preweighed centrifuge tubes. Cell pellets were resuspended in TEM buffer with 1 mM phenylmethylsulfonyl fluoride at 0.54–0.60 g (wet weight) of cells per ml and lysed by passage through a French pressure cell at 1010 atm (1 atm = 1.013×10^5 Pa). Cell debris was removed by centrifugation at 106,000 × *g* for 1 hr to yield a crude extract.

Rbu-P_2 Carboxylase/Oxygenase. Whole cell assays on *E. coli* plasmid-containing strains were performed by harvesting 10 ml of culture by centrifugation. Cell pellets were washed once in 5 ml of 50 mM Mops buffer (pH 7.2), repelleted, and finally resuspended in 200 μl of 50 mM Mops buffer. Cells

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Abbreviations: Rbu-P_2, ribulose-1,5-bisphosphate; kbp, kilobase pair(s); Cb, carbenicillin; Ap, ampicillin; Tc, tetracycline; IPTG, isopropyl β-D-thiogalactopyranoside; TYE, tryptone/yeast extract; ^{R}, resistant; Sm, streptomycin.

Table 1. Bacterial strains and plasmids

	Genotype or phenotype	Source
Bacteria		
E. coli JM105	Δ(*pro-lac*) *strA hsdR* F'*traD*36 *proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* ΔM15	D. T. Gibson; ref. 15
E. coli JM103	Δ(*pro-lac*) *thi strA supE endA, sbcB dsdR*$^-$ F'*traD*36 *proAb lacI*^{q} ΔM15	C. R. Somerville; ref. 14
Plasmids		
pANP1155	Tc^R, containing *A. nidulans* Rbu-P_2 carboxylase/oxygenase gene fragment	K. Shinozaki; ref. 11
pUC9	Ap^R *lacZ* (ΔM15), unique *Pst* I site	J. R. Walker; ref. 15
pCS75	Ap^R *lac*$^-$ (pUC9), containing *A. nidulans* DNA in orientation I	This study
pCS152	Ap^R *lac*$^-$ (pUC9), containing *A. nidulans* DNA in orientation II	This study

^{R}, Resistant; *A. nidulans, Anacystis nidulans*.

were permeabilized with 200 μl of toluene. The extracted cells were withdrawn from the aqueous (lower) phase and assayed for Rbu-P_2-dependent carboxylase activity by the whole-cell $^{14}CO_2$ fixation assay (20). Cell extracts were assayed as described (21).

Purification of Rbu-P_2 Carboxylase/Oxygenase from Plasmid-Containing *E. coli* Strains. The crude extract was treated with powdered ammonium sulfate to yield the fraction precipitating between 25% and 45% saturation as described (22). The precipitate resulting from the ammonium sulfate treatment contained nearly all of the Rbu-P_2 carboxylase/oxygenase and was resuspended in a 10-ml volume of TEM buffer and dialyzed overnight against 2 liters of TEM buffer. After dialysis, the ammonium sulfate fraction was loaded onto a 2.5 × 35 cm DEAE-cellulose column equilibrated in the same buffer. After passage of at least 1 liter of buffer, 1.25 liter of a gradient of 0–0.2 M NaCl in TEM buffer was passed through the column and this was followed by a 600-ml gradient of 0.2–0.35 M NaCl in TEM buffer. The protein content of column fractions was estimated by the absorbance at 280 and 260 nm (23) and Rbu-P_2 carboxylase/oxygenase assays were performed on each fraction. Fractions containing the majority of the carboxylase activity also coincided with the maximal absorbance at 280 nm. These fractions were pooled. The pooled fractions were concentrated by ultrafiltration to a volume of 2 ml. This material was then loaded onto a 0.2–0.8 M sucrose step gradient as described (24). The sucrose gradient tubes were centrifuged for 22 hr in a Beckman SW28 swinging bucket rotor at 25,000 rpm. Fractions of about 1.0 ml were collected after puncturing the bottom of the tubes with a 22-gauge needle. Each fraction was analyzed for protein and Rbu-P_2 carboxylase/oxygenase activity. $NaDodSO_4$ gel electrophoresis of each active fraction in 0.75-mm 11% acrylamide slab gels (25) was employed to assess purity. Densitometric scans of stained gels were performed with a Joyce–Loebl model 201 densitometer.

