Inhibition of Ribulose Bisphosphate Carboxylase Assembly by Antibody to a Binding Protein

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Abstract. We have developed an assay to monitor in vitro the posttranslational assembly of the chloroplast protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Most of the newly synthesized 55-kD catalytic ("large") subunits of this enzyme occur in a 29S complex together with 60- and 61-kD "binding" proteins. When the 29S complex is incubated with ATP and $MgCl_2$ it dissociates into subunits, and the formerly bound large subunits now sediment at 7S (still faster than expected for a monomer). Upon incubation at 24°C, these large subunits assemble into RuBisCO. The minority of newly made large subunits which are not bound to the 29S complex also sediment at 7S. When endogenous ATP was removed by addition of hexokinase and glucose, the dissociation of the 29S complex was inhibited. Nevertheless, the 7S large subunits assembled into RuBisCO, and did so to a greater extent than in controls retaining endogenous ATP. Thus the 7S large subunits are also assembly

competent, at least when ATP is removed. Apparently, in chloroplast extracts, ATP can have a dual effect on the assembly of RuBisCO: on the one hand, even at low concentrations it can inhibit incorporation of 7S large subunits into RuBisCO; on the other hand, at higher concentrations it can lead to substantial build-up of the 7S large subunit pool by causing dissociation of the 29S complex, and stimulate overall assembly. At both high and zero concentrations of ATP, however, antibody to the binding protein inhibited the assembly of endogenous large subunits into RuBisCO. Thus it appears that all assembly-competent large subunits are associated with the binding protein, either in the 7S complex or in the 29S complex. The involvement of the binding protein in RuBisCO assembly may represent the first example of non-autonomous protein assembly in higher plants and may pose problems for the genetic engineering of RuBisCO from these organisms.

R IBULOSE-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[1] (E.C. 4.1.1.39) catalyzes CO_2 fixation in the Calvin cycle of photosynthesis. It also catalyzes the competing oxygenation of ribulose bisphosphate to initiate photorespiration (22). In most photosynthetic organisms the enzyme consists of eight "large" subunits (M_r 55,000) and eight "small" subunits (M_r 14,000) (4). In higher plants and green algae, the large subunits are encoded in chloroplast DNA (24) and synthesized in chloroplasts, while the small subunits are synthesized as precursor polypeptides in the cytoplasm, whence they are taken up by the chloroplasts, processed to their final molecular weight, and assembled into intact enzyme molecules (10). The active sites of the enzyme are borne on the large subunit (22). Recently, however, Andrews and his co-workers (1, 2) have found that the small subunit is also required for catalytic activity. Because atmospheric CO_2 is not saturating for the enzyme, and oxygen inhibits the fixation of CO_2, there is interest in genetic engineering of RuBisCO in order to study its biochemical

functions or even to develop plants with higher photosynthetic rates (34). Large and small subunit genes from *Anabaena* have been introduced into *Escherichia coli* on expression vectors. Large subunits were synthesized in excess over small subunits, and a small amount of RuBisCO was formed. It was suggested that active enzyme (LS_8SS_8) could only have been formed if assembly proceeded via an L_1S_1 intermediate; otherwise small subunits would have been bound by separate large subunit aggregates and would have been prevented from forming LS_8SS_8 complexes (16). However, almost all of the large subunits in the cells were insoluble, and so it is not clear whether trace amounts of soluble large or soluble small subunits were limiting for assembly. When higher plant large subunit genes were cloned on expression vectors in *Escherichia coli*, even though large subunits of the enzyme were synthesized, these polypeptides were found in insoluble precipitates (13, 14).

Normally this insolubility should be circumvented; indeed, ~75% of large subunits synthesized *in organello* (i.e., in isolated pea chloroplasts) occur in a soluble ~720-kD particle in extracts of the chloroplasts (6, 32). Although there is some uncertainty about the sedimentation coefficient, we

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1. *Abbreviation used in this paper:* RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

rely for the moment on the value of 29S, determined by sucrose gradient analysis. The 29S particle consists of one large subunit and probably six 60- and six 61-kD subunits each (18). The association of large subunits with this complex is not an artefact of *in organello* protein synthesis, because the same result occurs when the subunits are synthesized in vivo before chloroplast isolation (32). The complex is specific: other proteins in the chloroplast extract do not bind to the 29S complex, and the newly synthesized large subunits are not associated with any other proteins (18, 32). The incorporation of large subunits derived from this complex into intact RuBisCO was demonstrated with an in vitro reaction developed in this laboratory (9, 25–27). The reaction mechanism involves release of large subunits from the 29S complex, followed by their incorporation into RuBisCO, presumably dependent on the endogenous small subunit pools. The dissociation of the 29S particle is accomplished in vitro by adding ATP and $MgCl_2$, and is believed to be reversible in isolated chloroplasts (20, 32). In addition, since the released large subunits remain in solution and sediment at about 7S (30, 31), they may well be associated with either the 60- or 61-kD proteins or they may occur in dimers. If the large subunits are indeed equilibrating between the 7S and the 29S forms, large subunits from these two complexes should be functionally equivalent. The data we present here are consistent with this prediction.

In tobacco, newly synthesized large subunits bind to a high molecular weight complex like the one reported to occur in peas (12). As in the case of pea, the addition of ATP and $MgCl_2$ causes dissociation of the complex. In a mutant which lacks RuBisCO, the binding protein appears to be more abundant than in normal tobacco (12). The binding proteins are immunologically detectable in tobacco, wheat, and barley leaves, in extracts of plastids from castor bean endosperm (20), and in a large number of photosynthetic bacteria which contain RuBisCO (18). We have detected the protein in maize, and it has been localized in maize bundle sheath but not mesophyll tissue, and in spinach leaves (S. M. Hemmingsen, personal communication).

Schmidt and Mishkind (33) have reported that when *Chlamydomonas* cells are treated with chloramphenicol, small subunits of RuBisCO can be detected in free pools. Because they are fully processed to their native molecular weight, it is clear that they are localized in the chloroplasts. They do not accumulate; during chase periods they soon become undetectable. The authors concluded from these and additional experiments that a constitutive protease degrades small subunits. Roy et al. (32) observed a similar degradation of large subunits in isolated intact pea chloroplasts. Newly synthesized proteins in isolated pea chloroplasts are subject to light-dependent proteolysis (21, 23). These observations appear to fit well with those of Schmidt and Mishkind, and with earlier proposals by Bennett (7) and by Grossman et al. (15), all of which suggest that proteases regulate the accumulation of chloroplast proteins by degrading those which cannot be properly assembled into appropriate macromolecular complexes. Data we present here indicate that endogenous ATP in chloroplast extracts limits the extent of assembly of large subunits into RuBisCO.

The binding protein interacts with most of the newly synthesized large subunits of RuBisCO (6, 32). We are interested

in the following questions: What are the molecular characteristics of the intermediates in RuBisCO assembly? How does the chloroplast regulate their assembly? Does the binding protein affect the rate of assembly of RuBisCO? To help answer the first question, we sought alternative conditions under which the assembly of RuBisCO could be monitored, and then investigated the inhibitory effect of an antibody to the binding protein on the in vitro assembly of large subunits into RuBisCO.

Materials and Methods

Plant Growth

Pisum sativum plants (Agway, var. Progress #9) were grown in vermiculite at a constant temperature of 25°C under a 12-h light/12-h dark cycle. The plants were watered every 2–3 d.

