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Pathway of assembly of ribulosebisphosphate carboxylase/oxygenase from *Anabaena* 7120 expressed in *Escherichia coli*

(enzyme assembly)

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ABSTRACT **We have placed the genes encoding ribulosebisphosphate carboxylase/oxygenase from the *Anabaena* 7120 operon under transcriptional control of the *lac* promoter carried on the *Escherichia coli* plasmid pUC19. The genes encoding both the large and small subunit polypeptides (*rbcL* and *rbcS*) are transcribed and translated so that ≈0.6% of the soluble protein in *E. coli* extracts is a fully functional holoenzyme with a sedimentation coefficient of approximately 18S, which contains stoichiometric amounts of the two subunits. However, expression of the large subunit polypeptide vastly exceeds that of the small subunit because the majority of transcripts terminate in the intergenic region between the *rbcL* and *rbcS* genes. As a result, excess large subunit is synthesized and accumulates in *E. coli* as an insoluble and catalytically inactive form. Because small subunit is found only in the high molecular weight soluble form of ribulosebisphosphate carboxylase/oxygenase, we propose that the small subunit promotes assembly of the hexadecameric form of the enzyme via heterodimers of large and small subunits.**

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the enzyme responsible for photosynthetic CO_2 fixation in all photosynthetic organisms. The most widespread form of the enzyme is composed of eight large subunits of ≈55 kDa and eight small subunits of 12–18 kDa (1). From preliminary x-ray diffraction analysis, the large subunits appear to be arranged so that the geometric center of the folded polypeptides describe the eight corners of a slightly flattened cube (2). The arrangement of the small subunits in this structure is not known. In photosynthetic eukaryotes, RuBisCO is located in the chloroplast and the *rbcL* genes for the large subunit are chloroplast encoded, whereas the small subunit genes (*rbcS*) are nuclear. Thus, the small subunit polypeptide is translated as a precursor that carries an amino-terminal transit peptide required for import of the small subunit polypeptide into the chloroplast (3). There is some uncertainty about the sequence of events that intervene between transport of the small subunit through the membrane and the formation of the hexadecameric L_8S_8 holoenzyme. However, from studies of the sedimentation properties of putative intermediates labeled *in vivo*, it has been suggested that an assembly protein may be involved in formation of the holoenzyme (3–6).

The RuBisCO from cyanobacteria is very similar to the eukaryotic form of the enzyme in that it is composed of eight large and eight small subunits. Both the *rbcL* and *rbcS* genes have been cloned and sequenced from *Anabaena* 7120 and *Anacystis nidulans* (7, 8). The homology between the *rbcL* genes of higher plants and cyanobacteria is ≈75% at the nucleotide sequence level and ≈85% at the protein sequence level (1). The cyanobacteria appear to have satisfied the requirement for stoichiometric synthesis of large and small subunits by arranging the *rbcL* and *rbcS* genes contiguously in a single transcriptional unit (9). The absence of organelles in the cyanobacteria obviates the need for a transit peptide so that the *rbcS* gene product appears to be translated in the mature form.

We are interested in developing methods that would permit analysis of RuBisCO subunit interactions and holoenzyme assembly and elucidation of RuBisCO function by directed mutagenesis of cloned genes. Toward this end, we have previously described the construction of an expression plasmid that directs high level expression of the *Rhodospirillum rubrum* RuBisCO in *Escherichia coli* (10). We and others have also obtained high level expression of the *Zea mays rbcL* gene in *E. coli* (11, 12). However, under these circumstances, the *Z. mays* large subunit polypeptide is insoluble and catalytically inactive, presumably because of the absence of the small subunit. We have, therefore, investigated the potential utility of the RuBisCO operon from *Anabaena* 7120 as a suitable system for the production of a hexadecameric RuBisCO in *E. coli*. We report here the construction of an expression plasmid for this operon, and an analysis of the properties of the transcription and translation products in *E. coli*. Our results indicate that the enzyme assembles via an ordered pathway that requires the formation of heterodimers (L_1S_1) as an obligate first step.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strains used were HB101 (F⁻ *pro leu thi lacY hsdR endA recA rpsL ara galK xyl mtl supE44*), JM83 [F⁻Δ(*lac-pro*) *ara rpsL φ80dlacZM15*] and JM109 [F′(*traD36 proAB*⁺ *lacZΔM15I*^{q})Δ(*lac-pro*) *recA thi rpsL endA gyrA hsdR supE44 relA*]. Plasmids pUC18 and pUC19 have been described (13). Plasmid pAn601, which carries the *rbcL* and *rbcS* genes of *Anabaena* 7120 on a 6.6-kilobase (kb) *Eco*RI/*Hin*dIII partial digest fragment (7), was generously provided by R. Haselkorn.

