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Synthesis of active *Olisthodiscus luteus* ribulose-1,5-bisphosphate carboxylase in *Escherichia coli*

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Abstract

The ribulose-1,5-bisphosphate carboxylase (Rubisco) large- and small-subunit genes are encoded on the chloroplast genome of the eukaryotic chromophytic alga *Olisthodiscus luteus*. Northern blot experiments indicate that both genes are co-transcribed into a single (>6 kb) mRNA molecule. Clones from the *O. luteus rbc* gene region were constructed with deleted 5′ non-coding regions and placed under control of the *lac* promoter, resulting in the expression of high levels of *O. luteus* Rubisco large and small subunits in *Escherichia coli*. Sucrose gradient centrifugation of soluble extracts fractionated a minute amount of carboxylase activity that co-sedimented with native hexadecameric *O. luteus* Rubisco. Most of the large subunit synthesized in *E. coli* appeared insoluble or formed an aggregate with the small subunit possessing an altered charge: mass ratio compared to the native holoenzyme. The presence in *O. luteus* of a polypeptide that has an identical molecular mass and cross reacts with antiserum generated against pea large-subunit binding protein may indicate that a protein of similar function is required for Rubisco assembly in *O. luteus*.

Introduction

The synthesis of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) (Rubisco) differs between prokaryotes and eukaryotes. The *rbc*L and *rbc*S genes that code for large-subunit (LS) and small-subunit (SS) polypeptides respectively, differ in organelle coding site, organization and number, when prokaryotic and eukaryotic organisms are compared [5]. For example, both Rubisco subunits are arranged as a 5′-*rbc*L-*rbc*S-3′ operon on the chromosome in photosynthetic bacteria and cyanobacteria (blue green algae) [28] and are transcribed into a single mRNA molecule [22]. Additionally, when the cloned *rbc*L and *rbc*S genes of prokaryotes are expressed in *E. coli*, native holoenzyme of apparently normal stoichiometry (LS_8SS_8) and activity is recovered [7, 10, 14, 32].

Most studies on Rubisco in eukaryotes have been done using chlorophytes, organisms containing chlorophyll a and b that include all terrestrial plants and green algae. In this taxon, the *rbc*L gene is located on chloroplast DNA [19], while the *rbc*S genes are encoded in the nucleus [13]. Nuclear encoded small subunit genes contain transit sequences that enable the precursor SS polypeptide to be transported into the plastid [33]. Newly synthesized LS polypeptides appear to interact with the large-subunit binding protein (LSBP) [15] prior to assembly into native LS_8SS_8 holoenzyme [4]. This protein may be actively involved in the assembly process of chlorophytic plant Rubisco or exert its effect by maintaining the

solubility of the LS polypeptide [4]. Expression of active chlorophytic plant Rubisco in *E. coli* from cloned DNA has been unsuccessful, perhaps due to the requirement for LSBP.

When Rubisco from eukaryotic plant taxa other than chlorophytes is examined, novel combinations of molecular [26] and biochemical characteristics are observed [21, 24, Newman and Cattolico, in press]. Based on morphological criteria it has been postulated that the rhodophytic (chl a, phycobilin-containing) and chromophytic (chl a, c-containing) algae represent ancient evolutionary lineages that are quite distinct from chlorophytic plants [5]. Our recent studies on Rubisco gene organization add support to this hypothesis. Rubisco genes in the chromophyte *Olisthodiscus luteus* are arranged in a 5′-*rbc*L-*rbc*S-3′ prokaryotic-like array on the chloroplast genome [26]. Similar *rbc* gene organizations may be found in other eukaryotic algae. Fine structure mapping and sequence analysis document a single Rubisco operon on the chloroplast DNA of the evolutionary enigmatic organism *Cyanophora paradoxa* [30] and *in vitro* translation studies provide evidence of Rubisco small subunit coding within the chloroplast of the true rhodophytes *Cyanidium caldarium* and *Porphyridium aeruginium* [31].

Extensive biochemical analysis of the enzyme provides additional evidence that supports the close affinity between chromophytic and rhodophytic algal Rubiscos. Rubisco holoenzyme and constituent subunits display unique structural properties [21], functional parameters [21], and immunological determinants [21, 24] that differ substantially from the chlorophytic plant Rubisco. In summary, chromophytes and probably rhodophytes encode Rubisco in a prokaryotic fashion, yet both synthesize an enzyme with a characteristic set of biochemical properties. In this study, the expression of *O. luteus* Rubisco at the level of transcription and assembly is analyzed. *O. luteus rbc*L and *rbc*S genes are cotranscribed in the alga, both polypeptides can be expressed in *E. coli* from cloned chloroplast DNA and holoenzyme activity can be detected. This communication is the first report that describes the synthesis of active eukaryotic Rubisco in *E. coli*. The mechanism of *O. luteus* Rubisco expression *in vivo* and in *E. coli*, appears to possess properties common to both prokaryotes and eukaryotes.

