Journal of Cellular Biochemistry 24:153–162 (1984)
Biosynthesis of the Photosynthetic Apparatus:
Molecular Biology, Development and Regulation 143–152

ATP-Released Large Subunits Participate in the Assembly of RuBP Carboxylase

Patrice Milos and Harry Roy

Biology Department, Rensselaer Polytechnic Institute, Troy, New York 12181

Preincubation of ^{35}S-methionine-labeled chloroplast extracts with ATP at 0°C potentiates the subsequent assembly of labeled large subunits into RuBPCase. This is correlated with the dissociation of newly synthesized large subunits from the 29S large subunit binding protein complex. These released large subunits then assemble into RuBPCase in a second, nucleotide-stimulated reaction. The data demonstrate that the 29S complex can play an active role in the assembly of RuBPCase.

Key words: chloroplast protein synthesis, assembly

The enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase [RuBPCase; 3 phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is an abundant, bifunctional enzyme [1]. The enzyme catalyzes the first step in the Calvin cycle of photosynthetic carbon reduction [2] and the first step in the competing process of photorespiration [3]. The ratio of these two activities is important in determing the efficiency of net carbon dioxide fixation. In higher plants, RuBPCase (M_r = 550 kilodaltons [kD]) contains eight large catalytic subunits (M_r = 55 kD) and eight small subunits (M_r = 14 kD) [4].

The enzyme's small subunit is synthesized on cytoplasmic ribosomes as a 20-kD precursor that is taken up by chloroplasts and processed to ca 14 kD [5] by endoproteolytic cleavage near the amino terminus. The processing activity is believed to reside in the soluble stromal fraction of the chloroplast [6]. A free pool of small subunits, sedimenting at 3–4S in sucrose gradients, exists within the chloroplast [7]. Large subunits are synthesized on chloroplast ribosomes and represent the major soluble product of protein synthesis in isolated intact pea chloroplasts [8]. Newly synthesized large subunits are found in a 7S free pool [7] and also associate specifically with a large subunit binding protein complex having M_r = 600–700 kD and a sedimentation coefficient of 29S. This 29S complex consists of 10–12 large subunit

Abbreviations used: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) N,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazineN′-2-ethanesulfonic acid; RuBPCase, ribulose-1,5-bisphosphate carboxylase; SDS, sodium dodecyl sulfate.

Received June 2, 1983; accepted October 1, 1983.

binding proteins (M_r = 60 kD) associated with perhaps one newly synthesized large subunit [7,9,10].

In vitro studies of isolated intact chloroplasts have been used to monitor assembly of RuBPCase. Incorporation of radioactive large subunits into RuBPCase lags behind that of the 7S free pool and the 29S complex by about 30–40 min. A similar lag occurs in vivo. During an in vitro illuminated chase period radioactive large subunits are lost from both the 7S free pool and the 29S complex and appear in newly assembled RuBPCase [7].

The assembly of RuBPCase has been shown to be light dependent in isolated chloroplasts [9]. Nucleotides such as ATP, GTP, and a nonhydrolyzable ATP analog, adenosine 5′ - [β,γ- methylene]-triphosphate, can partially substitute for light in promoting RuBPCase assembly in chloroplast extracts. In the presence of Mg^{2+}, ATP causes dissociation of the 29S complex into the individual 60-kD large subunit binding proteins and free radioactive large subunits.

In the present work we show that the large subunits released from the 29S complex are competent to participate in the assembly of RuBPCase. The effects of ADP and adenosine 5′ - [β, γ-methylene]-triphosphate subsequent to dissociation of the 29S complex were also investigated.

METHODS

Plant Growth

Pea plants (Pisum sativum var. Progress No. 9) (Agway Inc., Buffalo, NY) were grown in vermiculite under a 12 hr light/12 hr. dark cycle at 24°C. Plants used for experimental purposes were between 8–11 days old.

Chloroplast Isolation

Chloroplasts were isolated by the method of Fish and Jagendorf [11]. Twenty grams of apical leaves were cut from plants and homogenized in a blender with 100 ml of grinding buffer containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1mM $MgCl_2$, 50 mM HEPES·KOH (pH 8.3), 3 mM EGTA, and 0.5% BSA. The homogenate was filtered through a layer of Miracloth and centrifuged at 4,000 g momentarily. The supernatant was poured off and the pellets were gently resuspended in the remaining buffer, using a fine paint brush.