RESULTS

Plasmid pANP1155, a hybrid pBR322 molecule containing a 2.3-kilobase-pair (kbp) fragment from *A. nidulans* DNA that codes for the large and small subunit of Rbu-P_2 carboxylase/oxygenase (11), was digested with *Pst* I to liberate the cloned *A. nidulans* DNA. This material was then ligated into pUC9 to study the possible translation products while under *lac* promoter control. Restriction endonuclease analyses revealed subclones with the *A. nidulans* DNA inserted in opposite orientations. Orientation I (pCS75) contains the fragment with the large subunit region proximal to the *lac* promoter, whereas orientation II has the small subunit coding region proximal to the *lac* promoter (Fig. 1). It is thought the two genes are cotranscribed in a large subunit to small subunit direction (11, 12). Therefore, orientation I was presumed to be positioned properly for proper transcription from the *lac* promoter.

E. coli strains with plasmid subclones in both orientations were examined for possible expression of Rbu-P_2-dependent carboxylase activity. Plasmids pCS75 and pCS152 were examined under induced and noninduced conditions with the gratuitous inducer IPTG. Plasmid pCS75, with the fragment in orientation I, showed substantial activity with assays of whole cells and crude extracts, whereas pCS152, with the fragment in the opposite orientation, yielded negligible activity (Table 2). Studies were initiated to examine the optimal harvest times after induction with IPTG, and in all cases the amount of activity obtained was directly related to the optical density of the culture. To minimize the potential for proteolysis, cultures were usually harvested at late-logarithmic phase.

After cells were disrupted, the crude extract had a specific activity of 0.37 μmol of CO_2 fixed per min/mg of protein. This material was further fractionated by ammonium sulfate precipitation and DEAE-cellulose chromatography to yield a single distinct peak of activity that coeluted with the major protein peak (Fig. 2*A*). Peak fractions were pooled and yielded an average specific activity of between 3.7 and 4.5. Portions of this material were either concentrated or applied directly to a 0.2–0.8 M sucrose gradient. Purified spinach Rbu-P_2 carboxylase/oxygenase was centrifuged alongside

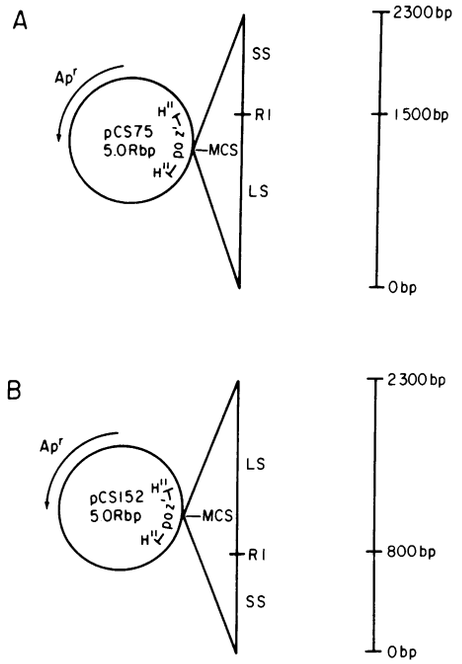

FIG. 1. Restriction maps of hybrid plasmids pCS75 and pCS152. The 2.3-kbp insert DNA in plasmid pANP1155 was subcloned into the *Pst* I site of pUC9. The pUC9 hybrids with insert DNA in orientation I and orientation II (pCS75 and pCS152, respectively) are shown with insert DNA offset in *A* and *B*. MCS is a multiple cloning site (15). LS, large subunit of Rbu-P_2 carboxylase/oxygenase; SS, small subunit of Rbu-P_2 carboxylase/oxygenase; RI, *Eco*RI. bp, Base pairs.

Table 2. Expression of the cloned Rbu-P_2 carboxylase/oxygenase genes in *E. coli*

Hybrid plasmid	Orientation of insert	Specific activity of Rbu-P_2 carboxylase/oxygenase, units/liter·OD_{650}
pCS75	I, induced*	54.0
pCS75	I, uninduced	2.0
pCS152	II, induced	0.02
pCS152	II, uninduced	0.02

*Cells were induced with 1 mM IPTG and harvested after reaching the late-logarithmic phase of growth.

the *E. coli* (pCS75) cyanobacterial enzyme to determine the relative size of the latter preparation. Fractions from both gradients were collected and a large peak of activity, corresponding to the major protein peak, was eluted at the bottom half to lower third of the gradient (Fig. 2*B*). Spinach Rbu-P_2 carboxylase/oxygenase migrated slightly faster than the *E. coli* (pCS75) enzyme. The specific activity of peak sucrose gradient fractions of the *E. coli* (pCS75) enzyme ranged from 3.6 to 4.5 and showed a single stained band on nondenaturing polyacrylamide gels.