Materials and methods

Reagents

Restriction and modifying enzymes were from BRL (Bethesda, MD). Guanidinium thiocyanate was from Fluka Chemical Co. (Germany). Formaldehyde was from Merck and Co. (Rahway, NJ), Bal 31 exonuclease from Boehringer Mannheim and ^{32}p dATP was obtained from New England Nuclear. Nitrocellulose was from Schliecher and Schull (Keene, NH). All other chemicals (including ribulose bisphosphate, 4-chloro-1-naphthol, ampicillin [sodium salt], X-gal, lysozyme, and CNBR activated Sepharose) were from Sigma Chemical Co. (St. Louis, MO). Antiserum generated against pea LSBP was generously provided by Harry Roy (Rensselaer Polytechnic Institute, Troy, NY).

Bacterial strains

The *E. coli* strains used were HB101 (F$^-$ *pro leu thi lac*^{4} *hsd*R *end*A *rec*A *rps*L *ara gal*K *xyl mtl sup*E44), JM83 [F$^-$ **(lac-pro ara rps*L @80dlacZM15] and GD32 (a *rec*$^-$ derivative of JM83).

Plasmid preparation

pOCXE3.2, containing the 3.2 kb *Xba* I-*Eco* RI fragment cloned into pUC19, and pOCE3.8, which contains a 3.8 kb *Eco* RI fragment cloned into pUC9, were generated from pOCBH5.0 which has previously been shown to contain the entire *Olisthodiscus luteus* (Carter) *rbc* operon and over two kb 5′ flanking sequence [26]. pOCBH0.7 (containing the 700 bp *Bam* HI-*Hin*d III fragment) and pOCPE0.8 (containing the 800 bp *Pvu* II-*Eco* RI fragment) were subcloned from pOCXE3.2 into pUC19 digested with either *Bam* HI-*Hin*d III or *Sma* I-*Eco* RI (products of *Pvu* II and *Sma* I catalyzed reactions are blunt ended). Deletion clones that contained only the *rbc*L and *rbc*S structural genes and minimal upstream non-coding DNA were obtained as follows. pOCE3.8 was linearized with *Xba* I and treated with Bal 31 exonuclease which digests bidirectionally from both ends. The reaction was terminated by the addition of EDTA to 0.1 M pri-

or to generating blunt ends by repair synthesis of protruding single strands. DNA was religated and transformed in *E. coli* GD-32. Screening of deletion clones was performed as described in the immunological screening and enzyme analysis section. Plasmid DNA from deletion clones expressing increased levels of *O. luteus* LS and SS were restricted with *Eco* RI to confirm the presence of a 2.4 kb insert in pUC9. Therefore, 1.4 kb of pOCE3.8 plasmid DNA was deleted between the *Eco* RI site and the 5′ end of *rbc*L to generate pOC*E2.4 pANOA contains the *Anacystis rbc*L and *rbc*S genes cloned into pUC9 and was a gift from B. Ramage and H. Bohnert (University of Arizona, Tucson). Plasmid DNA was isolated using the alkaline lysis method of Birnboim and Doly [2].

RNA isolation and Northern blot analysis

One to two liters of *O. luteus* cells at a density of 0.5 to 2×10^5 [20] cells/ml were collected by centrifugation (2000 *g*, 5 min 3 °C) and washed quickly with 15 ml of 50 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), pH 7.5, 330 mM sorbitol, 1 mM $MgCl_2$. The cells were pelleted (3000 *g*, 5 min, 3 °C) and lysed by vortexing in 3 ml guanidinium thiocyanate (50% w/v in 50 mM sodium acetate, pH 7.0, 0.5% SDS, 0.5% 2-mercaptoethanol). RNA was further processed by centrifugation through a cesium chloride pad according to Chirgwin *et al.* [6]. The 260/280 and 260/230 absorbance ratios of the RNA were routinely 1.8 or greater. RNA (15 μg) was denatured with 6% formaldehyde [18], electrophoresed through 1.2% agarose gels and transferred to GeneScreen Plus according to the methods provided by the supplier (New England Nuclear). DNA markers were processed identically. Plasmid DNA (0.5 μg) was nick-translated [8] using ^{32}P-dATP as the radioactive nucleotide (600 Ci/mmol) and was separated from the unincorporated nucleotides by the spun-column method [18]. After an overnight incubation at 60 °C in 1 M NaCl, 1% SDS, the blots were washed sequentially with 2×SSC at 20 °C for 5 min, 2×SSC, 1% SDS at 60 °C for 30 min and 0.1×SSC at 20 °C for 3 min prior to autoradiography using an intensity screen with Kodak X-Omat AR film.

Preparation of antibodies

Polyclonal antisera was prepared in rabbits against purified *Olisthodiscus* Rubisco holoenzyme or guanidine hydrochloride denatured large and small subunits (Newman, DeRocher and Cattolico, manuscript submitted). To minimize the level of background signal from rabbit antibodies which recognize *E. coli* proteins, the recovered holoenzyme and small subunit serum was slowly chromatographed through an affinity column that contained total *E. coli* protein immobilized to CNBr-activated Sepharose [29].