DNA Manipulations. Plasmid DNA preparations, ligations, transformations, and all routine nucleic acid manipulations were carried out by established methods (14). DNA sequence analysis was performed according to the method of Maxam and Gilbert (15).

DNA/RNA Hybridizations. Total RNA from *E. coli* was isolated as described (16). Aliquots of RNA were partially hydrolyzed in Tris·HCl (pH 9.0) at 60°C for 30 min; they were

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Abbreviations: CABP, 2-carboxyarabinitol bisphosphate; RuBisCO, D-ribulose 1,5-bisphosphate carboxylase/oxygenase; *rbcS*, small subunit gene of RuBisCO; *rbcL*, large subunit gene of RuBisCO; kb, kilobase(s); bp, base pair(s).

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then phenol-extracted and ethanol-precipitated. The hydrolyzed RNA was end-labeled with ^{32}P by the polynucleotide kinase reaction (14). DNA restriction fragments were resolved in 1% (wt/vol) agarose gels and transferred to nitrocellulose filters. The filters were then probed with the labeled RNA in 4× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate) containing 50% formamide at 42°C, dried, and autoradiographed (14).

Immunological Detection of RuBisCO. RuBisCO was purified from *Anabaena* 7120 essentially as described (17) and was used to induce production of antibodies against both large and small subunit polypeptides in rabbits (18). The presence of antigen in bacterial colonies was determined by immunohistochemical staining of nitrocellulose replicas of lysed bacterial colonies essentially as described (19). Identification of RuBisCO subunits resolved in $NaDodSO_4$/polyacrylamide gels was accomplished by electrophoretically transferring the proteins to nitrocellulose filters (20), allowing the filters to react with anti-RuBisCO antibody, then visualizing bound antibody with alkaline phosphatase-linked protein A (Sigma).

Quantitation of RuBisCO Subunits. *E. coli* cells were labeled by growing 30- to 50-ml cultures to midlogarithmic phase (OD_{600nm} = 0.5) in L broth (14) containing ampicillin (50 mg/ml) then adding [^{35}S]methionine (2–3 μCi/ml; 1320 μCi/μmol; 1 Ci = 37 GBq). Cells were harvested after 3 hr, washed, resuspended in assay buffer (50 mM Tricine, pH 8.0/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride), sonicated, and the lysate was centrifuged at 100,000 × *g* for 30 min. The RuBisCO in a 300-μl aliquot of the supernatant was immunoprecipitated with 100 μl of crude antiserum and 10 mg of formalin-fixed *Staphylococcus aureus* cells (21). The immunoprecipitated proteins were then resolved in $NaDodSO_4$/polyacrylamide gels and visualized by autoradiography. The radioactivity in specific bands was determined by cutting out the region of the gel and infiltrating it with scintillation fluid for 24 hr before measuring radioactivity by scintillation counting.