Chloroplast Isolation

Chloroplasts were isolated on Percoll (Pharmacia, Upsalla, Sweden) gradients at 4°C as described previously (11, 27, 29), using unfolding apical leaves of healthy 9–11-d-old plants. Intact chloroplasts were collected with a Pasteur pipette and placed into ~12–14 ml of ice cold resuspension buffer consisting of 50 μM EDTA, 0.2 mM $MgCl_2$, 375 mM sorbitol, 35 mM Hepes–KOH (pH 8.3), 0.96 mM dithiothreitol (DTT), 200 μM isoleucine, and 200 μM threonine. This mixture was again brought up to 4,000 g, immediately braked, and the supernatant removed. The pellet was resuspended in a minimal volume of the same resuspension buffer and the chlorophyll concentration determined. The chlorophyll concentrations of samples were determined by the method of Arnon (3). Resuspension buffer was used to bring the chloroplasts to appropriate concentrations for each experiment.

Purification of 29S Complex

A crude chloroplast preparation was obtained by homogenizing 60 g *Pisum sativum* apical leaves in 300 ml ice cold grinding buffer (330 mM sorbitol, 50 mM Hepes–KOH [pH 8.3], 0.5% BSA, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The homogenate was filtered through Miracloth (Calbiochem-Behring Corp., La Jolla, CA) and the chloroplasts pelleted by centrifuging up to 4,000 g and immediately braking. The pellet was resuspended gently in 40 ml resuspension buffer (2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 375 mM sorbitol, 35 mM Hepes–KOH [pH 8.3], 0.96 mM DTT). This mixture was again centrifuged up to 4,000 g, immediately braked, and the supernatant removed. The pellets were redissolved in 40 ml lysis buffer (10–25 mM Tris-HCl [pH 7.6], 1 mM Benzamidine, 1 mM ε-amino-n-caproic acid, 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 12,000 g for 10 min.

The supernatant was then pressure concentrated through a Diaflo Ultrafiltration membrane (Amicon Corp., Lexington, MA) with a molecular mass cut off of 300,000 D to a final volume of 6–9 ml. This sample was then dialyzed against the column buffer (0.96 mM DTT, 50 mM Tris HCl [pH 7.0], 5 mM $MgSO_4$, 1 mM Na_2 EDTA) for 2–2½ h. After dialysis, the sample was layered on a 28.5- × 1.5-cm DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column which had been packed with column buffer for a period no less than 12 h. The sample was washed through the column with two 10-ml washes of buffer and then 50 ml of column buffer was passed through the column. After this wash procedure, a 400-ml 0–0.6 M NaCl gradient in column buffer, generated by a Pharmacia Fine Chemicals gradient former, was passed through the column and 2-ml fractions were collected with an Instrumentation Specialties Model 328 Fraction collector. These fractions were placed in an ice bath. Aliquots of every fourth sample were taken and analyzed by nondenaturing PAGE. Those fractions containing the 29S complex were pooled and pressure concentrated as before. 1-ml aliquots of the concentrated sample were layered on 11.4-ml 5–20% sucrose gradients containing 1 mM PMSF, 50 mM Tris (pH 7), 1 mM benzamidine, 1 mM ε-amino-n-caproic acid, and 7 mM 2-mercaptoethanol, and centrifuged at 95,000 g for 17 h. The gradients were then fractionated into 25 samples using an Instrumentation Specialties Company (Lincoln, Nebraska) fractionation system. Aliquots of each were taken for SDS PAGE analysis.

Isolation of Pea RuBisCO Small Subunits

RuBisCO was purified by ammonium sulfate fractionation and sucrose gradient centrifugation (5), followed by DEAE-Sephacel chromatography as described above. It was kept frozen as an ammonium sulfate suspension at −80°C until needed, when drops were thawed, centrifuged, and the pellets dissolved in 10 mM sodium phosphate, 1 mM Na₂EDTA, 500 mM NaCl and dialyzed against the same buffer overnight. The protein concentration was adjusted to 0.61 mg/ml and the pH lowered to exactly 5.1 as described by Andrews and Lorimer (2). The sample was chilled and centrifuged to remove insoluble material, and the supernatant containing small subunits was returned to pH 7.6. The small subunits were kept in a centrifuge tube at 4°C overnight before use. Samples were checked by SDS PAGE and found to contain small subunits but not large subunits of RuBisCO.

In Organello Protein Synthesis

Chloroplasts isolated on 10–80% (wt/vol) Percoll (Pharmacia) gradients were diluted in resuspension buffer as described under *Chloroplast Isolation*, pelleted, resuspended, and illuminated for 24 min in the presence of 400–600 μCi [³⁵S]methionine (>1,000 Ci/mmol). They were resuspended in a 10-fold excess of resuspension buffer, and chloroplasts were pelleted and lysed in an equivalent amount of lysis buffer (10 mM Tris-HCl [pH 7.6], 1 mM benzamidine, 1 mM ε-amino-n-caproic acid, 7 mM 2-mercaptoethanol, 1 mM PMSF). After removal of membranes by centrifugation, the cleared lysate was divided into aliquots which were brought to 50 mM Hepes-KOH (pH 7.6), 220 mM KCl, 6 mM MgCl₂, 20 mM DTT. Some samples were brought to 5 mM glucose and mixed with 75 U hexokinase (Sigma Chemical Co., St. Louis, MO). Others were mixed with AMP (Sigma Chemical Co.) to give a final concentration of 20 mM. After 30 min at 0°C, the samples were brought to room temperature for 60 min and electrophoresed on 7.5% polyacrylamide gels overnight at 4°C. In antibody experiments, DTT was omitted without apparent deleterious effects.

PAGE

All buffers and gel solutions were made as described (19) except that SDS was omitted for nondenaturing gels. For the latter, samples containing equal volumes of chloroplast extract were loaded onto gels consisting of a 7.5% (wt/vol) polyacrylamide slab gel and a 4% (wt/vol) stacking gel, without the addition of SDS cocktail. If the samples were not dense enough to remain in the wells, 60% glycerol was added to a final concentration of 10% (vol/vol). The gels were run at a constant current of 2.5 mA until the voltage reached 150–200 V. At that time, the gels were switched to constant voltage and run at 150–200 V for 18–24 h, respectively.

Fluorography

Gels were soaked in EN³HANCE (New England Nuclear, Boston, MA), dried, and exposed to x-ray film as described earlier (25).

Staining

Gels were rehydrated in 60% glycerol and were stained overnight at room temperature or at 62°C for 30 min with Coomassie Blue. The gels were then destained by soaking them in several changes of an 8:1:1 water/ETOH/glacial acetic acid solution.

Antibody

The antibodies were the generous gift of S. M. Hemmingsen and R. J. Ellis (University of Warwick) (18).

Anti-29S Complex

One batch of antiserum was prepared in rabbits injected with purified 29S large subunit-binding protein complex. The serum reacted strongly with the binding protein and weakly with large subunits. In the presence of the high concentrations of RuBisCO found in chloroplast extracts, the antibodies against large subunit would be expected to be saturated with RuBisCO, so that under these conditions, the serum should be a specific agent for reacting with large subunits associated with the binding protein. Although in principle this serum could remove significant stainable RuBisCO from the soluble fraction, in the one case where this serum was used (Fig. 4), this effect was negligible.

Anti-60-kD Protein

To avoid the last-mentioned problem, we routinely used a second batch of antiserum, which had been raised against the pure binding protein subunits. This reacts strongly with the binding protein but not with large subunits (18). It cross-reacts weakly (<1%) with small subunits (18), as judged by a sensitive immunoblotting assay using ¹²⁵I-conjugated protein A (18). This extent of cross-reactivity would be negligible when the serum is used at equivalence with the binding protein, as in the experiments described here. Control experiments (Fig. 3) confirmed this expectation.