Immunological screening and enzyme analysis

Bal 31 generated deletion clones were streaked onto fresh ampicillin plates, replica plated and screened with holoenzyme and small subunit antisera using the method of Young and Davis [34]. Clones giving a positive immunological reaction were grown overnight, harvested, resuspended in 50 mM Tris, pH 8.0, 2% SDS, 5 mM DTT, 10% glycerol and boiled for three min. Following electrophoresis through 12% SDS-polyacrylamide gels and transfer to 0.15 μM nitrocellulose [3], polypeptides were immunostained with horseradish peroxidase and 4-chloro-1-naphthol as previously described (Newman, DeRocher and Cattolico, manuscript submitted).

Soluble and insoluble protein fractions from *E. coli* were obtained as follows: *E. coli* cells were treated with 1 mg/ml lysozyme in a buffer that contained 25 mM Tris pH 8.1, 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM EGTA, 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin, for 30 min at 5 °C. Cells were then disrupted at 5 °C by sonication (3×20 sec) or with a French pressure cell (100 MPa) and fractionated by centrifugation (18000 *g*, 20 min). Further centrifugation (100000 *g*, 40 min) was performed prior to analysis on a 10–40% linear sucrose gradient. Rubisco (from *O. luteus* and *E. coli*) was iso-

lated, activated and assayed as previously described [20]. Proteins were separated on nondenaturing gels using the buffer system of Laemmli [16] minus SDS.

Results

Northern blot analysis

Studies on the organization of the *O. luteus* chloroplast genome indicate that *rbc*S and *rbc*L [26] are located within a large inverted repeat region (Fig. 1A). To determine if *rbc*L and *rbc*S are co-