Preparation and Assay of RuBisCO. Cells were grown to midlogarithmic phase in L broth containing ampicillin (50 μg/ml) at 37°C, harvested by centrifugation, and resuspended in assay buffer. The cell suspension was disrupted by sonication and cell debris was removed by centrifugation at 30,000 × *g* for 20 min. The extract was then dialyzed against assay buffer. RuBisCO was activated before assay by adjusting the sample to 10 mM $NaH^{14}CO_3$ (1 μCi/μmol) and incubating for 20 min at 22°C, then initiating the assay by adding ribulosebisphosphate to 0.4 mM (22). The assay was terminated after 1–3 min with 100 μl of 6 M acetic acid, dried at 85°C, and acid-stable radioactivity was determined by scintillation counting. The amount of active RuBisCO was determined by adjusting extracts in assay buffer to 20 mM $MgCl_2$/20 mM $NaHCO_3$/15 μM 2-[^{14}C]carboxyarabinitol bisphosphate ([^{14}C]CABP) (56.5 μCi/μmol). After 1 hr at 22°C the amount of [^{14}C]CABP bound as enzyme–CO_2–Mg^{2+}–CABP quaternary complex was determined by chromatography on a 0.8 × 28 cm Sephadex G-50 column (22). CABP was a generous gift from G. Lorimer and J. Pierce (DuPont).

RESULTS

Construction of an Expression Plasmid. The *rbc* operon from *Anabaena* occupies ≈3.1 kb of chromosomal DNA, which includes a 474-base-pair (bp) leader sequence and a 552-bp noncoding spacer between the *rbcL* and *rbcS* genes (9) (Fig. 1). A DNA fragment containing these genes and ≈4.6 kb of flanking sequence was obtained by partial digestion of plasmid pAn601 (7) with *Kpn* I and complete digestion with *Bam*HI. The *Kpn* I/*Bam*HI fragment was isolated from an agarose gel and partially digested with *Hpa* I to remove noncoding 5′ sequences. A DNA fragment of the desired length was isolated from an agarose gel, ligated into the unique *Hin*cII/*Bam*HI site of plasmid pUC19, and transformed into *E. coli* strain JM83. The resulting transformants carried a plasmid with the expected restriction map, which has been designated pAnH27 (Fig. 1). In this plasmid, the 5′ noncoding sequence of the *Anabaena rbcL* gene is fused to the *Hin*cII site of the polylinker in pUC19 so that the amino-terminal ATG codon of the *rbcL* gene is 204 bp from the *Hin*cII site.

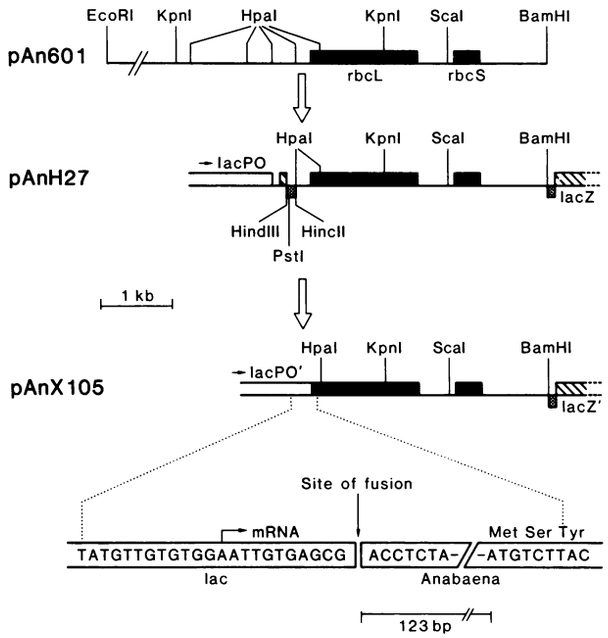

FIG. 1. Construction of the RuBisCO expression plasmid pAnX105. The coding regions of the *rbc* genes are indicated by heavy lines. Plasmid pAn601 is pBR322 in which the small *Eco*RI/*Hin*dIII fragment has been replaced with 6.6 kb of *Anabaena* DNA. Plasmid pAnH27 is pUC19 with a *Hpa* I (partial)/*Bam*HI fragment from pAn601 inserted into the *Hin*cII/*Bam*HI sites. Plasmid pAnX105 was obtained from pAnH27 by introducing a bidirectional deletion with BAL-31 nuclease which removed 145 bp from the 5′ side of the *Hin*cII site in the *lacZ′* gene and 81 bp of the 5′ noncoding *Anabaena* DNA.

