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Light-dependent assembly of ribulose-1,5-bisphosphate carboxylase

(chloroplast/ATP/protein synthesis)

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ASBTRACT **Ribulose-1,5-bisphosphate carboxylase [RuP_2Case; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is composed of eight small subunits (M_r, 14,000) and eight large subunits (M_r, 55,000). Newly synthesized large subunits are associated with two complexes having sedimentation coefficients of 7 and 29 S. Assembly of RuP_2Case occurs in isolated intact chloroplasts in the light but not in the dark. When extracts of chloroplasts are treated with ATP or GTP, RuP_2Case assembly is accelerated while the 29S large subunit complex is maintained. In the presence of Mg^{2+}, ATP brings about almost complete dissociation of the 29S complex, whereas GTP and a nonhydrolyzable analog of ATP are without effect. These results indicate the existence of a complex set of reactions involving nucleotides, Mg^{2+}, and several putative intermediates in RuP_2Case assembly. It is postulated that these reactions at least partly account for the light dependence of RuP_2Case assembly. In particular, ATP and GTP promote the assembly of large subunits into RuP_2Case.**

Ribulose-1,5-bisphosphate carboxylase/oxygenase [RuP_2Case; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] catalyzes the fixation of carbon dioxide in the photosynthetic carbon reduction cycle (1) and the competing fixation of oxygen into the photorespiratory substrate 2-phosphoglycolate (2). The balance between these reactions is critical for net carbon assimilation in photosynthesis. The enzyme is composed of two different subunit types: the "large" subunits (M_r, 55,000) and the "small" subunits (M_r, 12,000–16,000, depending on species) (1, 3). The small subunit is synthesized in the cytoplasm as a precursor, which is taken up by illuminated chloroplasts posttranslationally, cleaved, and assembled into RuP_2Case molecules (4). The large subunit is synthesized within the chloroplast, and it binds to a high molecular weight chloroplast protein consisting of 10–12 subunits of M_r 60,000 (5, 6). The complex between the large subunit and this relatively abundant oligomeric protein behaves as a particle of M_r 600,000–700,000 during nondenaturing electrophoresis (5) and sediments as a 29S complex in sucrose gradients (6). It is therefore clearly distinguishable from fully assembled RuP_2Case (M_r, 550,000; $s_{20,w}$ = 18 S). Large subunits appear to equilibrate between the 29S complex and a pool of 7S large subunits (6, 7). The 7S subunits, for example, bind *in vitro* to isolated 29S protein (7); large subunits dissociate from the 29S complex *in vitro* (6); and the 7S and 29S large subunits behave coordinately in pulse–chase experiments with illuminated isolated intact chloroplasts during the assembly of RuP_2Case (6).

Although the uptake of proteins by the chloroplast appears to be light dependent (8), it is not clear whether the assembly of macromolecular complexes such as RuP_2Case is also light dependent. The assembly of RuP_2Case occurs in extracts of chloroplasts that have been provided with supernatants from *in vitro* protein synthesis reactions using leaf poly(A)$^+$ RNA as messenger (9). In view of the likely presence of ATP in such supernatants, we considered it possible that the assembly of RuP_2Case might be partially dependent on ATP.

Recent work in this laboratory has concentrated on the behavior of RuP_2Case large subunits in isolated chloroplasts (6). Here we report that the assembly of these into RuP_2Case is indeed light dependent, and that nucleotides, Mg^{2+}, and monovalent cations have dramatic effects on the reactions of the large subunit complexes *in vitro*.

MATERIALS AND METHODS

Plant Growth. Pea seeds (*Pisum sativum* var. Progress no. 9) were purchased from Agway (Buffalo, NY). Plants were grown under a 12-hr light/dark cycle at 25°C. Plants used for experimentation were grown for 9–12 days, by which time they had attained a height of 5–6 cm.