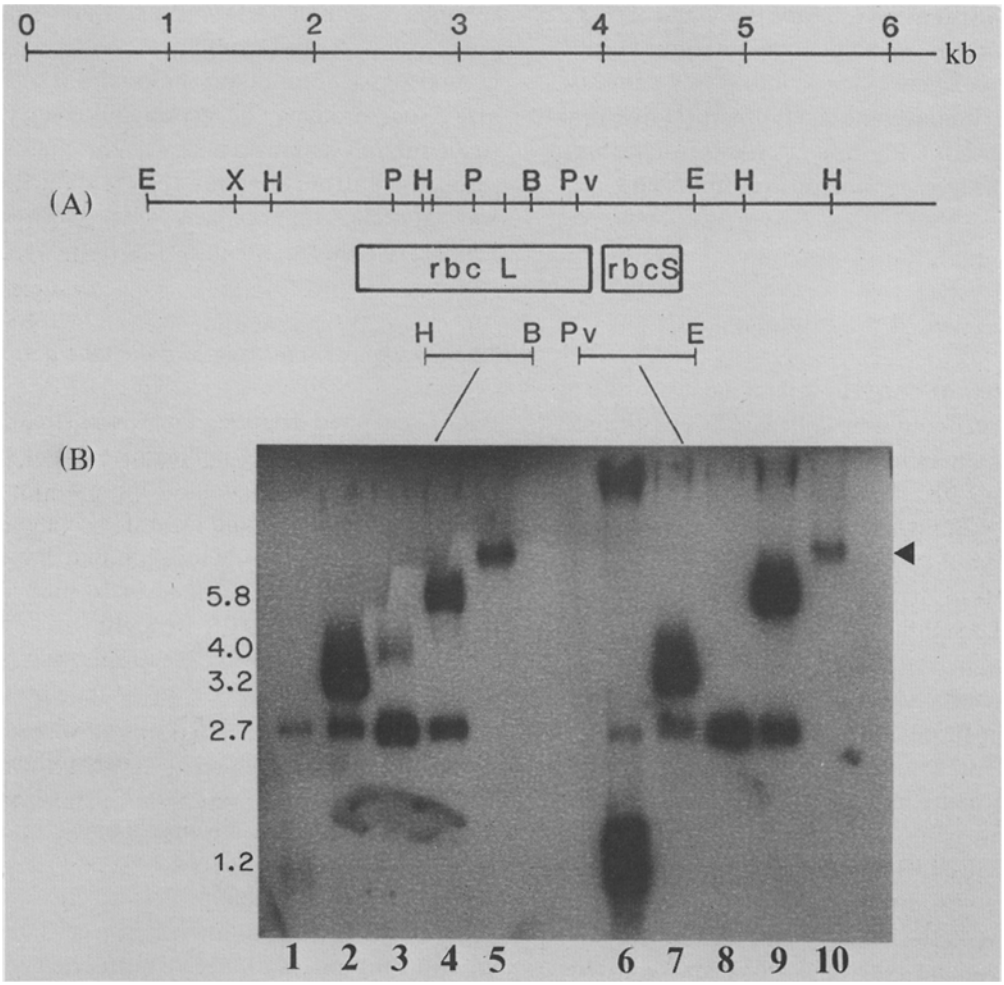

Fig. 1. (A) Fine structure map of the *O. luteus rbc* gene region. The positions of the plasmid inserts (the 700 bp *Bam* HI-*Hin*d III and 800 bp *Pvu* II-*Eco* RI fragments) that were cloned for use as gene probes are indicated. Restriction enzyme sites are designated as follows: *Eco* RI (E), *Xba* I (X), *Hin*d III (H), *Pst* I (P), *Bam* HI (B) and *Pvu* II (Pv). (B) Total *O. luteus* RNA (lanes 5, 10) was isolated, electrophoresed and probed with pOCBH0.7 (lanes 1–5) or pOCPE0.8 (lanes 6–10) as described in the Materials and methods. The size of the DNA markers are indicated in kilobases (kb) and include the 1.2 kb *Bam* HI-*Eco* RI fragment (encoding all of the *O. luteus rbc*S and the 3′ end of *rbc*L) cloned in pUC19 and digested with *Bam* HI and *Eco* RI (lanes 1, 6), pOCXE3.2 (encoding the entire *O. luteus rbc* operon) digested with *Xba* I and *Eco* RI (lanes 2, 7), a 4.0 kb *Bam* HI-*Eco* RI fragment (encoding *Chlamydomonas reinhardtii rbc*L) cloned into pUC8 and digested with *Bam* HI and *Eco* RI (lanes 3, 8) and the 5.8 kb *Pst* I fragment (extending from the middle of *rbc*L, through *rbc*S and 4.5 kb of DNA 3′ to the end of the *rbc* operon) cloned into pUC8 and digested with *Pst* I (lanes 4, 9) [26]. The arrowhead on the right indicates the position of the hybridizing RNA band.

transcribed in *O. luteus*, clones pOCBH0.7 (internal to the *rbc*L gene) and pOCPE0.8 (containing the entire *rbc*S gene and some 3′ coding sequence of *rbc*L) were used to probe size fractionated total RNA (Fig. 1B). A single large RNA transcript is recognized by both probes. This RNA transcript is greater than 6000 nucleotides. No other RNA species is found to hybridize with either probe even after long autoradiographic exposures. No hybridization was detected between pOCPE0.8 and the *Chlamydomonas rbc*L gene (Fig. 1B, lane 8).

Expression of O. luteus rbc*L and* rbc*S genes in* E. coli*: immunological analysis*

Attempts to express the *rbc* polypeptides from pOCE3.8 (containing the 3.8 kb *Eco* RI fragment encoding the *O. luteus rbc* operon and over 1.5 kb of 5′ non-coding DNA) in *E. coli* strain GD-32, failed to yield immunoreactive polypeptides. Contrasting data is obtained when pOCXE3.2 is expressed within the same *E. coli* host. This plasmid encodes the *O. luteus rbc* operon and approximately 0.7 kb of 5′ non-coding sequence. Polypeptides that co-migrate with native LS and SS are immunologically recognized (Fig. 2), but are minimally visible after Coomassie blue staining.

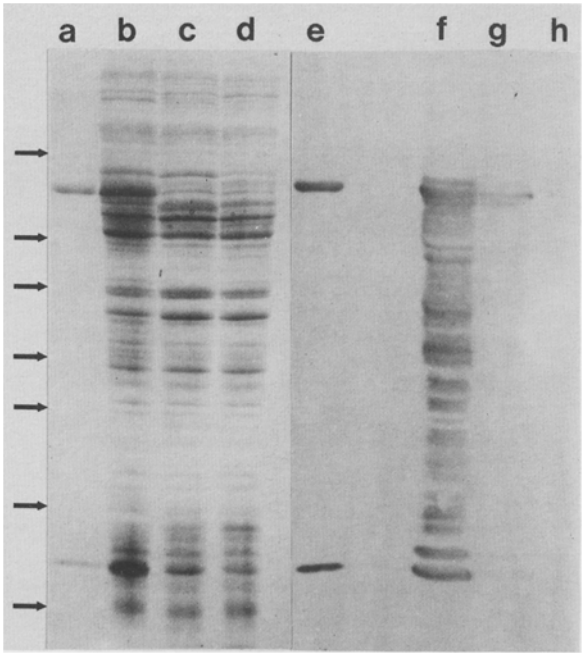

Fig. 2. Immunological identification of *O. luteus* proteins expressed in *E. coli*. Samples of purified *O. luteus* Rubisco (lanes a, e) and total protein from *E. coli* containing pOC*E2.4 (lanes b, f), pOCXE3.2 (lanes c, g) and pUC9 (lanes d, h) were electrophoresed through 12% SDS-polyacrylamide gels and stained with Coomassie brilliant blue (lanes a – d) as described. An identical unstained gel was transferred to nitrocellulose and immunostained with 0.2 and 0.4% solution of antisera generated against *O. luteus* Rubisco holoenzyme and small subunit respectively (lanes e – h). The position of molecular weight markers (66, 45, 36, 29, 24, 20 and 14 kDa) are indicated by arrows.

In an effort to further decrease the noncoding 5′ *rbc*L flanking region and increase expression of the distally encoded polypeptides [14], a series of pOCE3.8 deletion clones were generated using Bal 31 exonuclease. Approximately 100 clones were assayed for expression of LS and SS with holoenzyme and small subunit antisera in a colony screening experiment [34]. Ten clones were selectively chosen and further analyzed by SDS-PAGE and Western blotting. Several clones synthesized high levels of LS and SS (data not shown). One representative clone was designated pOC*E2.4 and analyzed further.