To evaluate whether plasmid pAnH27 would direct the synthesis of RuBisCO in *E. coli*, we assayed whole cell extracts of JM83 carrying the plasmid for ribulosebisphosphate-dependent $^{14}CO_2$ fixation, but we did not detect activity (Table 1). Also, comparison of the polypeptide pattern of whole cell extracts from *E. coli* cells with and without plasmid pAnH27 in $NaDodSO_4$/polyacrylamide gels failed to

Table 1. RuBisCO activity in crude extracts of *E. coli* strains carrying various plasmids

Strain	Plasmid	Inducer	RuBisCO activity, nmol·min^{-1} per mg of protein
JM83	None	–	0
JM83	pAnH27	–	0
HB101	pAnX105	–	23.6
JM109	pAnX105	–	1.3
JM109	pAnX105	+	1.4

The cultures were grown to late logarithmic phase in L broth at 37°C. *lac*-promoted transcription was induced, where indicated, with 0.1 mM isopropyl β-D-thiogalactoside. JM109 cells were grown to early logarithmic phase and this is, in part, responsible for the lower specific activity recorded.

show any difference that might suggest expression of either *rbc* gene (results not presented). We assumed that the lack of expression of RuBisCO from pAnH27 was most probably due to a lack of transcription of the coding sequences of the *rbc* operon in *E. coli*, presumably because of the presence of a transcription termination signal in the 5′ noncoding region of the *rbc* operon DNA. Therefore, we undertook to remove 5′ noncoding sequences in order to delete possible termination sequences and bring the coding region of the *rbcL* gene closer to the start site of *lac*-promoted transcription. Plasmid pAnH27 was linearized with *Pst* I, which cleaves at a site 208 bp in the 5′ direction from the *rbcL* ATG codon, and a family of deletions was obtained by treating with BAL-31 exonuclease. The resulting deletion clones were then screened for expression of RuBisCO in *E. coli* JM83 by lysing replicas of the colonies containing the family of plasmids on nitrocellulose filters then screening the protein bound to the filters for the presence of RuBisCO by immunohistochemical methods (19).

Among ≈600 colonies screened for the expression of RuBisCO, 4 exhibited a positive reaction. The plasmid in one of these colonies, designated pAnX105 (Fig. 1), was examined in detail because it exhibited the strongest positive immunological reaction. When extracts from cultures of HB101 or JM83 carrying pAnX105 were assayed for activity, a relatively high level of RuBisCO activity was evident (Table 1). Although we did not attempt to examine all possible variables of culture conditions, the highest amount of activity was generally obtained in late logarithmic phase cultures (OD_{600nm}, >2) grown in L broth at 37°C. The amount of enzyme in crude extracts was measured by quantitating the binding of ^{14}CABP, a transition state analog that exhibits very tight binding to RuBisCO (22, 23). By this method, the amount of RuBisCO accumulated by strain HB101 (pAnX105) or JM83 (pAnX105) was estimated to be ≈0.6% of the soluble protein in a crude whole cell extract. On this basis, it was also possible to estimate the specific activity of the enzyme as ≈3.7 μmol per mg of protein per min, which is slightly higher than that obtained previously for *Anabaena* (24).