Plastid Isolation. The chloroplast isolation procedure was based on that of Fish and Jagendorf (10), who adapted the procedures of Chua and Schmidt (11) and Morgenthaler *et al.* (12). All steps were carried out at 4°C. Twenty grams of leaves was homogenized in a Waring blender fitted with razor blades in 100 ml of buffer (330 mM sorbitol/2 mM EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$/50 mM Hepes, pH 8.5/5 mM ascorbic acid/3 mM EGTA). The homogenate was filtered through one layer of Miracloth and the filtrate was centrifuged at 4,000 × *g* by bringing the sample up to speed and down again as quickly as possible. The pellets were resuspended in the residual buffer remaining in the centrifuge tubes with the aid of a fine paint brush.

The resuspended chloroplasts were layered on linear 10–80% (wt/vol) Percoll (Pharmacia) gradients containing 1% bovine serum albumin, 1% Ficoll (Pharmacia), 3% polyethylene glycol 4000 (wt/vol), 330 mM sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbic acid, 5.5 mM glutathione, and 50 mM Hepes/KOH (pH 8.5). The gradients were centrifuged at 10,000 × *g* for 8 min. Intact chloroplasts (90% as judged by phase-contrast microscopy) migrated to a position 0.5–1.0 cm from the bottom of the gradient. The chloroplasts were removed with a Pasteur pipette. They were diluted approximately 1:5 with resuspension buffer (330 mM sorbitol/50 mM Hepes, pH 8.5) and centrifuged by being brought to 4,000 × *g* momentarily. The chloroplast pellet was resuspended with a minimum of resuspension buffer.

***In Organello* Protein Synthesis with [^{35}S]Methionine.** Chloroplasts were incubated with [^{35}S]methionine (New England Nuclear, 700–1,200 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) at concentrations up to 500 μCi/ml of chloroplast resuspension. Incubations were carried out at 20°C in a constant-temperature circulating water bath under 10,000 lux of filtered red light, as indicated in figure and table legends. After incubation, the chloroplasts were diluted with resuspension buffer and centrifuged

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Abbreviation: RuP_2Case, ribulose-1,5-bisphosphate carboxylase.

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momentarily at 4,000 × *g* to pellet intact chloroplasts. The pellet was lysed with at least the original sample volume of a hypotonic buffer containing 50 mM Tris·HCl (pH 8.5), 7 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 12,000 × *g* for 10 min to pellet the membranes. The supernatants were subjected to sucrose gradient sedimentation (6) or electrophoresis as described below.

Polyacrylamide Gel Electrophoresis. Samples were analyzed on a discontinuous $NaDodSO_4$ slab gel system as described by Laemmli (13). The gels consisted of a 7.5–15% linear gradient of polyacrylamide with a 4.5% polyacrylamide stacking gel. Electrophoresis was at 25 mA at constant current until the voltage reached 150 mV, after which electrophoresis continued at constant voltage.

The same system was used for nondenaturing electrophoresis except that $NaDodSO_4$ was omitted from the gel solutions. The nondenaturing gels were composed of 7.5% polyacrylamide with a 4.5% stacking gel. Electrophoresis was at 100 mV for 20 hr. Both types of gels were stained with 6.25% Coomassie blue R/45% ethanol/9% acetic acid (wt/vol/vol) either at 62°C for 15 min or overnight at room temperature. Gels were destained at room temperature in ethanol/acetic acid/water, 1:1:8 (vol/vol), dried, and fluorographed as described (6).

Peptide Digestion. Peptide mapping was accomplished by limited proteolysis in $NaDodSO_4$ followed by separation on $NaDodSO_4$/polyacrylamide gels (14). Excised bands from polyacrylamide gels were equilibrated in 125 mM Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/1 mM EDTA at 4°C. The proteins were placed in the wells of a 5-cm stacking gel, together with 0.7 μg of *Staphylococcus aureus* protease per well. Samples were electrophoresed until the dye front reached the running gel. Electrophoresis was stopped for 30 min to permit continued proteolysis, then resumed as usual.