The fact that higher levels of LS and SS are produced in some 5′ deletion clones is illustrated by studies done with pOC*E2.4 (Fig. 2, lanes b, f). Polypeptides that co-migrate with native *O. luteus* LS and SS are readily visible (lane b) when total protein is recovered from *E. coli* JM83 containing pOC*E2.4, electrophoretically separated through SDS-polyacrylamide gels and stained with Coomassie blue. Immunoblot studies confirm the identification of co-migrating LS and SS. The numerous other polypeptides that are recognized by *O. luteus* holoenzyme antiserum (lane f) may represent premature translation products [9, 26].

Expression of O. luteus rbc*L and* rbc*S in* E. coli*: enzymatic studies*

Several analyses were performed on *E. coli* containing pOC*E2.4 to determine if the expressed *O. luteus* LS and SS interact and assemble into an active LS_8SS_8 holoenzyme. Although high concentrations of immunoreactive LS and SS were synthesized in *E. coli* JM83 (Fig. 2), no Rubisco-dependent CO_2 fixa-

tion could be detected in either soluble or insoluble cell fractions. Desalting or dialysis of the 100000 *g* supernatant did not enhance the recovery of enzyme activity. However, further purification of the *E. coli* expressed Rubisco from pOC*E2.4 in JM83, by centrifugation of the 100000 *g* supernatant through a 10–40% linear sucrose gradient, fractionates a protein peak that displays carboxylase activity (Fig. 3). The level of enzymatic activity synthesized from pOC*E2.4 is quite low, yet noticeably above the activity generated from *E. coli* JM83 cells harboring the pUC9 cloning vector that had no *O. luteus* chloroplast DNA insert. The low levels of carboxylase activity expressed from pOC*E2.4 co-sediment with native *O. luteus* Rubisco (Fig. 3). As a control, pANOA (encoding the *rbc*L and *rbc*S genes from the blue green alga *Anacystis* cloned into a pUC9 vector), was expressed in *E. coli* JM83. Levels of *Anacystis* Rubisco activity were 250× higher than with *O. luteus* Rubisco (Fig. 3).

Solubility properties of expressed O. luteus *Rubisco polypeptides*

The data presented above demonstrate that (a) high concentrations of LS and SS are identified in pOC*E2.4 containing *E. coli* JM83 cells after electrophoresis of total cell protein (Fig. 4, lane d; also Fig. 2), but (b) only minimal carboxylase activity was detected (Fig. 3) despite using long experimental assays (5 min), high levels of radiospecific activity (3 Ci/mol $NaH^{14}CO_3$) and a sucrose gradient concentration step (Fig. 3). The lack of Rubisco activity may be caused by the sedimentation of insoluble LS during high-speed (100000 *g*) centrifugation. To de-

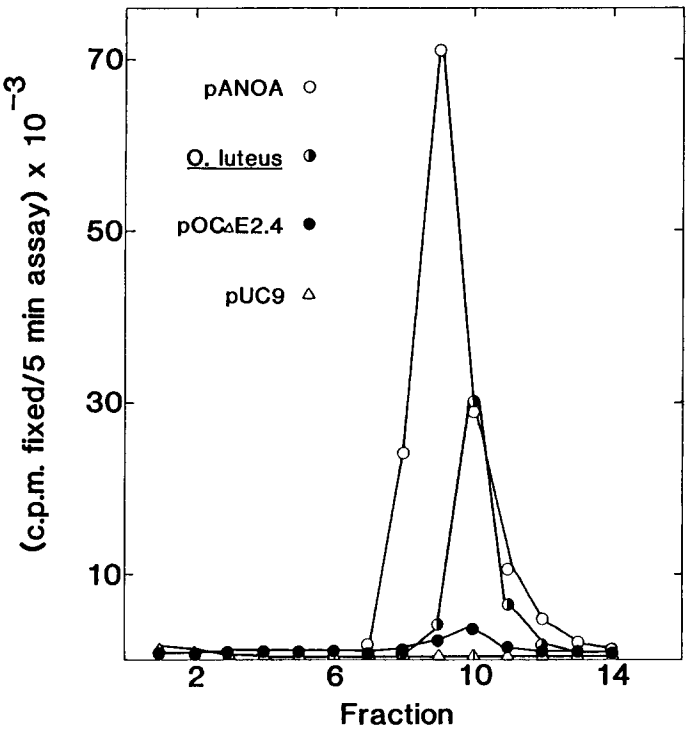

Fig. 3. Sedimentation studies of *O. luteus* Rubisco expressed in *E. coli.* One ml of extract from the supernatant following the 100000 *g* centrifugation was layered onto a 26 ml 10–40% linear sucrose gradient, centrifuged and eluted into 2 ml fractions as described [20]. 50 mM $NaHCO_3$ and 20 mM $MgCl_2$ was used to activate 0.05 ml of each fraction for 30 min. Catalytic assays were performed in 100 mM bicine-OH (N,N-bis[2-hydroxyethyl]glycine) (pH 8.0), 1 mM DTT, 10 mM $NaH^{14}CO_3$ (3 Ci/mol), 0.4 mM Rubisco for five min before terminating the reaction with acid, heating to dryness, solubilizing in 0.1 ml distilled H_2O and quantified by liquid scintillation spectroscopy.