The serum was stored frozen. For preincubation experiments, serum was thawed, and 4.3-μl aliquots were mixed with specified quantities of purified RuBisCO, purified 29S complex, or purified small subunits, adjusted to the same volume with the appropriate buffer, incubated for 1 h at 4°C, and stored at −80°C until the day of the experiment. This step was necessary in order to permit preparation of the perishable 29S complex, small subunits, or chloroplasts on different days.

Results

The large subunit of RuBisCO represents >90% of the radioactive protein recovered in the soluble fraction of pea chloroplasts after *in organello* protein synthesis in the presence of radioactive amino acid (8). This permits routine analysis of incorporation of large subunits into RuBisCO by one-dimensional PAGE and fluorography (9, 32). In this assay, the large subunits in the 29S complex migrate as a sharp, intensely radioactive band and can be resolved easily from intact RuBisCO. A disadvantage of the assay (compared with sucrose gradient analysis or gel filtration) is that the 7S large subunits migrate in a polydisperse fashion or precipitate at the top of stacking gel lanes (6, 32). The gel lanes therefore have background radioactivity which is due to the 7S large subunits. This behavior is not alleviated by altered buffers (6) or the addition of nonionic detergents to the electrophoresis media (our unpublished observations). Despite this disadvantage, the one-dimensional assay is used because it is the only assay which permits analysis of the effects of multiple treatments on the incorporation of radioactive large subunits into RuBisCO (9). The unassembled 7S and 29S large subunits have been identified previously by immunoprecipitation and two-dimensional electrophoresis (30, 31).

To find out what role the binding protein complex plays in the assembly of RuBisCO, we first asked whether large subunits in the 29S and 7S complexes are competent to assemble into RuBisCO. Data obtained by Milos and Roy (25) indicated that large subunits associated with the 29S binding protein complex were competent to assemble into RuBisCO. Although we have tried to investigate the 7S large subunit pool directly, it has not proven possible to reconstitute the in vitro assembly of RuBisCO after fractionating chloroplast extracts in sucrose gradients or gel filtration columns (28). The chloroplast extracts appear to contain components which are sensitive to manipulation and which are necessary for assembly. Therefore we undertook an alternative approach: to limit the dissociation of the 29S complex as much as possible, we controlled endogenous ATP by incubating labeled chloroplast extracts in the presence and absence of hexokinase and glucose, and monitored the subsequent incorporation of large subunits into RuBisCO. We found that labeled large subunits were indeed incorporated into RuBisCO under both these conditions. This suggested that at least some of the 7S large subunits were assembly competent. But this was not the most striking result. As shown in Fig. 1, the assembly of RuBisCO in the presence of hexokinase and glu-
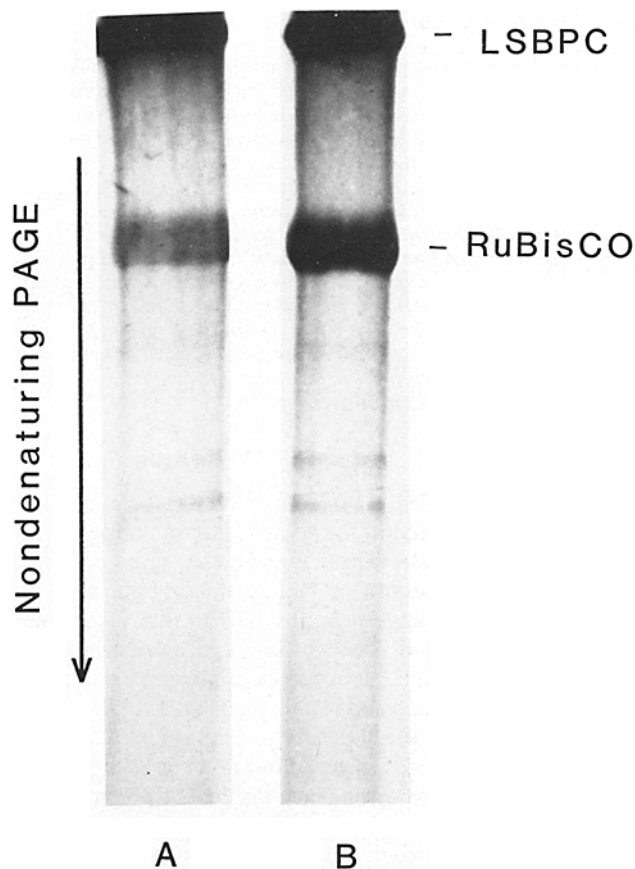
Figure 1. Stimulation of RuBisCO assembly by hexokinase and glucose. *Pisum sativum* chloroplasts were isolated on Percoll gradients, illuminated for 24 min in the presence of 400 μCi [³⁵S]methionine (>1,000 Ci/mmol) and lysed in 10 mM Tris-HCl (pH 7.6), 1 mM benzamidine, 1 mM ε-aminocaproic acid, 7 mM 2-mercaptoethanol, 1 mM PMSF. After removal of membranes by centrifugation, the cleared lysate was divided into 90-μl aliquots which were brought to 50 mM Hepes-KOH (pH 7.6), 220 mM KC, 6 mM MgCl₂, 20 mM DTT. Sample B was brought to 5 mM glucose and mixed with 30 U hexokinase. After 30 min at 0°C, the samples were brought to 24°C for 60 min and electrophoresed on 7.5% polyacrylamide gels overnight at 4°C. The gels were soaked in EN³HANCE, dried, exposed to x-ray film, and developed.

cose was more extensive than in the absence of these compounds. However, the assembly obtained this way (~20% of total large subunits) never achieved the maximum levels (48%) obtained by first dissociating the binding protein complex at 0°C in the presence of MgATP, and then incubating at 24°C (25).

The complete hexokinase and glucose effect occurred even at 0.5 μM added glucose, and some effect could be observed with no added glucose (data not shown). This is not surprising, since it is likely that the extracts contain some glucose. As expected, the effect was abolished by addition of EDTA, which would chelate Mg⁺⁺, a required cofactor in the hexokinase reaction (Table I, sample F vs. D). The addition of EDTA alone partially stimulated assembly (C vs. A), but this stimulation did not occur in the presence of inactive hexokinase (lanes F vs. C). This minor effect has not been studied further. Overall the data in Fig. 1 and Table I suggest that endogenous ATP in the extract may be responsible for limita-

tion of assembly. If so, then added AMP, via the endogenous adenylate kinase in chloroplast extracts, should mimic the hexokinase + glucose effect. Table I (samples E and G) also shows that AMP (20 mM) was as effective as hexokinase and glucose in stimulating assembly of large subunits into RuBisCO. Changes in the amount of radioactivity in the 29S complex were small by comparison with changes in radioactivity of 18S RuBisCO. For example, in the presence of 20 mM AMP (Table I, sample G) 18S radioactivity had increased over controls (samples A and B) by 2.9 to 3 × 10⁷ U, while 29S radioactivity had declined by only 0 to 1.1 × 10⁷ U.