$NaDodSO_4$/polyacrylamide slab gel electrophoresis and subsequent staining of the *E. coli* (pCS75) enzyme revealed the presence of large and small subunits (Fig. 3) of about M_r 56,000–57,000 and M_r 12,000, respectively. The sucrose gradient removed several lightly staining contaminants from the DEAE-cellulose fraction. The molar ratio of large subunits to small subunits is about 1:1 for the cyanobacterial enzyme (8). The ratio of the molecular weights of small subunits to large subunits of the recombinant cyanobacterial enzyme was found to be 0.21, which is slightly less than the ratio of 0.25 obtained by comparison of the molecular weights deduced by nucleotide sequencing (11). After densitometric scanning of the stained gel (Fig. 3) the ratio of the area of the small subunit peak to the area under the large subunit peak was found in several preparations to range from 0.20 to 0.22, similar to results obtained previously with other cyanobacterial enzymes (8). These results suggest that the *E. coli* (pCS75) enzyme is composed of equimolar ratios of large and small subunits. We have also found that the amount of small subunit observed in $NaDodSO_4$ gels may be dependent on the conditions of dissociation and digestion. Best results were obtained by using a 5-min digestion period in boiling water with the standard digestion buffer (25).

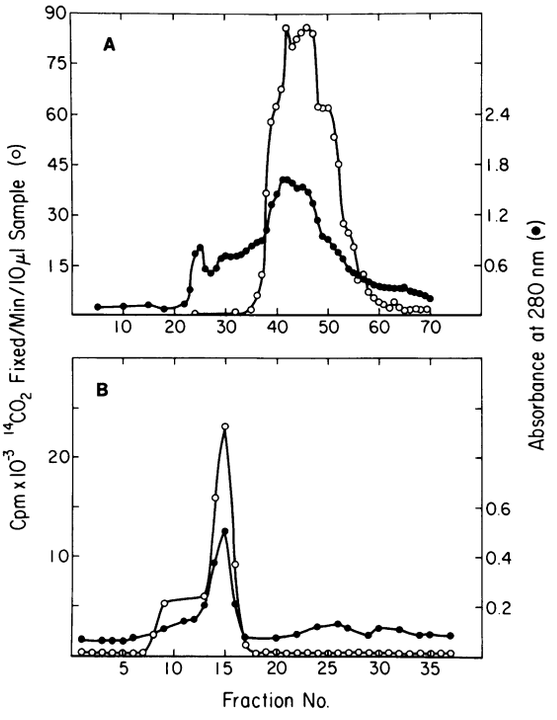

FIG. 2. (*A*) DEAE-cellulose chromatography of *E. coli* (pCS75) Rbu-P_2 carboxylase/oxygenase. The data represent only those fractions collected after the 0.2–0.35 M NaCl gradient was begun. (*B*) Sucrose gradient fractionation of a portion of the DEAE-cellulose fraction.

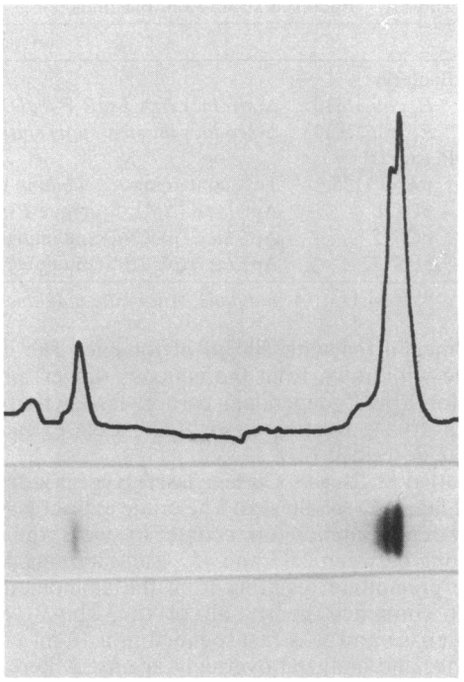

FIG. 3. $NaDodSO_4$/polyacrylamide slab gel of purified *E. coli* (pCS75) Rbu-P_2 carboxylase/oxygenase after sucrose gradient fractionation. Protein was stained with Coomassie blue. A densitometric trace is shown above the gel.