To determine whether RuBisCO activity was constitutively expressed from pAnX105, the plasmid was transformed into *E. coli* strain JM109. This strain carries a *lacI^q* mutation, which results in overproduction of the *lac* repressor, thereby causing effective repression of *lac*-promoted transcription from multicopy plasmids such as pUC19. Cultures of JM109 carrying the plasmid were found to have the same level of RuBisCO activity with and without the addition of the *lac* inducer isopropyl β-D-thiogalactoside to the growth medium (Table 1). The probable reason for this lack of transcriptional control was deduced by DNA sequence analysis of the *rbcL* 5′ flanking region in pAnX105 (Fig. 1). It appears that the BAL-31-induced deletion on this plasmid extends into the region that binds *lac* repressor (25). Thus, the plasmid pAnX105 has a *lac* operator-constitutive mutation.

Analysis of Transcription. The amount of functional RuBisCO accumulated in *E. coli* cells carrying pAnX105 was considered relatively low by comparison with other examples of *lac*-promoted gene expression (10). As a first step in the analysis of possible reasons for low levels of expression, we investigated the level of transcription of the two *rbc* genes in pAnX105. The plasmid was digested with *Hpa* I, *Sca* I and *Bam*HI in order to separate the *rbcL* and *rbcS* genes (Fig. 1). The DNA fragments were resolved by gel electrophoresis, transferred to nitrocellulose, and probed with labeled RNA from cells carrying pAnX105. The results of this experiment (Fig. 2) revealed that the *rbcL* gene was expressed at relatively high levels compared to the gene encoding β-lactamase, which is carried on the same plasmid. By contrast, relatively little RNA hybridized to the restriction fragment

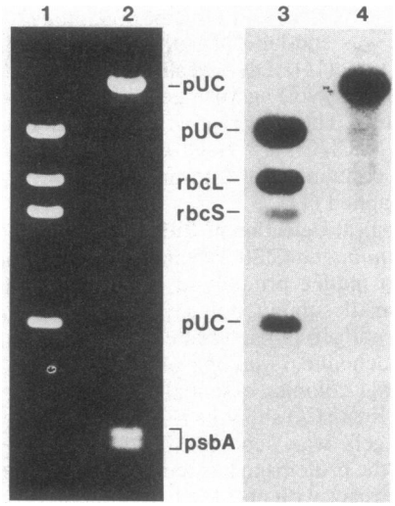

FIG. 2. Transcription of *rbc* genes in *E. coli*. Lane 1, restriction fragments from digestion of pAnX105 with *Hpa* I, *Sca* I, and *Bam*HI stained with ethidium bromide. Lane 2, *Hin*dIII digest of pUC9 derivative (included as a control). Lane 3, autoradiogram of a Southern blot of the restriction fragments resulting from digestion of pAnX105 with *Hpa* I, *Sca* I, and *Bam*HI. The filter was probed with total RNA from *E. coli* strain HB101 (pAnX105). Lane 4, control (identical to lane 2) showing similar intensity of hybridization of total RNA to pUC9 but with no hybridization to *psbA* sequences. The restriction fragments labeled pUC do not contain any of the *rbc* genes and carry the gene encoding ampicillin resistance. The position in pAnX105 of the restriction fragments designated *rbcL* and *rbcS* may be deduced from Fig. 1. The two fragments designated pUC are heavily labeled because each contains part of the gene encoding β-lactamase. The restriction fragments labeled *psbA* did not hybridize to RNA from pAnX105 and were included as a control.

carrying the *rbcS* gene. On the basis of amounts of radioactivity bound to appropriate regions of the filter (data not presented), it was estimated that the *rbcL* transcript was ≈50 times more abundant than the *rbcS* transcript. This observation suggests that the rate of transcription of the *rbcL* and *rbcS* genes is very different, presumably because of premature termination of transcription in the intergenic region.