Since the in vitro dissociation of the 29S complex in the absence of added ATP and MgCl₂ is rather slow (Table II; 32), it appears unlikely that 29S material could be the source of the large subunits which are assembling in the presence of AMP. Since the 7S large subunits are the only other major source of large subunits in the system (32), it appears more likely from the foregoing data that 7S large subunits not bound to the 29S complex can assemble into RuBisCO. To check this point, we examined the recovery of large subunit radioactivity in the 7S, 18S RuBisCO, and 29S binding protein complexes using a combination of sucrose gradient analysis, SDS PAGE, and nondenaturing PAGE. Inspection of the fluorograms in this experiment showed that during incubation in the presence or absence of 20 mM AMP, radioactivity in 7S large subunits decreased, radioactivity in 29S complexes did not decrease, and radioactivity in 18S RuBisCO increased. This perception was confirmed by densitometric analysis of the films (Table II): in the absence of added AMP, there is a 30% increase (0.6 × 10⁷ arbitrary units) in 18S RuBisCO over the ice control (B vs. A), a 20% decrease in 7S radioactivity (0.4 × 10⁷ U), and an 11% increase in 29S radioactivity (0.9 × 10⁷ U). The apparent increase in 29S radioactivity under these conditions is not reproducible, but comparable increases in 18S radioactivity have been observed repeatedly. Compared with the ice control lacking AMP (A), the incubation in the presence of AMP (D) at

Table I. Effect of AMP and EDTA on Assembly of Large Subunits into RuBisCO

Sample	Integrated density in each peak	
	RuBisCO	29S Binding Protein
A	1.7	13.1
B	1.8	12.0
C	2.5	12.7
D	4.7	12.4
E	4.2	11.3
F	1.8	12.8
G	4.7	12.0

Chloroplasts were isolated and labeled as described in legend to Fig. 2, and divided into aliquots which were brought to 50 mM Hepes-KOH (pH 7.6), 220 mM KC, 20 mM DTT. Additions were made to each sample: (A) MgCl₂ (6 mM); (B) no additions; (C) Na₂EDTA (2 mM); (D) MgCl₂ (6 mM), glucose (5 × 10⁻³ M), hexokinase (0.3 U/μl); (E) glucose (5 mM), hexokinase (0.3 U/μl); (F) glucose (5 mM), hexokinase (0.3 U/μl), Na₂EDTA (2 mM); (G) MgCl₂ (6 mM), AMP (20 mM). After 30 min at 0°C, the samples were brought to 24°C for 60 min and electrophoresed on 7.5% polyacrylamide gels overnight at 4°C. The gels were soaked in EN³HANCE, dried, exposed to x-ray film, and developed. The developed film was analyzed with an LKB laser densitometer, and the integrated autoradiographic density peaks above the background were quantitated. These values, reduced by a factor of 10⁷, were entered in the table for the 18S RuBisCO band and the 29S large subunit binding protein complex band.

Table II. Recovery of Radioactive Large Subunits in 7S, 18S, and 29S Material

Sample	Integrated density in each peak			
	7S	18S	29S	Total
A	2	2	7	11
B	1.6	2.6	7.9	12.1
C	1.8	3.5	7.3	12.6
D	0.7	4.7	7.2	12.6

Chloroplasts were isolated as described in legend to Fig. 1, and labeled in the presence of 600 μCi/ml [³⁵S]methionine for 24 min. After lysis and removal of membranes, the soluble material was divided into four 450-μl samples containing the same salts and buffer as in Fig. 1, and the following additions were made to each sample: (A and B) lysis buffer; (C and D) AMP to a final concentration of 20 mM and a lysis buffer to a final vol of 612 μl. The reaction mixtures were incubated for 90 min under the following conditions: (A and C) 90 min at 0°C; (B and D) 30 min at 0°C, 60 min at 24°C. At the end of the incubation period, 70 μl of each sample was loaded onto a nondenaturing gel and electrophoresed as described in Fig. 1 to resolve the 18S and the 29S material. The remainder of each was loaded onto one of four 5–20% sucrose gradients (25) and centrifuged at 95,000 g for 17 h. Each gradient was fractionated into 0.5-ml samples, and 150 μl of each of the top six samples (containing 7S material) were examined by SDS PAGE and fluorography. A laser densitometer was used to record the film density in autoradiographic density units essentially as in Table I. The values, corrected for sample volume differences and a difference in exposure time for the films, were reduced by a factor of 10⁷ and entered in the table.

room temperature yielded 135% more labeled RuBisCO. In the presence of AMP, incubation at 0°C led to a 75% increase in 18S RuBisCO radioactivity (C vs. A). (This is the most substantial assembly of RuBisCO we have ever observed at low temperature.) Further incubation of the AMP-supplemented extract at room temperature led to a 34% increase (1.2 × 10⁷ U) in 18S RuBisCO over the ice control (D vs. C), a 61% decrease in 7S radioactivity (1.2 × 10⁷ U), and a 1.3% decrease (0.1 × 10⁷ U) in 29S radioac-

tivity. The total recovered radioactivity was consistent in all cases, except that there may have been a ~9% decrease in total radioactivity in the absence of added AMP (C and D vs. A and B). It is apparent that assembly occurred at the expense of the 7S material in the presence of AMP at room temperature. In the absence of added AMP, the data do not allow a firm conclusion to be drawn, due to the 11% increase in 29S radioactivity during room temperature incubation. However, this change could not explain increased RuBisCO radioactivity since it occurred in the wrong direction. Further, the loss of 7S radioactivity in the absence of AMP was sufficient to account for 67% of the radioactivity incorporated into 18S RuBisCO at room temperature.

Taking the data in Table I and Table II into account, the working hypothesis we adopt is that in the absence of endogenous ATP, the 29S complex is relatively stable, and assembly of RuBisCO occurs at the expense of 7S large subunits already present in the extracts.

These experiments have revealed an apparently paradoxical situation, in which low concentrations of ATP limit assembly while at high concentrations the same molecule is a potent stimulator of assembly. From a practical standpoint, these observations permit us to carry out in vitro assembly reactions both in the presence and the absence of ATP, thereby focusing on one or the other of the two sedimentation classes of large subunits.
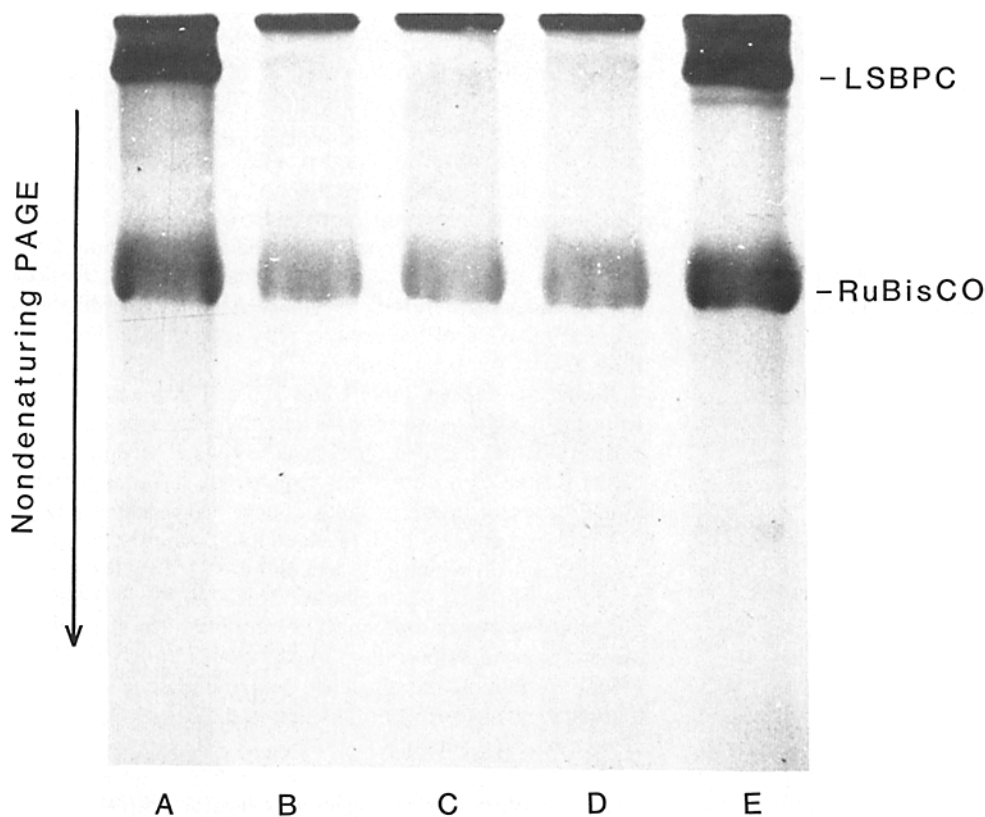
Inhibition of RuBisCO Assembly by Antibodies to the Large Subunit Binding Protein