The resuspended chloroplasts were layered on two Percoll gradients (Pharmacia) 10–80%(v/v), containing 1%(w/v)BSA, 1%(w/v)Ficoll 400, 3%(w/v) polyethylene glycol, 330 mM sorbitol, 2mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 50 mM HEPES·KOH (pH 8.3), 5 mM ascorbic acid, and 5.5 mM glutathione. The gradients were centrifuged at 10,000 g for 8 min. Chloroplasts migrating to a position between 0.5–1.0 cm from the bottom were removed with a Pasteur pipette. These were observed to be intact chloroplasts by phase contrast microscopy. The chloroplasts were resuspended in a buffer containing 330 mM Sorbitol and 50 mM HEPES·KOH, pH 8.3, and pelleted by centrifuging momentarily at 4,000 g. The chlorophyll concentration was determined by the method of Arnon [12] and adjusted to the concentration specified in each experiment.

In Vitro Protein Synthesis

Chloroplasts were incubated in a 21°C circulating water bath with ^{35}S-methionine (from New England Nuclear, 700–1,200 Ci/mmol.) The bath was illuminated

with 10,000 lux of filtered red light. Following incubation, the chloroplasts were diluted ten fold with resuspension buffer and pelleted by centrifuging at 4,000 g momentarily. The chloroplasts were then lysed with a volume of lysis buffer (10 mM Tris, pH 76) equal to the original suspension, and then centrifuged at 12,000 g for 10 min. The supernatant is referred to as a "chloroplast extract."

Monitoring Assembly in Chloroplast Extracts

The chloroplast extracts were incubated as described in figure captions in a buffer containing a final concentration of 50 mM HEPES (pH 7.6), 220 mM KCl, 6 mM $MgCl_2$, 20 mM dithiothreitol, and an excess of unlabeled methionine. Extracts were analyzed by sucrose density gradient ultracentrifugation [7,9] followed by electrophoresis of gradient fractions.

Polyacrylamide Gel Electrophoresis

Electrophoresis of samples was carried out using the discontinuous sodium dodecyl sulfate slab gel system of Laemmli [13] using a 8.5–18% running gel and a 4.5% stacking gel. Electrophoresis was carried out at 25 mA until the voltage reached 150 V, at which time the gels were switched to a constant 150 V and run until the tracking dye was 1 cm from the bottom of the running gel.

Nondenaturing gel electrophoresis was conducted using a 7.5% running slab gel and 4.5% stacking gel. SDS was eliminated from the buffers and the gels. Electrophoresis was carried out at 100 V for 18–22 hr.

Densitometry

Semiquantitative analysis of autoradiograms was done by scanning the autoradiograph film using a Gilford spectrophotometer equipped with a linear gel transporter. The integrated film density (IFD) was calculated from a formula: IFD = bhv $\times$ normalization factor, where normalization factor = ratio of lane width divided by standard band width; b = No. of chart units at base of peak, h = No. of chart units from top of peak to baseline, and v = volume of sample.

RESULTS

ATP has been shown to stimulate assembly of RuBPCase when incubated with chloroplast extracts at room temperature [9]. Little or no assembly of RuBPCase occurred when the extracts were incubated with ATP at 0°C. However, when we examined samples of extracts which had been treated with ATP at 0°C, we observed significant disaggregation of the stainable 29S large subunit binding protein complex (data not shown). For this reason, an ATP preincubation experiment was designed to resolve the dissociation of the 29S complex from the actual assembly of RuBPCase molecules.

Extracts were obtained from in vitro labeled chloroplasts and preincubated for 20 min on ice with or without 5 mM ATP; then the extracts were incubated for 60 min at 0°C or room temperature. The four extracts were then centrifuged on sucrose gradients, and gradient fractions were analyzed by nondenaturing gel electrophoresis to resolve the 18S holoenzyme from the 29S complex (Fig. 1), or by SDS gel electrophoresis to visualize the 7S free large subunit pool (Fig 2).