The Physical Properties of RuBisCO. Analysis of the polypeptide composition of soluble extracts from *E. coli* strains carrying pAnX105 in $NaDodSO_4$/polyacrylamide gels revealed the presence of an abundant 55-kDa protein that comigrated with a RuBisCO standard. However, the small subunit could not be resolved by this method from among the many polypeptides in the 12- to 18-kDa region of the gel (Fig. 3, lane E). The presence of the small subunit in cultures of *E. coli* HB101 (pAnX105) was demonstrated by immunoprecipitating RuBisCO from catalytically active soluble extracts (Fig. 3, lane G). The insoluble fraction, which remained as a pellet after centrifugation of cell-free extracts of cells carrying pAnX105, also contained large amounts of insoluble large subunit polypeptide (Fig. 3, lanes B and D). By contrast, no small subunit polypeptide was detected in the insoluble fraction by immunohistochemical staining of nitrocellulose replicas of a $NaDodSO_4$/polyacrylamide gel loaded with the proteins from the insoluble fraction (Fig. 3, lane B). Although it was difficult to obtain quantitative information, it appeared that most of the large subunit polypeptide in cells carrying pAnX105 was in the insoluble fraction. This observation is consistent with the evidence from analysis of the mRNA pool, suggesting that the large subunit is expressed at much higher levels than the small subunit. Insolubility of the large subunit when expressed in *E. coli* in the absence of the small subunit has also been noted in previous studies of an expression plasmid that produces large quantities of the *Z. mays* large subunit polypeptide in *E. coli* (11, 12).

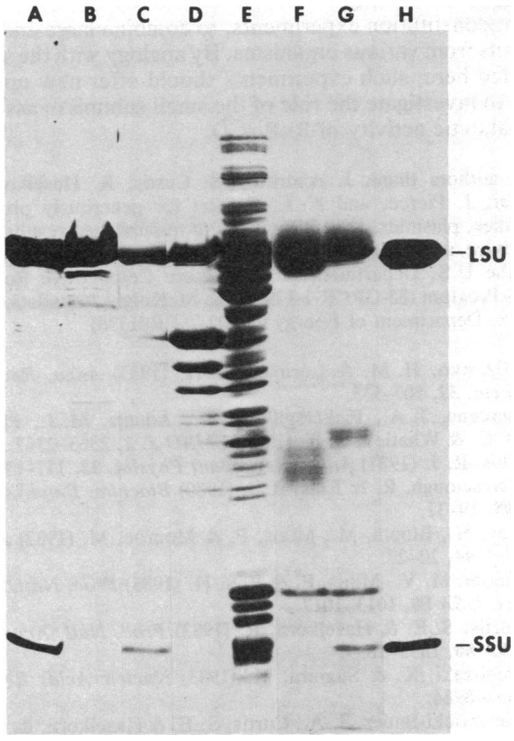

FIG. 3. Expression of *Anabaena* RuBisCO in *E. coli*. Lanes A and B, nitrocellulose filter replica of a $NaDodSO_4$/polyacrylamide gel (10–15% linear gradient) containing pure RuBisCO from *Anabaena* (lane A), and insoluble extract from HB101 (pAnX105) (lane B). The presence of RuBisCO was detected immunohistochemically. Lanes C–G are from a $NaDodSO_4$/polyacrylamide gel stained with Coomassie blue. Lane C, pure RuBisCO from *Anabaena*. Lane D, insoluble extract from HB101 (pAnX105). Lane E, supernatant from an extract of HB101 (pAnX105). Lane F, immunoprecipitate from treatment of extract of HB101 (pAnX105) with preimmune serum. Lane G, immunoprecipitate from treatment of extract of HB101 (pAnX105) with anti-RuBisCO antiserum. Lane H, autoradiograph of immunoprecipitate from ^{35}S-labeled extract of HB101 (pAnX105). LSU, large subunit; SSU, small subunit.

To determine whether the catalytically active soluble form of the enzyme produced in *E. coli* was physically similar to the enzyme isolated directly from *Anabaena*, we compared the sedimentation coefficient of the enzyme produced in *E. coli* with that extracted from *Anabaena*. The similar or identical rates of migration of the active fraction in the sucrose gradients (Fig. 4) indicated that the soluble RuBisCO activity had a comparable sedimentation coefficient to that of the 18S holoenzyme from *Anabaena*.