If assembly of RuBisCO requires the binding protein, an antibody to the binding protein should inhibit assembly. Antibody to the binding protein subunits was made available by S. M. Hemmingsen and R. J. Ellis (18). This antibody
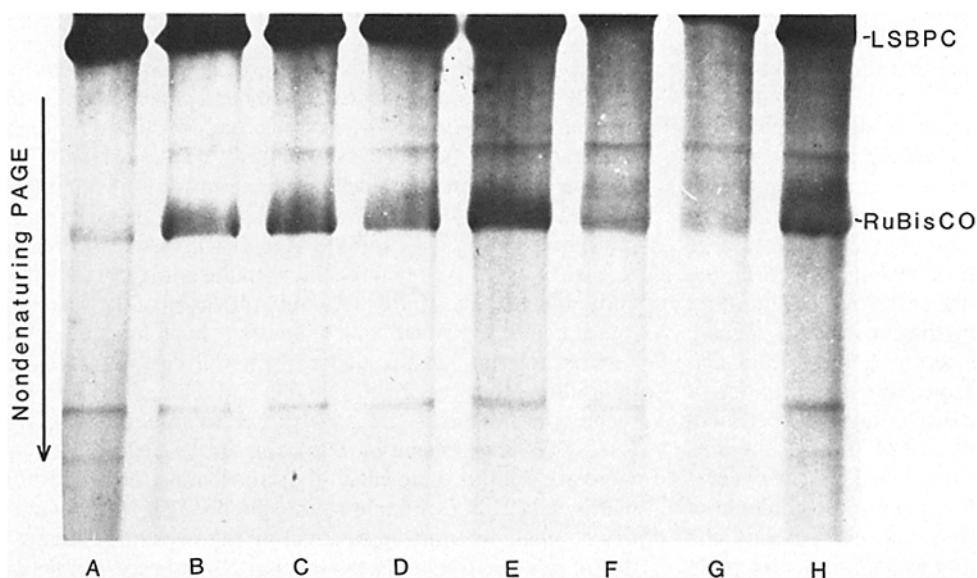
Figure 2. Inhibition of assembly by antiserum specific for the large subunit–binding proteins in the presence of AMP. Chloroplasts were prepared and labeled exactly as described in Fig. 1. An extract was prepared in the usual manner and 37-μl samples were set up so that final concentrations of 50 mM Hepes–KOH (pH 7.6), 220 mM KC, 6 mM MgCl₂, 1 mM unlabeled methionine would be achieved. The following additions were made to each sample: (A) no addition; (B) 20 mM AMP; (C) 20 mM AMP + 3.7 μg column-purified RuBisCO; (D) 20 mM AMP plus 0.67 μg column-purified 29S complex; (E) 20 mM AMP plus 4.3 μl preimmune serum; (F) 20 mM AMP plus 4.3 μl specific binding protein antibody; (G) 20 mM AMP plus 4.3 μl specific binding protein antibody which had been preincubated for 1 h at 0–4°C with 3.7 μg RuBisCO; (H) 20 mM AMP plus 4.3 μl specific binding protein antibody which had been preincubated for 1 h at 0–4°C with 0.67 μg 29S complex. After 30 min on ice the samples were incubated at 24°C for 60 min and analyzed by nondenaturing PAGE. The gel was soaked in EN³HANCE, dried, and exposed to x-ray film. The film density at the RuBisCO band in each lane, determined with the laser densitometer as in Table I and II, was determined as follows (×10⁻⁷): (A) 0.62; (B) 3.1; (C) 3.4; (D) 2.6; (E) 3.6; (F) 1.4; (G) 0.7; (H) 3.1.

Figure 3. Specific inhibition of antibody effect by 29S complex. Chloroplasts were prepared and labeled exactly as described in Fig. 2. An extract was prepared in the usual manner and 37-μl samples set up so that final concentrations of 50 mM Hepes-KOH (pH 7.6), 220 mM KC, 6 mM MgCl$_2$, 1 mM unlabeled methionine would be achieved. Additions were made as follows: (lane *A*) 4.3 μl preimmune serum, 20 mM AMP; (lane *B*) 20 mM AMP and 4.3 μl binding protein antibody prepared beforehand as described in Materials and Methods; (lane *C*) 20 mM AMP and 4.3 μl binding protein antibody preincubated with 0.48 μg pea RuBisCO small subunits; (lane *D*) 20 mM AMP and 4.3 μl binding protein antibody preincubated with 1.03 ng pea RuBisCO small subunits; (lane *E*) 20 mM AMP and 4.3 μl binding protein antibody preincubated with 0.5 μg column-purified, diafiltered, and dialyzed 29S complex. After 30 min on ice the samples were incubated at 24°C for 60 min and analyzed by nondenaturing PAGE. The gel was soaked in EN^{3}HANCE, dried, and exposed to x-ray film. Autoradiographic film densities for the RuBisCO band, determined as in Table I, were as follows ($\times 10^{-7}$): (*A*) 1.3; (*B*) 0.7; (*C*) 0.9; (*D*) 0.6; (*E*) 1.4.
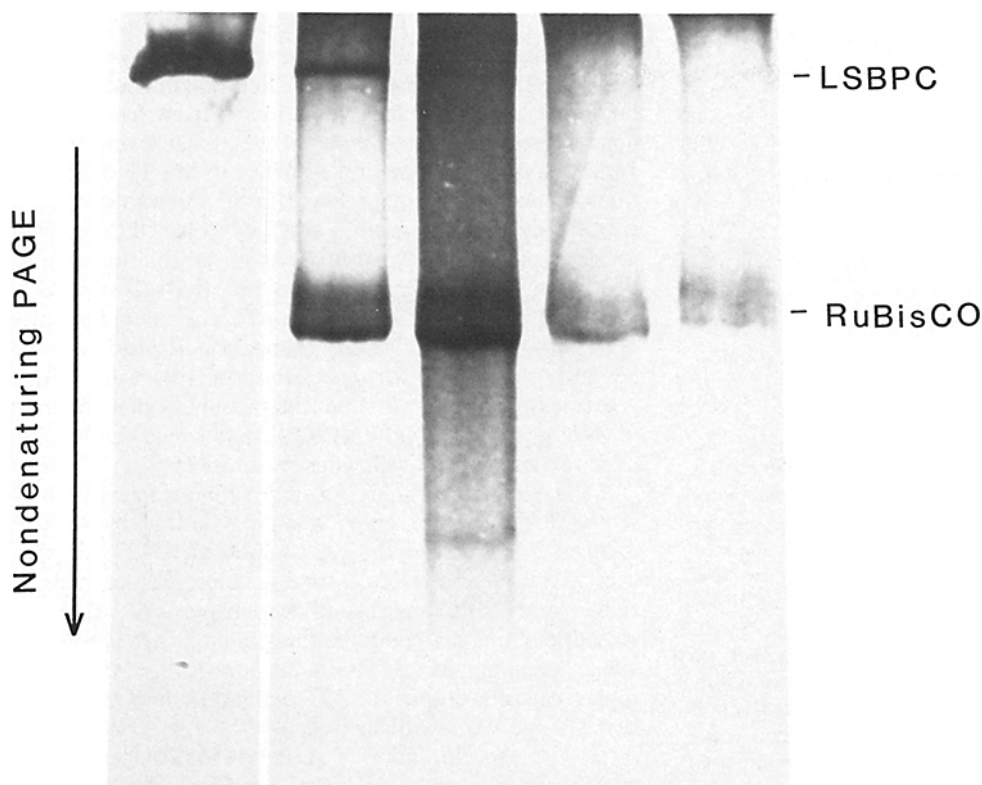
specifically inhibited the assembly of large subunits into RuBisCO.