RESULTS

A pulse–chase strategy was employed to determine whether the posttranslational assembly of RuP_2Case is itself dependent on light. Isolated chloroplasts were illuminated in the presence of [^{35}S]methionine for 20 min. Excess unlabeled methionine was then added, one sample was covered with aluminum foil, and the illumination was continued. At various times, aliquots of the chloroplasts were pelleted and lysed. Because we were interested primarily in the behavior of the 18S RuP_2Case molecule in multiple samples, we chose to analyze the supernatants by nondenaturing electrophoresis. Free (7S) large subunits are not resolved on nondenaturing gels (6). Their behavior is assessed by sucrose gradient analysis and $NaDodSO_4$ gel electrophoresis (see below; also ref. 6). All the radioactive material in these samples, including that which is arrested at the start of the gel lane, consists of large subunits, as shown by the fact that only large subunit bands appear when the samples are analyzed on $NaDodSO_4$ gels (5–7). As expected (5, 6), after illumination the 29S complex was intensely labeled, whereas very little radioactivity was found in the 18S RuP_2Case (Fig. 1). During such an illuminated chase period, no incorporation of radioactive methionine occurs (7); despite this, radioactivity increased significantly in the 18S RuP_2Case band. Radioactivity also declined in the 29S band. The 29S complex is sufficient, but not the only possible, posttranslational source for the increased radioactivity in the 18S RuP_2Case (see ref. 6 for discussion). In contrast to the illuminated sample, the sample kept in the dark after the first 20 min failed to accumulate label in RuP_2Case over the level found at the beginning of the chase period. Thus, light appears to stimulate the *in organello* assembly of RuP_2Case.

The apparent light dependence of RuP_2Case assembly might be due to ATP produced by photosynthetic phosphorylation. To investigate this possibility, soluble supernatants were prepared from chloroplasts that had been illuminated in the presence of [^{35}S]methionine. Unlabeled methionine and salts were added, with or without ATP. The samples were incubated and subjected to nondenaturing electrophoresis (Fig. 2). In the absence of ATP (samples 7–11), the labeling pattern remained largely the same throughout the incubation. In the presence of ATP (samples 2–6), radioactivity in the 29S complex declined very rapidly at first and then more slowly throughout the 90-min incubation. The stained band at the 29S position also declined proportionately with radioactivity, but the stained band at the 18S RuP_2Case position was not affected (data not shown). Despite this, radioactivity increased significantly in the RuP_2Case band. This increase appeared to continue throughout the incubation period. Thus ATP substitutes for light in promoting the assembly of RuP_2Case.

The specificity of the ATP effect was examined by incubating labeled chloroplast extracts with various salts or nucleotides. Optical densities at the 29S and 18S positions of the film from a representative experiment are shown in Table 1. In the presence of $MgCl_2$, ATP caused a decline in 29S peak optical density from 2 to about 0.66. This 67% depletion is expected for a 15-min incubation (Fig. 2); almost complete dissociation of the 29S

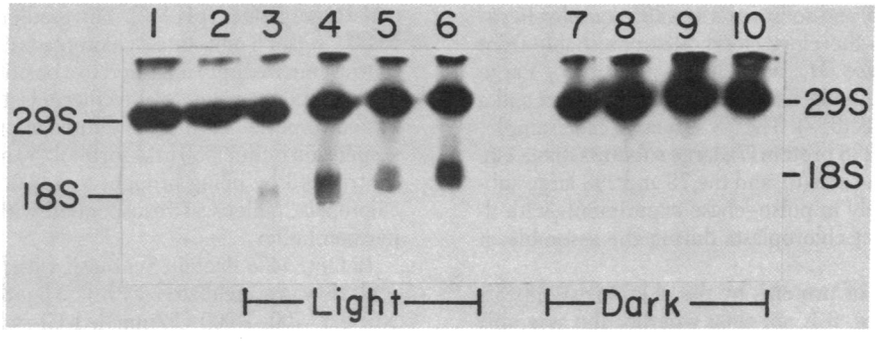

FIG. 1. Effect of light on RuP_2Case assembly *in organello*. Two aliquots of isolated chloroplasts (318 μg of chlorophyll) were illuminated in the presence of 360 μCi of [^{35}S]methionine. After 20 min a 2,000-fold molar excess of unlabeled methionine was added to one sample and it was covered with aluminum foil. The incubation continued for an additional 80 min. Aliquots from the light and dark samples were extracted and lysed and the cleared lysates were electrophoresed on a nondenaturing gel. The fluorogram is shown. Samples 1 and 2, zero time controls; samples 3–6, illuminated, sampled at 40, 62, 84, and 105 min; samples 7–10, dark, sampled at 40, 62, 84, and 105 min. Labels 29S and 18S indicate positions of the large subunit binding protein complex and RuP_2Case, respectively. These positions change from left to right because the samples were loaded immediately at the end of each incubation as a precaution against *in vitro* proteolysis. High exposure at the 29S position results from compensating for the isotopic dilution of radioactivity at the 18S position. The 29S radioactivity is strictly localized to a very sharp band of protein (5, 6).