DISCUSSION

Several recent investigations have reported and stressed the importance of the small subunit of Rbu-P_2 carboxylase/oxygenase to the activity of the large catalytic subunit (9, 10, 26). These studies take advantage of the acid-stimulated removal of the majority of the small subunits of the cyanobacterial protein (9), the alkali-dependent removal of small subunits from the *Chromatium* enzyme (10, 26), or the lowering of the ionic strength of preparations of Rbu-P_2 carboxylase/oxygenase from the halophile *Aphanothece* (27). In each case, removal of small subunits correlates with a loss of activity, which can be restored by the addition of small subunits to reconstitute the holoenzyme. At this time, however, little is known of the mechanism of subunit assembly and nothing is known of the precise function of small subunits, since isolated large subunits still contain unaffected activator and catalytic sites in the absence of small subunits (26, 28). To probe the function of the small subunit as well as discern the sites on both subunits necessary for assembly, we have begun a program of investigation based on recombinant DNA procedures. In this report, we demonstrate the insertion and expression in *E. coli* of a 2.3-kbp region containing the genes of the large and small subunits of Rbu-P_2 carboxylase/oxygenase from the cyanobacterium *A. nidulans*. These genes, under *lac* control in the expression vector pUC9, have been shown to yield substantial levels of Rbu-P_2-dependent carboxylase activity upon induction with the gratuitous inducer IPTG. Although optimal levels of enzyme were

produced when IPTG was added, in the absence of inducer, there still were low, but significant, levels of activity. This low basal level of activity is probably due to the high copy number of pUC9 and consequent titration of *lac* repressor molecules produced by a single chromosomally located gene. Similar results were obtained when the *Rhodopseudomonas sphaeroides* form II gene was expressed in *E. coli* (29). The enzyme from *Anacystis* is a hexadecamer consisting of eight identical large subunits and eight identical small subunits. Both genes are present on a 2.3-kbp region and are thought to be cotranscribed in a large subunit to small subunit direction (11). In fact, only in orientation I, with the large subunit gene proximal to the *lac* promoter, was the production of highly active enzyme detected. To determine if the enzyme was assembled in a proper stoichiometry of large and small subunits in *E. coli*, we purified the protein and examined its subunit structure.

The ratio of the molecular weights of the *E. coli* (pCS75) Rbu-P_2 carboxylase/oxygenase subunits as well as the ratio of the masses of the subunits indicated that assembly to the holoenzyme proceeded normally in *E. coli*. Certainly, the very fact that enzyme of high specific activity is formed and is easily isolated strongly suggests that an enzyme molecule close to or identical to the hexadecameric structure is formed, since activity is directly dependent on the proper arrangement of subunits (8–10). These results, combined with the finding that the native *E. coli* (pCS75) enzyme essentially comigrates with the spinach enzyme in sucrose gradients, indicate that the normal L_8S_8 structure (with a native M_r of about 500,000) is formed. The heterogeneity of large subunits seen in our preparation is a common occurrence with Rbu-P_2 carboxylase/oxygenase and perhaps may be eliminated by the use of additional proteolytic inhibitors or the employment of a more rapid purification regimen (8).

It appears that the contiguous location of large and small subunit genes on the cyanobacterial chromosome may be a general characteristic of these organisms, providing a rationale for seeking to obtain expression of these genes in *E. coli* for subsequent studies of the mechanism of assembly. Recently, the large and small subunit genes of the cyanobacterium *Spirulina platensis* were cloned and found to be closely placed on a 4.6-kbp DNA fragment. Convincing evidence for the expression of large subunits in *E. coli* minicells was obtained but the amount of small subunits produced was minimal, which may correlate with the lack of enzyme activity produced in this system (30). Gatenby *et al.* (31) cloned the 2.17-kbp *Pst* I fragment of *Synechococcus* 6301 DNA containing the large and small subunit genes into the *Pst* I site of plasmid pLa2311, which is under control of the P_L promoter. Immunoblotting studies, using anti-wheat Rbu-P_2 carboxylase/oxygenase, showed the synthesis of large and small subunits in *E. coli*, as well as a M_r 72,000 fusion protein, after temperature induction of cells containing the hybrid pLa2311 plasmid. In this study, it was also apparent that there was a lack of completely assembled enzyme of high specific activity in crude extracts. In the present investigation, we were able to obtain expression and assembly of the large and small subunits of *A. nidulans* Rbu-P_2 carboxylase/oxygenase to produce a highly active hexadecameric protein in *E. coli*, raising the tantalizing possibility that oligonucleotide site-specific mutagenesis might reveal site(s) within the small subunit necessary for reconstitution and subsequent catalysis. Requisite important sites for assembly that are found on the large subunit may also be probed by these procedures. It is also worth noting that a convenient *Eco*RI site exists between the large and small subunit genes on the 2.3-kbp *Pst* I fragment of the *Anacystis* chromosome. This *Eco*RI site has allowed us to separately subclone the large subunit gene in pUC9 and small subunit gene in pUC8. Thus, reconstitution studies of separately expressed large and small subunits may be initiated without the complication of residual contaminating small subunits commonly encountered with large subunit preparations of cyanobacterial Rbu-P_2 carboxylase/oxygenase prepared from holoenzyme by *in vitro* procedures (8, 32).

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