In the presence of 20 mM AMP, the highly specific binding protein antibody inhibited assembly ∼70%, as judged by densitometric analysis (Fig. 2; $[1-(F\text{-}A)/(B\text{-}A)] \times 100$). Densitometry of the stained gel verified that the amount of 18S RuBisCO was the same (within 5%) in all gel lanes (not shown). Addition of the specific antibody also prevented entry of the stainable radioactive 29S binding protein complex into the gel (lanes *F* and *G*). Preimmune serum did not affect the extent of assembly (compare lanes *E* and *B* with lane *A*). Preimmune serum seemed to increase the tendency of large subunits to enter the running gel (lane *E*), as seen by the increased background radioactivity compared to the other lanes. This effect was reversed by the presence of specific binding protein antibody in the serum (lanes *F* and *G*). Added 29S complex appeared to reduce the incorporation of radioactive large subunits into RuBisCO in this experiment (lane *D*). This may reflect the presence of 7S large subunits in the added material, which may dissociate from 29S complexes during overnight storage. When a minimal amount of 29S complex was mixed beforehand with the antibody, the antibody reproducibly lost its ability to inhibit the assembly reaction (Fig. 2, lane *H*; Fig. 3). Correspondingly, the binding protein complex once again migrated into the gel, and the background radioactivity returned to the level seen with preimmune serum. This shows that, at the concentration used, the serum was close to equivalence with the immunologically sensitive component of the assembly process, and

it was also close to equivalence with the binding protein. In the presence of 20 mM AMP there was no substantial decrease in radioactivity of the 29S complex in samples where increased assembly of RuBisCO occurred (Tables I and II; Figs. 2 and 3).

As pointed out earlier, there is a trace of cross-reactivity between the binding protein antibody and small subunits. In an attempt to control for the remote possibility that inhibition of assembly was mediated through this weak cross-reactivity, we tested the effect of preincubating the antiserum with intact RuBisCO. Added RuBisCO was without effect on the assembly reaction (Fig. 2, compare lane *C* with lane *B*). When RuBisCO was mixed beforehand with the antibody, the antibody retained its ability to inhibit the assembly reaction (lanes *F* and *G*). Thus, none of the superficial large or small subunit determinants on native RuBisCO can suppress the inhibition. However, it still remained possible that the cross-reacting determinants are not exposed to solution in native RuBisCO, as suggested by Hemmingsen and Ellis (18). We therefore examined the effect of preincubating the antiserum with pea RuBisCO soluble small subunits (Fig. 3). Quantities of small subunits approximating the in vivo concentration of free small subunits as well as a 20-fold excess of the in vivo concentration were used. In this experiment, the extent of inhibition of RuBisCO assembly by antibody (∼50%) was slightly less than normal for experiments conducted in the presence of AMP. However, added 29S complex completely prevented inhibition of assembly by the binding protein antiserum, while added small subunits did not.

Nondenaturing PAGE (vertical label with downward arrow)

−LSBPC

−RuBisCO

Figure 4. Inhibition of assembly by antiserum specific for the large subunit binding proteins in presence of ATP and MgCl₂. Chloroplasts were isolated on Percoll gradients and illuminated at a concentration of 400 µg chl/ml in the presence of 250 µCi/ml [^{35}S]methionine for 24 min at 22°C. An extract was prepared in the usual manner and four 90-µl samples were set up so that final concentrations of 50 mM Hepes–KOH (pH 7.6), 220 mM KCl, 6 mM MgCl₂, 1 mM unlabeled methionine would be achieved. Additions were made to each sample at 0°C as follows: (*A*) no addition; (*B*) 5 mM ATP at t=0 min; (*C*) 5 mM ATP at t=0 min and 15 µl preimmune serum added at t=20 min; (control experiments showed that this interval is sufficient to cause maximal assembly of large subunits into RuBisCO); (*D*) 5 mM ATP at t=0 min and 15 µl anti–29S complex serum at t=20 min; (*E*) 5 mM ATP at t=0 min and 15 µl anti–60 kD protein at t=20 min. At t=30 min, samples were incubated at 24°C for 60 min and analyzed by nondenaturing PAGE. The gel was soaked in EN³HANCE, dried, and exposed to x-ray film. This fluorogram is slightly underexposed in order to bring out the detail in lane *C*. Background levels in other lanes on the original film are comparable to those in other figures. LSBPC is the 29S large subunit binding protein complex.

Antibody to the binding protein also inhibited assembly when the assembly reaction was carried out after incubation in the presence of 5 mM ATP (Fig. 4, compare lanes *D* and *E* with lane *B*). As in Figs. 2 and 3, the preimmune serum seemed to increase the tendency of large subunits to enter the running gel (lane *C*), as seen by the increased background radioactivity compared to the other lanes. Because the concentration of 7S complexes is several-fold higher in the presence of ATP than in its absence (25), this creates a very high background compared with lane *E*, Fig. 2. However, as in Fig. 2, the binding protein–specific serum reversed this effect (compare lanes *E* and *C* in Fig. 4). This is consistent with the idea that all large subunits are complexed to the binding protein.
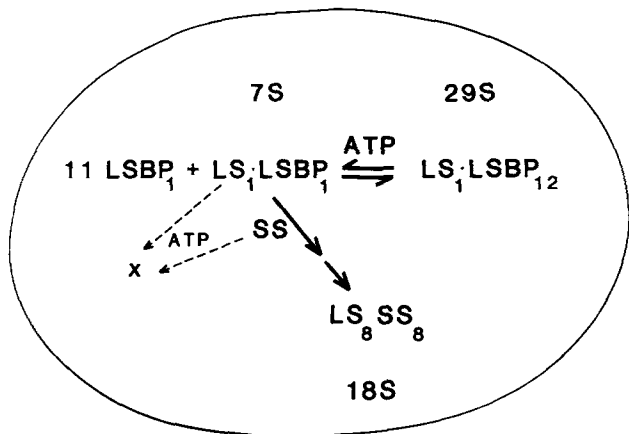
Discussion

This project was initiated because there was preliminary quantitative evidence for a role of the large subunit binding protein in assembly of RuBisCO (25). Since then, other researchers have detected and partly characterized this protein in other organisms, confirming its widespread occurrence (12, 18, 20, 34); our group has repeated the demonstration that the 29S complex contains active, assembly-competent large subunits (25, 27); we have obtained evidence that large subunits not associated with the high molecular weight form of the binding complex are also assembly competent; and we have found that antibody to the binding protein inhibits the in vitro assembly of large subunits into RuBisCO under two

quite different sets of conditions. We have also made several observations, some of which show that the assembly process in vitro is more complex than previously believed. The most important of these include the unexpected limitation of assembly caused by low levels of endogenous ATP; the fact that the 29S complex exhibits a characteristic ultrastructure (Koretz, J. F., S. C. Cannon, S. J. Tumminia, and H. Roy, manuscript in preparation); and the occurrence of the binding protein in maize.