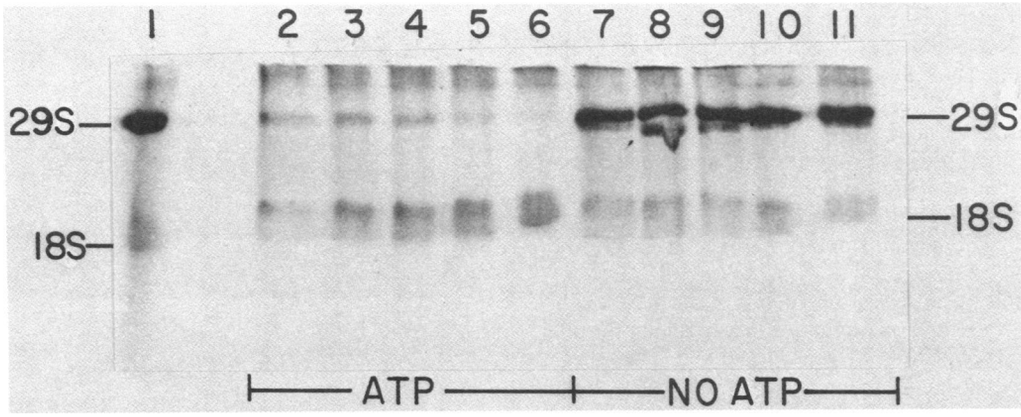

FIG. 2. ATP dependence of assembly of RuP_2Case. Isolated chloroplasts were illuminated in the presence of 450 μCi of [^{35}S]methionine for 30 min. The cleared lysate was incubated with salts in the presence or absence of 5 mM ATP. Aliquots were removed at various times and electrophoresed on a nondenaturing gel. The fluorogram is shown. Sample 1, control placed on ice; samples 2–6, 0, 5, 10, 30, and 60 min in presence of 5 mM ATP; samples 7–11, 0, 5, 10, 30, and 60 min with no ATP added. Samples were loaded as in Fig. 1. Apparent band splitting at the 29S position did not occur reproducibly.

complex occurs after longer incubation (Fig. 3). Neither GTP nor the nonhydrolyzable ATP analog adenosine 5′-[β,γ-methylene]-triphosphate caused a significant change in the optical density at the 29S position. This experiment suggests that ATP hydrolysis is required to destabilize the 29S complex.

Samples containing ATP, GTP, or a nonhydrolyzable ATP analog all stimulated RuP_2Case assembly. The optical density values at the 18S position in these samples were approximately 2-fold over those for the control held on ice or the sample incubated at room temperature in the absence of nucleotides and the presence of salt at high concentrations (Table 1). These results indicate that ATP hydrolysis is not required in order to stimulate RuP_2Case assembly. We attribute the "stimulation" observed in the absence of added nucleotides and KCl to a salt effect discussed below.

The effects of ATP on both the 29S complex and RuP_2Case assembly were found to depend on the salt composition. $MgCl_2$ was found to be required for ATP-dependent dissociation of the 29S complex; ATP alone resulted in only a minimal decrease in optical density from 2 to 1.86 (Table 1). Therefore a Mg^{2+}–ATP complex may be required for this reaction. Elimination of KCl from the salts buffer impaired the ability of ATP to dissociate the 29S complex, but it increased RuP_2Case assembly, even in the absence of added nucleotide. This second effect of KCl is in accord with a report by Barraclough and Ellis (5), which indicates that high KCl concentrations inhibit assembly of RuP_2Case in isolated chloroplasts.