Since the relatively crude centrifugal methods would probably not resolve enzymes composed of eight large subunits and eight small subunits (i.e., L_8S_8) from those with eight large subunits and one or more small subunits, we undertook to measure the stoichiometry of the subunits in the high molecular weight soluble form of the enzyme. *E. coli* cells carrying pAnX105 were labeled with [^{35}S]methionine and the labeled soluble RuBisCO specifically immunoprecipitated from crude extracts. The subunits were resolved in $NaDodSO_4$/polyacrylamide gels and were unambiguously identified by immunohistochemical development of nitrocellulose replicas of the gels (Fig. 3). The relative abundance of each subunit was quantitated by measuring the radioactivity in each band, then correcting for the molar ratio of methionine in each subunit, which was deduced from the DNA sequence (7, 9). In six independent repetitions of this experiment, the amount of radioactivity in the large subunit band was between 7.3 and 7.7 times that found in the small subunit.

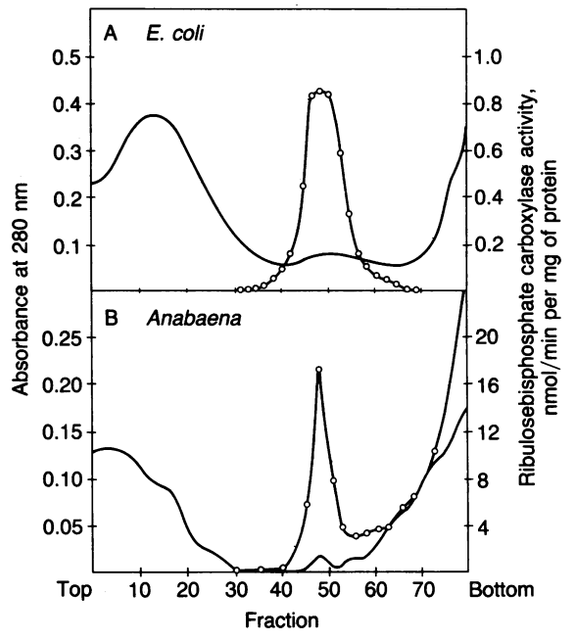

FIG. 4. Sucrose density gradient centrifugation of ribulosebisphosphate carboxylase from extracts of *E. coli* HB101 (pAnX105) and *Anabaena*. Extracts were centrifuged at 100,000 × *g* for 30 min to remove insoluble material, and then applied to 36-ml linear sucrose gradients (0.2–0.8 M sucrose in assay buffer) and centrifuged at 240,000 × *g* for 135 min in a vertical rotor. The activity at the bottom of the gradient in *B* is due to the presence of a particulate form of RuBisCO, "carboxysomes," in *Anabaena* cells (26).

Since there are eight methionine residues in the *Anabaena* large subunit but only one in the small subunit (7, 9), this observation indicates that the subunits are present in equimolar ratios in the soluble form of RuBisCO produced from plasmid pAnX105. In conjunction with the evidence that the enzyme is 18S, we conclude that the soluble form is hexadecameric.

DISCUSSION

At least one gene from *Anabaena* 7120 has been found to be functional in *E. coli* without any modification of transcriptional or translational signals (27). Therefore, we considered it probable that the technical problem in obtaining high levels of expression of the *Anabaena* RuBisCO operon in *E. coli* would be primarily associated with ensuring sufficiently high levels of transcription. This was accomplished by deleting most of the noncoding 5′ flanking region of the *rbcL* gene and fusing the remainder to the *lac* promoter. This plasmid construction resulted in high levels of transcription and translation of the *rbcL* gene but apparently low levels of transcription of the *rbcS* gene. The simplest interpretation of this observation is that there is a site or a region of secondary structure in the 552-bp intergenic region separating the *rbcL* and *rbcS* genes that is recognized as a transcriptional termination signal in *E. coli* but not in *Anabaena*. In support of this concept, there are several putative stem and loop structures in the intergenic region immediately following the *rbcL* gene that might cause termination. The fact that small subunit polypeptide accumulates in *E. coli* indicates that some transcripts continue through the intergenic region and are correctly translated, or that transcription reinitiates at a low rate in the intergenic region. Thus, future attempts to increase the amount of expression of the *rbcS* gene are expected to necessitate restructuring of the intergenic region or expression of the *rbcS* gene from a separate promoter.