Figure 1A shows that at 0°C the bulk of the radioactivity remained associated with the stainable 29S complex. We have previously shown that all this radioactivity

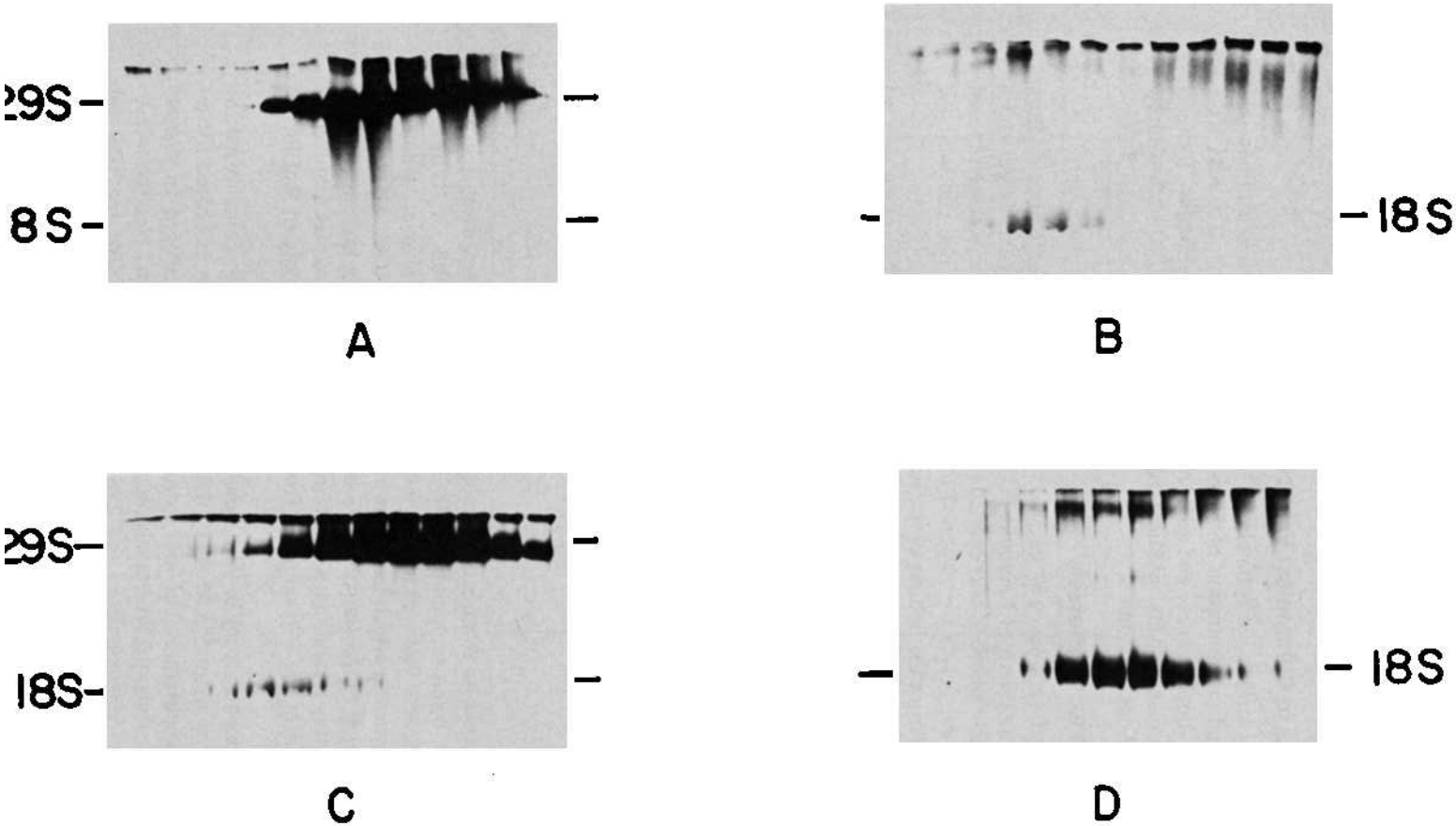

Fig. 1. Chloroplast extract preincubation with ATP on ice. Lower gradient fractions. Chloroplasts (1,060 μg chlorophyll) were illuminated in the presence of 1,176 μCi ^{35}S-methionine for 30 min. A soluble chloroplast lysate was obtained. Excess unlabeled methionine was added and the ionic conditions were adjusted as described in Methods. Four aliquots were subjected to the various conditions indicated below. After incubation, samples were layered on sucrose gradients and centrifuged overnight. Lower gradient fractions were analyzed on nondenaturing polyacrylamide gels. The flurogram is shown. Left to right in panel, direction of sedimentation; top to bottom, direction of electrophoresis. 18S marks positions of stained RuBPCase band; 29S marks position of stained 29S complex. A) Incubated at 0° (t = 90 min). B) Incubated with 5 mM ATP at 0°C (t = 90 min). C) Incubated at 0°C (t = 30 min) + incubation at 24°C (t = 60 min). D) Incubated with 5 mM ATP at 0°C (t = 30 min) + incubation at 24°C (t = 60 min).