termine if the *O. luteus* LS and SS became insoluble after their synthesis in *E. coli,* the following experiment was performed. When the 100000 *g* supernatant from pOC*E2.4 is monitored on 12% SDS-polyacrylamide gels and stained with Coomassie blue, no LS is observed (Fig. 4, lane e). Although quantitative estimates are difficult to obtain, most of the LS synthesized in *E. coli* cells containing pOC*E2.4 is present in an insoluble form in the 100000 *g* pellet (data not shown). Therefore only trace quantities of Rubisco are present in sucrose gradient fractions corresponding to the peak of CO_2-fixation activity (Fig. 4, lane f). In contrast, the LS polypeptides synthesized in *O. luteus* (Fig. 4, lane b) and those expressed from pANOA (containing the cloned *Anacystis rbc*L and *rbc*S genes) in JM83 (Fig. 4, lane h) remain in the supernatant following high speed centrifugation. Sucrose gradient fractionation of the 100 000 *g* supernatant from *O. luteus* and pANOA substantially enriches for the LS and SS polypeptides in fractions containing enzymatic activity (Fig. 4, lanes c and i, respectively).

Non-denaturing polyacrylamide gel analysis

An experiment was designed to compare the structure of the Rubisco holoenzyme that is expressed in *E. coli* with that of the native holoenzyme. Soluble extracts (20000 *g*, 20 min) of pOCXE3.2 were subjected to electrophoresis through a 7% polyacrylamide gel that lacked SDS [16]. Separated proteins were transferred in the presence of SDS to nitrocellulose paper and then probed with *O. luteus* Rubisco holoenzyme antiserum. Results in Fig. 5 demonstrate that Rubisco synthesized in *O. luteus* has a different electrophoretic mobility than the immunoreactive form synthesized from pOCXE3.2 (Fig. 5). The data obtained using *O. luteus* small subunit antiserum are identical to those presented in Fig. 5.

Immunological cross reactivity of large-subunit binding protein antisera

Requirement of the large-subunit binding protein

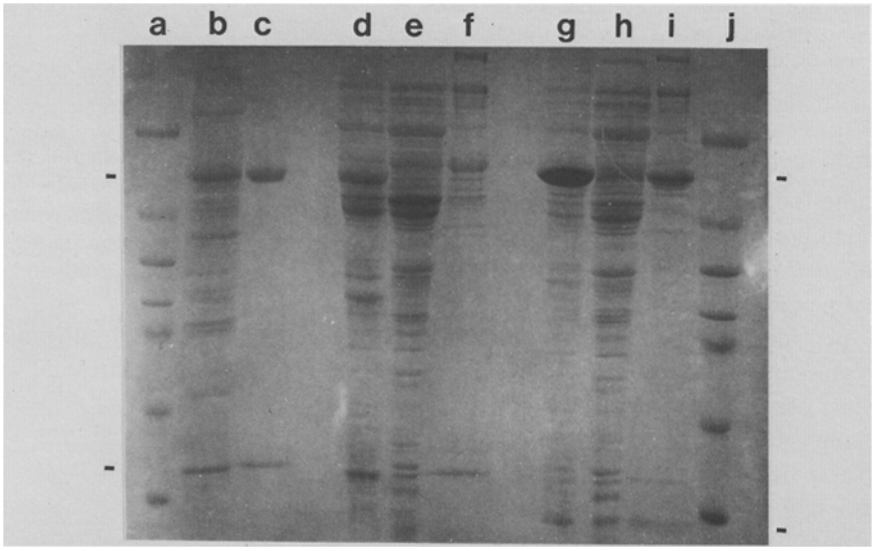

Fig. 4. Solubility analysis of *O. luteus* Rubisco expressed in *E. coli.* Total protein from *O. luteus,* pOC*E2.4 in *E. coli* JM83 (lane d) and pANOA in *E. coli* JM83 (lane g) were centrifuged at 100000 *g*. Aliquots of the three supernatants (lanes b, e, h, respectively) were removed prior to rate sedimentation centrifugation. Samples of sucrose gradient fractions containing peak levels of activity [No 10 from *O. luteus* (lane c), No 10 from pOC*E2.4 (lane f) and No 9 from pANOA (lane i)] were electrophoresed on 12% SDS-polyacrylamide gels, followed by Coomassie blue staining. Molecular weight markers (66, 45, 36, 29, 24, 20 and 14 kDa) were co-electrophoresed in lanes a and j and the positions of the *O. luteus* and *Anacystis* Rubisco LS and SS polypeptides are indicated on the left and right side of the figure, respectively.