Densitometry can be a useful aid in assessing the relative amounts of radioactive RuBisCO large subunits in various complexes in chloroplast extracts (25). The recovery of total radioactivity in Table II from one sample to the next is reasonably constant under a given set of conditions, which indicates that the data are internally consistent. In the presence of 20 mM AMP, or in the presence of hexokinase and glucose, the large subunit radioactivity in the 29S complex does not decrease, while that in the 7S pool decreases by a substantial amount (61%). The extent of this decrease is comparable in magnitude to the increase in radioactivity in 18S RuBisCO. These data support the inference that 7S large subunits, in the fresh state, are assembly competent. Considering our earlier data showing that large subunits derived from the 29S complex are also assembly competent (25), it appears that the large subunits in both the 7S and 29S complexes are functionally equivalent, as expected (20, 32). But the 29S complex does not serve as a direct donor of large subunits to small subunits, since assembly of large subunits derived from the 29S complexes appears to require dissocia-

$$7S \qquad 29S$$
$$11\,LSBP_1 + LS_1LSBP_1 \underset{}{\overset{ATP}{\rightleftharpoons}} LS_1 \cdot LSBP_{12}$$
$$X \xleftarrow{\quad ATP \quad} SS$$
$$LS_8 SS_8$$
$$18S$$

Figure 5. Current model of assembly of RuBisCO in pea chloroplast extracts. In this model, large subunits are bound to monomers of the binding protein in 7S complexes (LS_1LSBP_1), or in dodecamers of binding protein in 29S complexes (LS_1LSBP_{12}). ATP in the mM range (large letters) can mediate the release of 7S complexes and binding protein monomers ($LSBP_1$). Since antibodies to LSBP inhibit assembly, and radioactive large subunits sediment at 7S, and since under no circumstances have we observed direct transfer of radioactivity from the 29S complex into 18S RuBisCO (LS_8SS_8), assembly of RuBisCO is depicted as a small subunit (SS) reaction with LS_1LSBP_1, followed by more than one unknown step. To account for the effects of hexokinase and glucose on assembly we propose an ATP-dependent step (arrows pointing to X, designating unknown products) which inhibits the participation of small subunits or LS_1LSBP_1 in the assembly process. Since this occurs at very low ATP concentrations, the ATP is printed in smaller capitals in the drawing. We think that at low ATP, the restriction mechanism dominates and little assembly is observed. At zero ATP the restriction mechanism is not operative and assembly can be seen. At high ATP, the large subunit pool is so much enlarged that assembly outpaces the restriction mechanism. Although the mechanism of this restrictive activity is not known, one possibility is that it is due to an ATP-dependent protease.

tion of the 29S complex into 7S subunits and binding protein monomers (25).

Since we find that large subunits not associated with the 29S complex assemble to the greatest extent when the low level of endogenous ATP in the chloroplast extract has been removed, it appears that ATP functions as both a positive and negative regulator of the in vitro assembly of RuBisCO. At high (e.g., 5 mM) concentrations, ATP is responsible for the dissociation of the 29S complex. The resulting large increase in the size of the 7S pool leads to stimulation of assembly of the released large subunits into RuBisCO. At the low (micromolar) concentrations of ATP present in our chloroplast extracts, the 7S pool is small, and assembly is minimal, but it can be stimulated by removal of this ATP. This indicates the existence of an ATP-dependent reaction which limits the extent of in vitro assembly of large subunits into RuBisCO. Probably this reaction always occurs, but is only noticeable when the 7S pool is small (i.e., when the 29S complex is prevented from dissociating into 7S material) (Fig. 5). We do not know whether the mechanism of the ATP-dependent limitation on RuBisCO assembly is related to the turnover of polypeptides reported in *Chlamydomonas* (33) or higher plant chloroplasts (7, 15, 21, 23), but we are investigating this possibility. However, there are some inferences which can be drawn from the discovery of this limitation, considering the fact that the posttranslational assembly of large subunits into RuBisCO is light dependent in intact chloroplasts (9). Since the limitation on in vitro assembly is dependent on ATP, presumably it is dependent on some enzyme activity. Since the limitation occurs at ATP concentrations far lower than those found in the chloroplast stroma (17), the enzyme responsible would be expected to be active in the dark in vivo. Thus, it seems reasonable to postulate that assembly of 7S large subunits into RuBisCO would be restricted in the dark, and that the ATP-dependent limitation of in vitro assembly of 7S large subunits described here may be partly responsible for this restriction. However, the data presented here do not rule out the possibility that assembly of 7S large subunits into RuBisCO could occur slowly in the dark in vivo, for the following reasons.

(a) The immediate source of large subunits for in vitro assembly is the 7S pool, either when it is built up by MgATP-induced dissociation of the 29S complex (9, 25-27), or when it is investigated in its fresh state in chloroplast extracts, as shown here. In the presence of endogenous ATP, when dissociation of the 29S complex is negligible, there is some residual assembly of large subunits into RuBisCO in chloroplast extracts, despite the ATP-dependent limitation (Table II; Cannon, S., unpublished data).

(b) The concentrations of ATP, any ATP-dependent enzymes, and unassembled RuBisCO subunits must be several hundred-fold higher in vivo than in the chloroplast extracts. It is not clear how the high in vivo concentrations of all these components would affect the relative rates of RuBisCO assembly on the one hand, and the limiting, ATP-dependent activity we have detected in the extracts on the other.

After dialysis of chloroplast extracts against buffers containing 0.5 mM ATP, the binding protein migrates primarily as a monomer during nondenaturing PAGE. Since this concentration of ATP is present even in the dark (17), it has been argued that the monomeric form of the binding protein complex would predominate over the high molecular weight or 29S form in vivo (20). This conclusion seems premature, since the association of binding protein subunits to form the dodecameric complex would be expected to be proportional to the 12th power of the monomer concentration. We are investigating this possibility.

Because some assembly of RuBisCO takes place during *in organello* protein synthesis, and there can be some assembly of RuBisCO even at 0°C in the presence of 20 mM AMP, the baseline from which one measures in vitro assembly can be elevated in some experiments. Because this is accompanied by depletion of large subunit (and presumably small subunit) pools, there is a limited quantity of large subunits or small subunits remaining to permit continued assembly. Despite this, as shown in each of the three experiments in Figs. 2, 3, and 4, further assembly of RuBisCO does take place during incubation at room temperature, and the binding protein antibody consistently inhibits this assembly; as shown in both Figs. 2 and 3, this inhibition is specifically and completely blocked by preincubating the antiserum with 29S complex.

The inhibitory effect of the binding protein–specific antibody on assembly of RuBisCO ranges from 50 to 70% in the experiments presented in this paper. There are at least three possible interpretations of this, any or all of which could contribute to the observed effects.

(a) It could be that between 50 and 70% represents the proportion of large subunits associated with the binding protein. This is unlikely based on analysis of the physical properties of the large subunits, which do not show a major component sedimenting in the 4S region expected of monomeric large subunits, for example (25, 30–32).

(b) It could be that large subunit–binding protein complexes which have reacted with antibody still retain from 30 to 50% of their ability to interact with small subunits and assemble into RuBisCO.

(c) The equivalence point for each chloroplast extract could vary, accounting for variation between 50 and 70% inhibition. Since it is not practical to titrate the equivalence point for each chloroplast preparation, we simply chose the lowest concentration of antiserum which gave a consistent, visually (and densitometrically) detectable inhibition of assembly from one experiment to the next.

The most reasonable interpretation of the data, therefore, is that the great majority of assembly-competent large subunits are associated with binding protein. It is clear therefore that no model for assembly of RuBisCO in pea chloroplasts can exclude the binding protein. Our current working hypothesis is illustrated in Fig. 5.