The assembly of an active heteromeric RuBisCO gene in *E. coli* is informative in several respects. First, it indicates that no enzyme-specific factors are apparently required to organize the assembly of a functional hexadecameric holoenzyme. However, this does not, by itself, negate the possibility that additional factors may be required in eukaryotes where the *rbcL* and *rbcS* genes are transcribed and translated in separate compartments. The insolubility of the large subunit, noted here and in previous studies with *Z. mays* RuBisCO expression vectors (11, 12), may necessitate the existence of a companion protein that keeps the large subunit in solution until it assembles with small subunit into holoenzyme or another soluble form (4). Perhaps such a companion protein is not required in cyanobacteria because the two subunit polypeptides are cotranslated and able to interact directly. Second, the fact that the RuBisCO produced in *E. coli* sediments at about 18S in sucrose gradients indicates that it has a high molecular weight, characteristic of the enzyme extracted from native sources. In conjunction with the observation that this enzyme has stoichiometric amounts of large and small subunits, this indicates that the enzyme from *E. coli* is in the L_8S_8 hexadecameric form.

The observation that all of the soluble RuBisCO is in the L_8S_8 form under circumstances where there is a vast excess of large subunit being produced has important implications for understanding the pathway of enzyme assembly. In this respect, there seem to be two possibilities. First, the large subunits could assemble to form L_4 or L_8 complexes, which would then compete for free small subunits. In this case, one would expect to find the RuBisCO produced from pAnX105 primarily in the L_4S_1 or L_8S_1 form because the excess of large subunit complexes would compete for free small subunits. By contrast, if the first step in assembly of holoenzyme was the formation of L_1S_1 heterodimers that subsequently aggregated to the stable L_8S_8 form, one would expect to see primarily hexadecamers. Our results strongly favor the latter model. One caveat to this conclusion is that it might be argued that if only the L_8S_8 form is soluble, all other conformers (i.e., L_8S_1) would be in the insoluble fraction. However, we have been unable to detect small subunit antigen in the insoluble fraction by immunohistochemical staining of nitrocellulose replicas of $NaDodSO_4$/polyacrylamide gels of the polypeptides in this fraction (Fig. 3). The other obvious possibility is that binding of small subunit could be cooperative so that the L_8S_1 form is rapidly converted to L_8S_8 and intermediate forms are not observed. However, analysis of the kinetics of reassociation of free small subunits with L_8 cores, prepared by stripping small subunits from holoenzyme, have not indicated cooperative binding of small subunits to the L_8 core (28, 29). The observation that small subunits can be reversibly stripped from the hexadecamer without disrupting the L_8 core suggests that, once formed, the large subunit core is no longer dependent on the presence of small subunits to maintain the L_8 configuration. Thus, we propose that, in addition to a role in catalysis (17, 28, 29) and possibly solubilization of the large subunit, a specific function of the small subunit is to catalyze hexadecamer formation.

Finally, the amount of expression of active RuBisCO from pAnX105 is probably too low to make it ideally suited for enzymological studies that require relatively large amounts of readily purified protein. To improve the suitability of the plasmid in this respect we are attempting to modify the structure of the expression vector so as to increase the expression of the *rbcS* gene product. The high-level production of an active RuBisCO hexadecamer in *E. coli* should allow the application of genetic techniques, as opposed to *in vitro* reconstitution experiments, to combine large and small subunits from various organisms. By analogy with the studies reported here, such experiments should offer new opportunities to investigate the role of the small subunit in assembly and catalytic activity of RuBisCO.

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