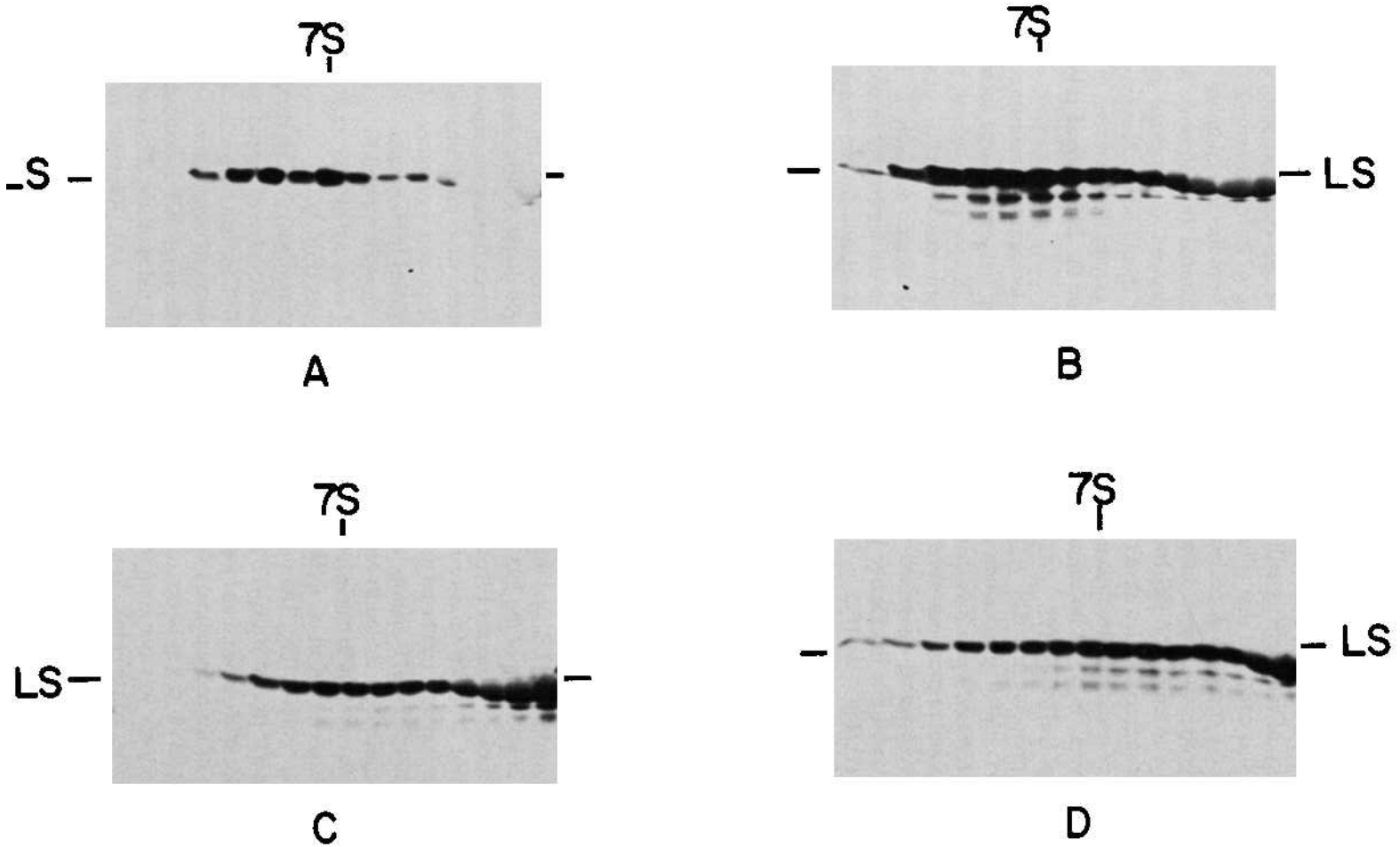

Fig. 2. Chloroplast extract preincubation with ATP on ice. Upper gradient fractions. Experiment described in Figure 1. Upper gradient fractions were analyzed on SDS-polyacrylamide gels.

is due to large subunits [7,9]. No radioactivity corresponded to the 18S RuBPCase. The ice control treated with ATP (Fig. 1B) showed essentially no radioactivity (or stain) associated with the 29S complex. A small amount of radioactivity was associated with the 18S RuBPCase. In the room-temperature, non-ATP control (Fig. 1C) the majority of radioactivity still was associated with the 29S complex. RuBPCase assembly also exceeded that which was seen on ice. The ATP-treated sample (Fig. 1D) showed a complete dissociation of the radioactivity from the 29S complex. The stainable protein was also dissociated from the 29S material (not shown). Most importantly, assembly of 18S RuBPCase was greatly enhanced as compared to the sample not preincubated with ATP or the ice controls. It should be emphasized that the extent of assembly observed under these conditions is greater than that seen in our previous work, in which no attempt was made to dissociate the 29S complex prior to assembly of RuBPCase [9].

The preincubation with ATP on ice prior to room-temperature incubation resulted in a complete dissociation of the 29S complex. The radioactive large subunits that were released upon dissociation were found in the 7S free large subunit pool (Fig. 2). Visual impressions were confirmed by densitometric analysis and calculation of the corresponding integrated film density (IFD units) as seen in Table I. This analysis showed that the 