Nondenaturing electrophoresis cannot resolve any free subunits released from the 29S complex (6). Therefore, we employed the "split gel" technique used earlier (6). Two aliquots of a labeled chloroplast lysate were incubated either with or without ATP. After incubation they were layered on sucrose gra-

Table 1. Specificity and salt dependence of the ATP effect

Solute conc., mM					Temp., °C	Optical density	
KCl	MgCl	ATP	GTP	β,γ-Methylene-ATP		29S	18S
220	6	—	—	—	0	2.07	0.27
—	6	—	—	—	20	2.01	0.51
—	6	—	5	—	20	1.83	0.51
—	6	5	—	—	20	1.11	0.66
—	6	—	—	5	20	1.98	0.66
—	—	5	—	—	20	1.86	0.54
220	6	—	—	—	20	1.89	0.21
220	6	—	5	—	20	2.07	0.57
220	6	5	—	—	20	0.66	0.54
220	6	—	—	5	20	2.13	0.45
220	—	5	—	—	20	1.95	0.36

Isolated chloroplasts (663 μg of chlorophyll) were illuminated in the presence of 750 μCi of [^{35}S]methionine for 30 min. Aliquots of the cleared lysate were incubated at 0°C or 20°C for 15 min. All samples contained at least 50 mM Hepes and 20 mM dithiothreitol plus a residual contribution from the original lysate, and variable solutes adjusted as indicated. The samples were subjected to nondenaturing gel electrophoresis and fluorography, and the peak optical density at the 29S and 18S positions was determined by scanning at 550 nm with a Gilford linear gel transporter. All peak values were at least 2-fold higher than the adjacent base line and are corrected for base line optical density.

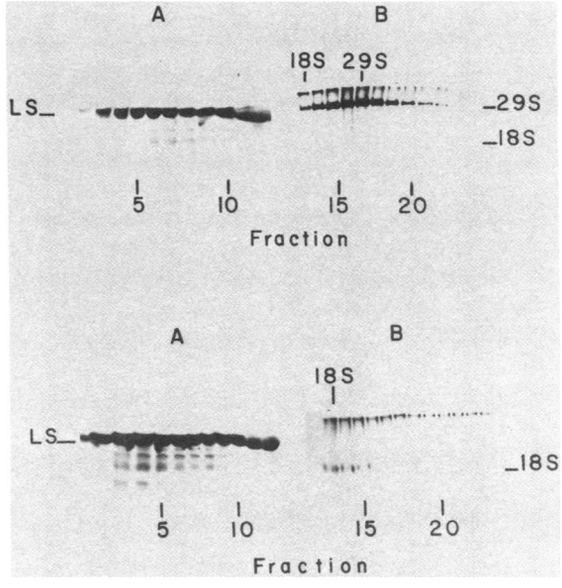

FIG. 3. Analysis of ATP effect on $NaDodSO_4$ and nondenaturing gels. Isolated chloroplasts (663 μg of chlorophyll) were illuminated in the presence of 750 μCi of [^{35}S]methionine for 30 min. Aliquots of the cleared lysate were incubated with (*Lower*) or without (*Upper*) ATP as in Fig. 2 and centrifuged on sucrose gradients. Upper sucrose gradient fractions were analyzed on $NaDodSO_4$ gels (*A*), while the lower fractions were electrophoresed on nondenaturing gels (*B*). This procedure permits the analysis of the 7S, 18S, and 29S large subunits in a single experiment (6). Fluorograms are shown. Labels 29S and 18S denote the positions of the 29S complex and the RuP_2Case bands, respectively, on the nondenaturing gels, and LS marks the position of the large subunit on the $NaDodSO_4$ gels.

dients and centrifuged. The top gradient fractions were electrophoresed on $NaDodSO_4$ gels, which permit resolution of the free large subunits. The bottom fractions were analyzed on nondenaturing gels (Fig. 3). Once again, in the sample treated with ATP, the label declined in the 29S complex. Radioactivity also increased in the RuP_2Case band. As expected, more labeled large subunits were found in upper fractions of the sucrose gradients after treatment with ATP. This confirms that large subunits are released from the 29S complex in the presence of $MgCl_2$ and ATP.