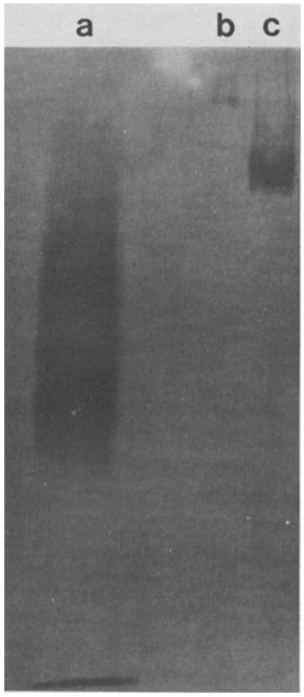

Fig. 5. Non-denaturing gel electrophoresis of *O. luteus* Rubisco. Purified *O. luteus* Rubisco (lane c), or treated with 0.5% SDS (lane a), and a 20000 *g* soluble extract from pOCXE3.2 (b) was electrophoresed on 7% polyacrylamide gels, transferred to nitrocellulose in the presence of SDS and immunostained with *O. luteus* Rubisco holoenzyme antiserum. In lane a, SS polypeptide migrated and stained near the dye front and LS appeared as a smear.

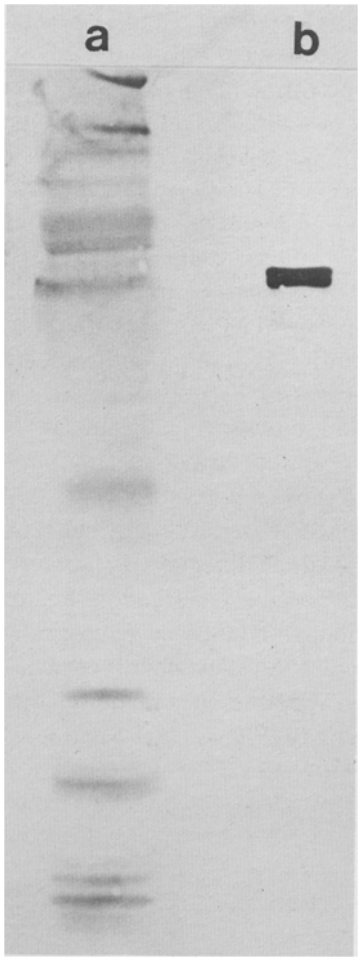

Fig. 6. Western blot of total protein from *O. luteus* (lane a) and pea chloroplasts (lane b) immunostained with antiserum generated against pea LSBP. Pea chloroplast protein was prepared as described by Cannon *et al.* [4]. Approximately 50 μg of *O. luteus* protein was used.

(LSBP) for the synthesis of land plant Rubisco is indicated by (A) the inability to synthesize chlorophytic plant Rubisco in *E. coli* and (B) the inhibition with LSBP antiserum of pea Rubisco assembly *in vitro* [4]. To test if a polypeptide that is immunologically related to LSBP is present in *Olisthodiscus,* antiserum generated against pea LSBP was used to probe Western blots of whole-cell SDS extracts of *O. luteus* and lysed chloroplasts from pea. Many *O. luteus* polypeptides cross react with pea LSBP antiserum (Fig. 6). However among the more strongly cross reacting polypeptides appears a doublet of approximately 60 kDa, which co-migrates with the two immunoreactive proteins in the pea chloroplast extract. These appear to correspond to the 60 and 61 kDa LSBP monomers [15]. The presence of an *O. luteus* polypeptide that has conserved antigenic determinants and a similar molecular mass as the pea LSBP, can suggest that a functionally related protein may exist in *O. luteus.*

Discussion

The localization, arrangement and transcription of *rbc*L and *rbc*S in *Olisthodiscus luteus* indicates several features have been conserved when this eukaryotic alga is compared to prokaryotes. For example, *Alcaligenes eutrophus* [1] and several species of *Rhodobacter* [12] possess two copies of the *rbc* ope-

ron, one on the bacterial chromosome and the other on a large plasmid. The *rbc* genes in *O. luteus* [25, 26] are duplicated because they are encoded on the large inverted repeat of the chloroplast genome. The *rbc*L loci in *Chlamydomonas eugametos* [17] and geranium [23] are also located on the inverted repeat of chloroplast DNA.

The *rbc* genes from prokaryotes, *Cyanophora* and *O. luteus* are organized and transcribed in a 5′-*rbc*L-*rbc*S-3′ gene array. Northern blot analysis indicates that *rbc*L and *rbc*S are cotranscribed in prokaryotes [22, 28], and *Cyanophora* [30]. The identification of a single mRNA species with *rbc*L and *rbc*S gene probes (Fig. 1, lanes 5 and 10, respectively) indicates that both genes are cotranscribed in *O. luteus*. Although chloroplast genes are frequently encoded in polycistronic messages, the size of this transcript in *O. luteus* is at least 3 kb larger than those that encode the blue green algal or *Cyanophora rbc* genes (2.2–3.1 kb). Introns cannot account for the large size increase of the *O. luteus rbc* mRNA since the full-length LS and SS are synthesized when cloned chloroplast DNA from this alga is expressed in *E. coli* (Fig. 2). The existence of an intron-processing mechanism in *E. coli* has not been described.