7S free large subunit pool seen in the ice control incubated without ATP (Fig. 2A, IFD = 4,813) was not enough to account for the increased 18S RuBPCase assembly seen in the extract incubated at room temperature after preincubation with ATP (Fig. 1D, IFD = 16,052) as compared to the levels of 18S RuBPCase assembly seen in the ice control incubated without ATP (Fig. 1A, IFD = 1,100) or the ice/room temperature control without ATP (Fig. 1C, IFD = 3,955). The 29S complex seen in the ice control incubated without ATP (Fig. 1A, IFD = 25, 201) contained sufficient material to account for the increased assembled 18S RuBPCase. Therefore, the 29S large subunits released into the 7S free large subunit pool by ATP treatment must be competent to participate in RuBPCase assembly. It is likely that the labeling of the 18S enzyme by large subunits represents de novo assembly of holoenzyme. Small subunit pools, labeled in vivo, have been detected in isolated chloroplasts [7,9] and these are depleted in the presence of ATP [9].

The dissociation of the 29S complex and the assembly of 18S RuBPCase appear to be two distinct processes [9]. To investigate these processes further, an experiment was designed in which in vitro labeled chloroplast extracts were preincubated with ATP on ice and then incubated at room temperature in the presence of either ADP or a nonhydrolyzable ATP analog, adenosine 5′ [β,γ-methylene]-triphosphate. Glucose and hexokinase were added to remove any residual ATP or ATP formed by reaction of ADP with endogenous adenylate kinase. Extracts were analyzed by sucrose density gradient ultracentrifugation, followed by gel electrophoresis (Fig. 3).