The 29S complex primarily is composed of M_r 60,000 subunits. These are present in sufficient quantity to render the 29S complex visible on stained gels. When ATP and $MgCl_2$ have been added, this protein no longer sediments at 29 S; however, it can be recovered from upper fractions of sucrose gradients and analyzed by partial proteolytic digestion (Fig. 4). The close correspondence of the peptide patterns is readily apparent, thereby confirming the identity of the M_r 60,000 protein in the top gradient fractions for the ATP-treated sample. Thus MgATP effects complete dissociation of the 29S complex into large subunits and free M_r 60,000 subunits.

As shown previously (6), chloroplasts isolated from pea plants labeled *in vivo* contain radioactive 7S and 29S large subunits and a pool of radioactive small subunits (Fig. 5 *Upper*). When an extract of such chloroplasts labeled *in vivo* was treated with ATP, there was a drastic decline in radioactivity in the 29S material. There was a substantial increase in radioactive 18S large subunits (Fig. 5 *Lower*), and some residual radioactivity in the 7S large subunits, as expected from the earlier result with the chloroplast extract labeled *in organello* (Fig. 3). It also should be noted that ATP brings about a substantial depletion of radioactivity in the 3S small subunit pool. This experiment establishes that the dissociation of the 29S complex by ATP can be effected whether the complex is labeled *in vivo* or *in organello*.

DISCUSSION

It has been known for a long time that light drives the synthesis of RuP_2Case large subunits in isolated chloroplasts (15). More recent observations (8) indicate that ATP produced by photosynthetic phosphorylation plays a role in the uptake of precursor polypeptides. The results presented here show that light can promote the posttranslational assembly of large subunits into RuP_2Case molecules and that ATP promotes the *in vitro* assembly of RuP_2Case from native subunits. The complexity of RuP_2Case assembly may account for the well-known failure of investigators to reconstitute RuP_2Case from its dissociated subunits. It is particularly interesting that large subunits bind to the 29S complex in a specific fashion (5, 6). Although the precise role of the 29S complex cannot be defined at present, some observations can be made:

The ATP effects on the 29S and 18S (RuP_2Case) complexes appear to involve at least two different reactions. This interpretation is supported by the different nucleotide and salt requirements for the stimulation of RuP_2Case assembly and the dissociation of the 29S complex. Results from several experiments such as that described in Table 1 indicate that hydrolysis of a Mg–ATP complex is necessary to destabilize the 29S complex. GTP will not work. On the other hand, GTP, ATP, and a nonhydrolyzable ATP analog all have been observed to stimulate RuP_2Case assembly. Furthermore, elimination of KCl from the salts buffer somewhat inhibits the breakdown of the 29S complex yet still stimulates RuP_2Case assembly.

These results could be imagined to suggest that the 29S complex may not serve as the final donor of large subunits in the assembly of RuP_2Case (5). It is possible that the 29S large subunit pool that is present before ATP addition functions directly or indirectly as a donor of large subunits for the final assembly step. If this role is indirect, one has to explain how the ATP-dependent dissociation of the 29S complex appears not to stimulate further RuP_2Case assembly. This could happen if there is

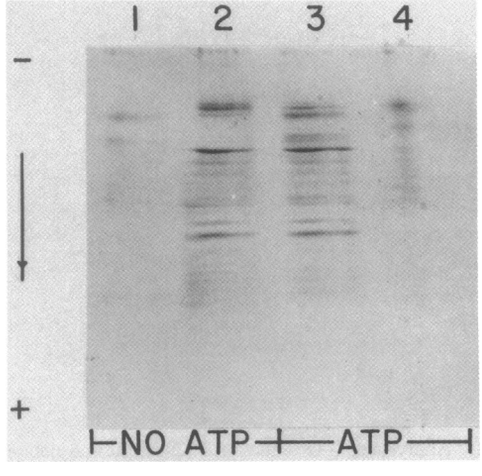

FIG. 4. Peptide map analysis of the M_r 60,000 binding protein. Aliquots of gradient fractions as in Fig. 3 representing the entire sedimentation profile were electrophoresed on $NaDodSO_4$ gels. Regions of the gels containing proteins of M_r 60,000 were excised, exposed to *Staphylococcus aureus* protease, and electrophoresed on a $NaDodSO_4$ gel. The stained gel is shown. Lane 1, M_r 60,000 band from upper sucrose gradient fractions, no ATP; lane 2, M_r 60,000 band from lower sucrose gradient fractions, no ATP; lane 3, M_r 60,000 band from upper sucrose gradient fractions, ATP-treated sample; lane 4, M_r 60,000 band from lower sucrose gradient fractions, ATP-treated sample.