*O. luteus rbc*L and *rbc*S appear to be maintained on a single transcript during translation because a single RNA species hybridized with the *O. luteus rbc*L and *rbc*S gene probes. No RNA species shorter than the primary transcript were detected (Fig. 1). Shorter transcripts would be expected if individual *rbc*L and *rbc*S messages were required for effective translation. It appears that *O. luteus rbc* mRNA processing differs from that of other algae. For example, *Cyanophora* contains *rbc*L and *rbc*S specific transcripts that are generated from a single larger mRNA molecule [30], whereas *Anabaena* contains multiple smaller mRNA species that hybridize to both the *rbc*L and *rbc*S gene specific probes [22].

The dual organellar coding site of *rbc*L and *rbc*S in chlorophytes results in extensive post-translational interactions and modifications of the LS and SS polypeptides that are required for the synthesis of active LS_8SS_8 Rubisco holoenzyme [cf. 5, 11, 33]. In contrast, the organization of *rbc* genes in prokaryotes lends itself to the stoichiometric production of the two subunits and a mechanism of holoenzyme synthesis that appears to require no other factors [7, 10, 14, 32]. It would be expected that if *O. luteus* and blue green algae possessed identical *rbc* gene organizations, then the pattern of synthesis of LS, SS and active holoenzyme from cloned DNA in *E. coli* should be similar. However, unlike the blue green algal LS and SS, which are capable of assembly into holoenzyme in *E. coli,* functional assembly of active *O. luteus* Rubisco is not efficient when the subunits are synthesized in *E. coli.* The greater insolubility of the *O. luteus* Rubisco subunits in aqueous buffers (Fig. 4) compared to the blue green algal polypeptides, may explain the minute levels of active *O. luteus* Rubisco holoenzyme synthesized in *E. coli.* The solubility of most proteins expressed in *E. coli* from cloned heterologous DNA is frequently decreased [27]. This observation is supported by the substantial insolubility of wheat [9], maize [11] and blue green algal LS [14] synthesized in *E. coli.*

Structural analyses of *O. luteus* expressed from pOC*E2.4 indicates that the enzymatic activity cosediments with native *O. luteus* Rubisco (Fig. 4) and possesses immunoreactive LS and SS that are of identical electrophoretic mobility as the native subunits. These data suggest that the active *O. luteus* enzyme synthesized in *E. coli* may contain an 8:8 ratio of LS:SS. However *O. luteus* LS and SS synthesized in *E. coli* apparently also interact to form complexes with an altered charge:mass ratio when compared to the native holoenzyme (Fig. 5). It should be noted that non-denaturing gel analysis fails to discriminate between the possibilities that (a) the subunits interact but incorrectly assemble and (b) that LS and SS are unable to interact with each other and only bind homologous subunits forming LS_x and SS_x oligomers. If these LS_x and SS_x homomers have similar charge:mass ratios to each other and to the native *O. luteus* Rubisco, their electrophoretic mobility in the absence of SDS will be similar. Furthermore, post-translational, non-proteolytic modifications would minimally alter the mobility of a polypeptide in SDS-polyacrylamide gels. However modifications could be identified as an altered charge:mass ratio on non-denaturing or isoelectric gels.

Participation of a large subunit binding protein (LSBP) in the assembly of land plant Rubisco appears certain [4]. Inhibition of pea Rubisco assembly is achieved with antisera generated against purified LSBP demonstrating the necessity of this protein in the synthesis of the chlorophytic plant enzyme. The absence of LSBP in *E. coli* precludes assembly of chlorophytic plant Rubisco from cloned DNA. Similarly, if an analogous protein were required for synthesis of *O. luteus* holoenzyme, no Rubisco assembly would be expected when *O. luteus rbc*L and *rbc*S genes are expressed in *E. coli*. The identification of a polypeptide in *O. luteus* (Fig. 6) that has conserved antigenic determinants and similar molecular mass as pea LSBP seems to indicate the presence of such a polypeptide in this alga, although a number of other polypeptides are equally cross reactive with this antiserum. The extensive insolubility of *O. luteus* LS polypeptide synthesized in *E. coli* supports the proposed function of LSBP in maintaining the LS in a soluble, assembly competent [4] form. The absence of LSBP in *E. coli* would minimize the amount of soluble LS available for assembly with SS and results in extensive insolubility of LS. However the immunological data (Fig. 6), the extensive insolubility of *O. luteus* LS (Fig. 4) and the low level of Rubisco activity synthesized in *E. coli* (Fig. 3) do not demonstrate the necessity or existence of this protein in *O. luteus*. Determining the role LSBP plays in modulating *O. luteus* Rubisco assembly requires analysis of the interaction between LSBP and newly synthesized LS both *in vivo* and in *E. coli*.

Blue green algae lack a polypeptide that is immunologically similar to LSBP and perhaps, not coincidently, are able to synthesize active Rubisco when *rbc*L and *rbc*S are expressed in *E. coli*. However, polypeptides immunologically related to pea LSBP have been identified in all eukaryotic organisms tested to date. Therefore LSBP may be ubiquitious in all plants including eukaryotes that encode *rbc*S on the nuclear or chloroplast genomes. If this were the case, then this protein may be quite ancestral and present in all plant cells regardless of the evolutionary origin of chloroplasts or *rbc*S localization.

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