The ice control (Fig. 3A) showed radioactivity associated with the 29S complex. The ice control treated with ATP (Fig. 3B) showed that dissociation of the radioactive 29S complex had occurred and a small amount of 18S RuBPCase had been assembled. In the case of ice preincubation with ATP, and subsequent room temperature incubation with ADP in the presence of hexokinase and glucose, (Fig. 3C) dissociation of the 29S complex was not complete. Radioactivity associated with the 29S complex was, however, considerably less than the ice control (Fig. 3A). Assembly of 18S RuBPCase (Fig. 3C) was greater than in either of the ice controls. In the case where the non-hydrolyzable ATP analog adenosine 5′-[β,γ-methylene]-triphosphate was

TABLE I. Densitometric Analysis*

Conditions	7S	18S	29S
0°C, 90′	4,813	1,100	25,201
0°C, 90′ (+ATP 5mM)	25,069	3,153	3,749
0°C, 30′; 25°C, 60′	6,579	3,955	26,496
0°C, 30′ (+ATP 5mM); 25°C, 60′	16,577	16,052	0

*Densitometry was carried out on the flurograms shown in Figures 1 and 2, as described in Methods. The numbers in the table represent integrated film density (defined in Methods).

substituted for the ADP, again, dissociation of the 29S complex was not complete (Fig. 3D); but the nonhydrolyzable ATP analog was able to stimulate the assembly of the 18S holoenzyme above the level seen in any other sample.

DISCUSSION

ATP has been shown to stimulate assembly of RuBPCase in chloroplast extracts [9]. At the same time ATP and Mg^{2+} also cause a dissociation of the 29S large subunit binding protein (LSBP) complex, leading to release of the radioactive large subunits associated with this complex into the free large subunit pool [9]. The results presented here show that the radioactive large subunits that are released from the 29S complex are indeed competent to participate in the assembly of RuBPCase.

From these results some working hypotheses for RuBPCase assembly can be formulated (Fig. 4). Isolated chloroplasts synthesize large subunits (LS), which occur either in a free pool or bound to LSBP. It is likely that an equilibrium exists between LS and $LSBP_n$ LS [7,9] mediated in part by ATP hydrolysis. RuBPCase (LS_8SS_8) is assembled in the chloroplast from these large subunits and the free small subunit pool (SS) [9].

The results presented here are consistent with two ideas about the role of the LSBP complex. First, the $LSBP_n$ LS may represent an obligatory intermediate in the assembly of LS_8SS_8; secondly, it could represent a nonobligatory storage site for large subunits (LS) that can be made available for holoenzyme assembly by release from the 29S complex when levels of chloroplast ATP are adequate. The data show that large subunits released from the 29S complex ($LSBP_n$ LS) enter the free LS pool and can then assemble into RuBPCase (pathway A). However, the question remains whether large subunits located in the 29S complex are directly able to participate in LS_8SS_8 assembly, without reappearing in the free LS pool (pathway B). Nor can we tell whether the participation of the 29S complex in assembly is mandatory.

The present work provides further evidence that the dissociation of the 29S complex by ATP and the promotion of RuBPCase assembly by various nucleotides are two distinctly different processes [9]. ADP and a nonhydrolyzable ATP analog are not able to dissociate the 29S complex [9]. However, they do produce enhanced levels of assembled 18S RuBPCase after extracts have been preincubated with ATP on ice. The nonhydrolyzable ATP analog promotes this reaction somewhat better than