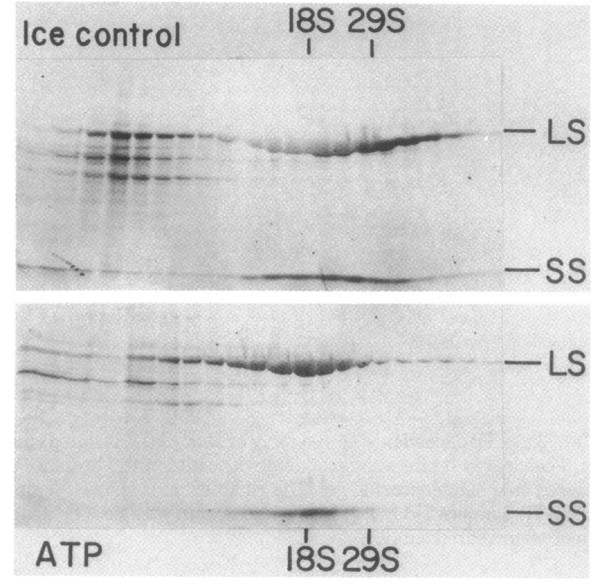

FIG. 5. Effect of ATP on RuP_2Case assembly from plants labeled *in vivo*. Chloroplasts were isolated from three plants, each of which had been labeled for 30 min *in vivo* by transpiration of 121 μCi of [^{35}S]-methionine. Aliquots of the pooled chloroplast lysate were incubated with ATP at room temperature (*Lower*) or without ATP on ice (ice control, *Upper*) for 90 min. The samples were centrifuged on sucrose gradients and the gradient fractions were analyzed on $NaDodSO_4$/polyacrylamide gels. The fluorograms are shown. The third lane from the left in *Lower* was underloaded, as judged by comparison of both staining intensity (not shown) and radioactivity levels with adjacent fractions. Free small subunit bands are visible in lower left region of *Upper*. LS, large subunit; SS, small subunit.

a physical difference between the large subunits in the 7S pool and those released from the 29S complex by ATP treatment; alternatively, it could also occur if large subunits are always in excess in isolated chloroplasts. The labeled small subunit pool can be abolished by illuminating *in vivo* labeled chloroplasts in the presence of unlabeled methionine or by treating the chloroplast lysate with ATP. Although the data do not permit the calculation of absolute pool sizes, it is certainly possible that the small subunit pool in isolated chloroplasts can limit the extent of assembly observed with large subunits labeled *in organello* (see also ref. 6). In view of these considerations, it would be premature to draw a firm conclusion about the immediate source of large subunits that are incorporated into RuP_2Case *in vitro*.

Our experiments indicate that ATP concentrations as low as 0.5 mM can cause significant dissociation of the 29S complex (data not shown). It has been estimated that the concentration of ATP in the illuminated chloroplast stroma is 1.5 mM (16). Thus, the sensitivity of the 29S complex to dissociation by ATP falls within the range of concentrations of ATP thought to exist in the chloroplast. We believe that MgATP hydrolysis exerts a direct effect on the 29S complex. This hypothesis is supported by the observation that the ATP effect on the 29S complex remains even after the complex has been purified by velocity sedimentation.

Nucleotides have been found to play important roles in the polymerization of other macromolecular complexes (17–19). The data presented here implicate light-induced changes in the posttranslational assembly of RuP_2Case in chloroplasts. The dissociation of the 29S complex and the promotion of assembly of RuP_2Case appear to be distinct reactions, each regulated by ATP and other molecules that are known to be affected by light in chloroplasts. We propose that the light effect on assembly *in organello* is mediated by ATP.

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