What is the function of the binding protein? It could be concerned primarily with regulating the supply of large subunits in the light and the dark. It could be required for assembly of RuBisCO in higher plant chloroplasts; for example, the binding protein might confer assembly competence on large subunits by maintaining their solubility until they are capable of interacting with small subunits. Since the 7S large subunits sediment more rapidly than the monomeric binding protein subunits (18), and have an estimated molecular weight of 117,000 (31), and since their ability to assemble is inhibited by antibody to the binding protein, it appears that they are heterodimers containing one large subunit and one binding protein subunit. If this is the case, perhaps the next step in the assembly process involves association of small subunits with the heterodimer, or release of large subunit from the heterodimer. Experiments are now under way to answer these questions.

References

1. Andrews, T. J., and B. Ballment. 1983. The function of the small subunits of ribulose bisphosphate carboxylase-oxygenase. *J. Biol. Chem.* 258:7514–7518.
2. Andrews, T. J., and G. Lorimer. 1985. Catalytic properties of a hybrid between cyanobacterial large subunits and higher plant small subunits of ribulose bisphosphate carboxylase-oxygenase. *J. Biol. Chem.* 260:4632–4636.
3. Arnon, D. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris. Plant Physiol. (Bethesda).* 24:1–15.
4. Baker, T. S., D. Eisenberg, and F. Eiserling. 1977. Ribulose bisphosphate carboxylase: a two-layered square-shaped molecule of symmetry 422. *Science (Wash. DC).* 196:293–297.
5. Barnard, G., H. Roy, and Y. P. Myer. 1983. Reactions of spinach ribu-

lose 1,5-bisphosphate carboxylase with te tranitromethane. *Biochemistry.* 22:2697–2704.
6. Barraclough, R., and R. J. Ellis. 1980. Assembly of newly synthesized large subunits into ribulose bisphosphate carboxylase in isolated pea chloroplasts. *Biochem. Biophys. Acta.* 608:19–31.
7. Bennett, J. 1981. Biosynthesis of the light-harvesting chlorophyll a/b protein. *Eur. J. Biochem.* 118:61–70.
8. Blair, G. E., and R. J. Ellis. 1974. Protein synthesis in chloroplasts. I. Light-driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts. *Biochem. Biophys. Acta.* 319:223–234.
9. Bloom, M., P. Milos, and H. Roy. 1983. Light-dependent assembly of ribulose 1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. USA.* 80: 1013–1017.
10. Chua, N. H., and G. W. Schmidt. 1978. Posttranslational transport into intact chloroplasts of a precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. USA.* 75:6110–6114.
11. Fish, L., and A. T. Jagendorf. 1982. High rates of protein synthesis by isolated chloroplasts. *Plant Physiol. (Bethesda).* 70:1107–1114.
12. Fromm, H., M. Devic, R. Fluhr, A. K. Mattoo, and M. Edelman. 1984. Protein metabolism and chloroplast regulatory functions. NATO Plant Meeting, Abstract L26, Renesse, The Netherlands.
13. Gatenby, A. A., J. A. Castleton, and M. W. Saul. 1981. Expression in *E. coli* of maize and wheat chloroplast genes for the large subunit of ribulose bisphosphate carboxylase. *Nature (Lond.).* 291:117–121.
14. Gatenby, A. A., and J. A. Castleton. 1982. Amplification of maize ribulose bisphosphate carboxylase large subunit synthesis in *E. coli* by transcriptional fusion with the lambda N operon. *Mol. Gen. Genet.* 185:424–429.
15. Grossman, A. R., S. G. Bartlett, G. W. Schmidt, J. E. Mullet, and N. H. Chua. 1982. Optimal conditions for post-translational uptake of proteins by isolated chloroplasts. *J. Biol. Chem.* 257:1558–1563.
16. Gurevitz, M., C. R. Somerville, and L. McIntosh. 1985. Pathway of assembly of ribulose bisphosphate carboxylase/oxygenase from *Anabaena* 7120 expressed in *Escherichia coli. Proc. Natl. Acad. Sci. USA.* 82:6546–6550.
17. Hall, D. O. 1976. The coupling of photophosphorylation to electron transport in isolated chloroplasts. *In* The Intact Chloroplast. J. Barber, editor. Elsevier/North Holland, the Netherlands. 135–170.
18. Hemmingsen, S. M., and R. J. Ellis. 1986. Purification and properties of RuBisCO LSU binding protein. *Plant Physiol. (Bethesda).* 80:269–276.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of phage T4 head. *Nature (Lond.).* 227:680–685.
20. Lennox, C. R., and R. J. Ellis. The carboxylase large subunit binding protein: photoregulation and reversible dissociation. *Biochem. Soc. Trans.* In press.
21. Liu, X.-Q., and A. T. Jagendorf. 1984. ATP-dependent proteolysis in pea chloroplasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 166:248–252.
22. Lorimer, G. H. 1981. The carboxylation and oxygenation of ribulose-1,5-bisphosphate: the primary events in photosynthesis and photorespiration. *Annu. Rev. Plant Physiol.* 32:349–383.
23. Malek, L., L. Bogorad, A. R. Ayers, and A. L. Goldberg. 1984. Newly synthesized proteins are degraded by an ATP-stimulated process in pea chloroplasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 166:253–257.
24. McIntosh, L., C. Poulsen, and L. Bogorad. 1980. Nucleotide sequence of the single gene for the large subunit of ribulose 1,5-bisphosphate carboxylase of maize. *Nature (Lond.).* 288:556–560.
25. Milos, P., and H. Roy. 1984. ATP-released large subunits participate in the assembly of ribulose bisphosphate carboxylase. *J. Cell. Biochem.* 24: 153–162.
26. Milos, P., and H. Roy. 1985. *In vitro* assembly of ribulose bisphosphate carboxylase/oxygenase. *In* Molecular Biology of the Photosynthetic Apparatus. K. E. Steinback, C. Arntzen, L. Bogorad, S. Bonitz, editors. Cold Spring Harbor, N.Y. 349–354.
27. Milos, P., M. Bloom, and H. Roy. 1985. Methods for studying the assembly of ribulose bisphosphate carboxylase. *Plant Mol. Biol. Rep.* 3:33–42.
28. Milos, P. 1984. An investigation of the roles of newly synthesized subunits in the assembly of RuBPCase. Ph.D. Thesis, Rensselaer Polytechnic Institute. 164 pp.
29. Nivison, H., and A. T. Jagendorf. 1984. Factors permitting prolonged translation by isolated pea chloroplasts. *Plant Physiol. (Bethesda).* 75: 1001–1008.
30. Roy, H., K. A. Costa, and H. Adari. 1978. Free subunits of ribulose bisphosphate carboxylase in pea leaves. *Plant Sci. Lett.* 11:159–168.
31. Roy, H., H. Adari, and K. A. Costa. 1979. Characterization of free subunits of ribulose bisphosphate carboxylase. *Plant Sci. Lett.* 16:305–318.
32. Roy, H., M. Bloom, P. Milos, and M. Monroe. 1982. Studies on the assembly of large subunits of ribulose bisphosphate carboxylase in isolated pea chloroplasts. *J. Cell Biol.* 94:20–27.
33. Schmidt, G. W., and M. L. Mishkind. 1983. Rapid degradation of unassembled ribulose-1,5-bisphosphate carboxylase small subunits in chloroplasts. *Proc. Natl. Acad. Sci. USA.* 80:2632–2636.
34. Somerville, C. R. 1984. The analysis of photosynthetic carbon dioxide fixation and photorespiration by mutant selection. *Oxford Surveys of Plant Molecular and Cell Biology.* 1:103–131.