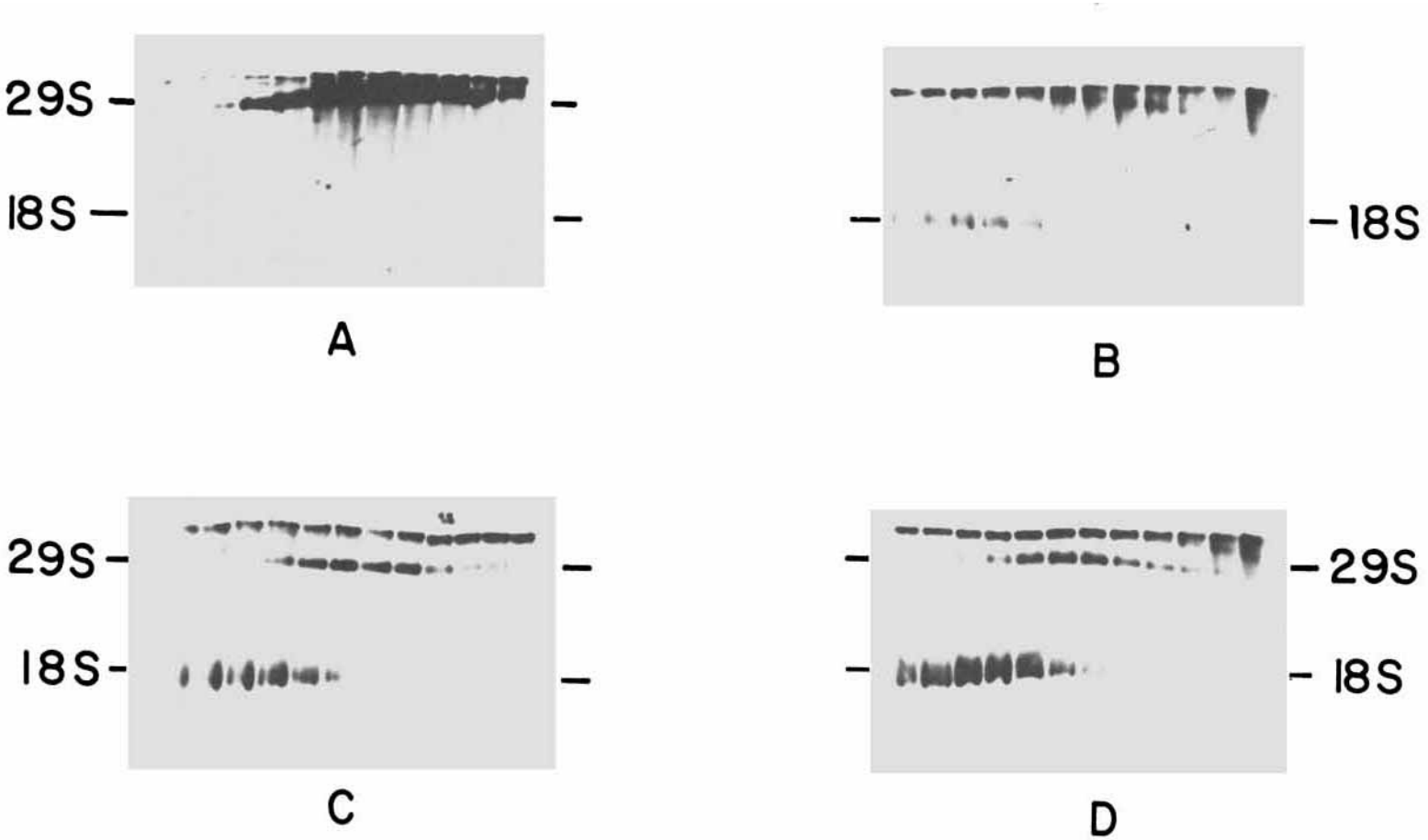

Fig. 3. Chloroplast extract preincubation with ATP followed by incubation with ADP or β, γ-methylene-ATP. Isolated chloroplasts (1,060 μg of chlorophyll) were illuminated in the presence of 1,078 μCi of ^{35}S-methionine for 25 min. Four aliquots of chloroplast extract were subjected to the various conditions indicated after excess unlabeled methionine was added and ionic conditions were adjusted. After incubation, the samples were centrifuged on sucrose gradients. Lower gradient fractions were analyzed on non-denaturing gels. Symbols in Figure 1. A) Incubated at 0°C (t = 90 min). B) Incubated at 0°C (t = 30 min). + incubation with 5 mM ATP at 0°C (t = 60 min). C) Incubated with 5 mM ATP at 0°C (t = 30 min) + incubation with 10 mM glucose, 25 U of hexokinase and 5 mM aDP at 24°C (t = 60 min). The extra ADP was added so that total nucleotide concentration would be the same in C and D. This precaution is not necessary, however. D) As in C except 5 mM β, γ-methylene ATP was substituted for 5 lmM ADP.

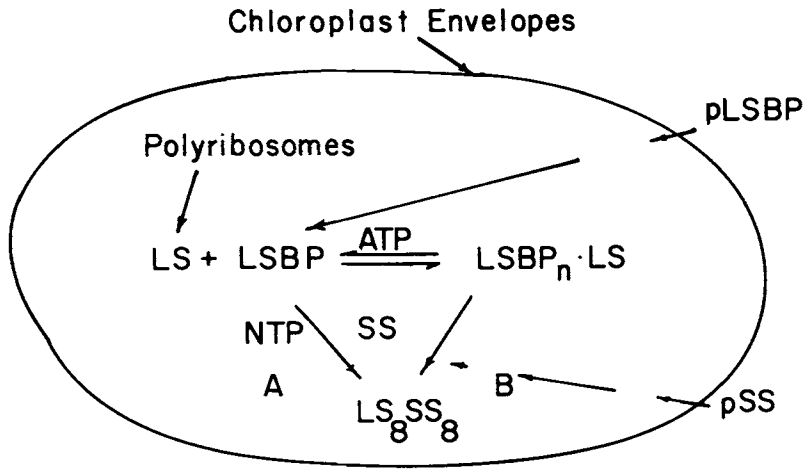

Fig. 4. Models of RuBPCase assembly. Postulated models for the assembly of RUBPCase (LS_8SS_8) from the newly synthesized free pool of large subunits (LS), large subunits associated with a 60-kD large subunit binding protein ($LSBP_n$ LS), and a free pool of small subunits (SS) that exists within the chloroplast. A and B represent unresolved alternatives for the final step. NTP, nucleoside triphosphate (either ATP, GTP, or β,γ-methylene ATP). Subscript n = 10 or 12 [7–9]. (See text for more detail.)

ADP. The assembly of RuBCase in vitro is thus supported by the ATP-dependent dissociation of the 29S $LSBP_n$ LS complex first, followed by a second, less specific nucleotide-dependent reaction. Studies are continuing on the mechanisms underlying these reactions.

The major conclusion we draw from the present information is that the 29S complex represents a real component of the RuBPCase assembly system in pea chloroplasts, since the large subunits derived from the 29S complex can indeed participate in assembly of RuBPCase in vitro. One possible role of the 29S complex can be related to known physical properties of the large subunit. This protein is notoriously insoluble when isolated from RuBPCase that has been dissociated with urea or SDS. Even under the relatively favorable circumstances seen in our studies, large subunits do tend to aggregate in the sample loading wells or at the start of the slab gel in nondenaturing electrophoresis. This is a concentration-dependent phenomenon and is more evident in our recent work, in which we have obtained higher protein synthesis rates than in our previous studies [7,9]. Thus, the 29S complex may represent a means of maintaining the large subunit pool in a soluble state, as originally suggested by Barraclough and Ellis [10]. This interpretation is consistent with either of the two assembly pathways depicted in Figure 4.

Grossman et al [14] have shown that ATP can substitute for light in promoting the uptake of precursor polypeptides by chloroplasts. Our observations underscore the need to examine in detail the posttranslational aspects of chloroplast biogenesis, because it appears that in more than one instance, the assembly of chloroplast proteins is at least potentially under the control of the energy supply in the leaf cell.

ACKNOWLEDGMENTS

This material is based on work supported by the US Department of Agriculture under agreement 59-2367-1-1-633-0 (Competitive Grants Office), and is part of the PhD thesis research of Patrice Milos. The authors thank Dr Henry Ehrlich, who suggested that we look at the effect of ATP on ice.

REFERENCES

1. Ellis RJ: Trends Biochem Sci 4:241, 1979.
2. Weissbach A, Horecker BL, Hurwitz J: J Biol Chem 218:795, 1956.
3. Bowes G, Orgren WL, Hagemann R: Biophys Biochem Res Commun 45:716, 1971.
4. Baker TS, Eisenberg D, Eiserling F: Science 196:293, 1977.
5. Chua NH, Schmidt G: Proc Natl Acad Sci USA 75:6110 1978.
6. Smith S, Ellis RJ: Nature 278:662, 1979.
7. Roy H, Bloom M, Milos P, Monroe M: J Cell Biol 94:20, 1982.
8. Blair GE, Ellis RJ: Biochem Biophys Acta 319:223, 1973.
9. Bloom M, Milos P, Roy H: Proc Natl Acad Sci USA 80:1013, 1983.
10. Barraclough R, Ellis RJ: Biochim Biophys Acta 608:19, 1980.
11. Fish L, Jagendorf AT: Plant Physiol 70:1107, 1982.
12. Arnon DI: Plant Physiol 24:1, 1949.
13. Laemmli UK: Nature 227:680, 1970.
14. Grossman A, Bartlett S, Chua NH: Nature 285:625, 1980.