Studies on the Assembly of Large Subunits of Ribulose Bisphosphate Carboxylase in Isolated Pea Chloroplasts

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ABSTRACT Ribulose bisphosphate carboxylase consists of cytoplasmically synthesized "small" subunits and chloroplast-synthesized "large" subunits.

Large subunits of ribulose bisphosphate carboxylase synthesized in vivo or *in organello* can be recovered from intact chloroplasts in the form of two different complexes with sedimentation coefficients of 7S and 29S. About one-third to one-half of the large subunits synthesized in isolated chloroplasts are found in the 7S complex, the remainder being found in the 29S complex. Upon prolonged illumination of the chloroplasts, newly synthesized large subunits accumulate in the 18S ribulose bisphosphate carboxylase molecule and disappear from both the 7S and the 29S large subunit complexes. The 29S complex undergoes an in vitro dissociation reaction and is not as stable as ribulose bisphosphate carboxylase.

The data indicate that (*a*) the 7S large subunit complex is a chloroplast product, that (*b*) the 29S large subunit complex is labeled in vivo, that (*c*) each of these two complexes can account quantitatively for all the large subunits assembled into RuBPCase *in organello*, and that (*d*) excess large subunits are degraded in chloroplasts.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39) catalyzes the CO_2 fixation step in photosynthetic carbon reduction and the cleavage of ribulose bisphosphate by oxygen in a key reaction in photorespiration (1). In higher plants and green algae, and in some photosynthetic bacteria, the enzyme consists of eight 55,000-dalton "large" subunits and eight ~12,000-dalton "small" subunits. The large subunit bears the catalytic site and the small subunit is of unknown function. The fully assembled enzyme has a molecular weight of ~550,000 and a sedimentation coefficient of 18S (2). In higher plants and green algae both biochemical and genetic data have firmly established that the large subunit is synthesized in chloroplasts and that the small subunit is synthesized in the cytoplasm (3). The small subunit is taken into the chloroplasts in the form of a precursor polypeptide, which is cleaved by a soluble endoprotease within the chloroplast before integration into the 18S ribulose bisphosphate carboxylase holoenzyme (4).

Free subunits of ribulose bisphosphate carboxylase have been detected in extracts of barley (5) or pea seedlings (6, 7) provided with radioactive amino acids. The large subunits behave as a dimer or heterodimer with a sedimentation coefficient of 7S, and the small subunits sediment at 3S. These free subunits turn over in vivo and appear to represent the subunit pools from which ribulose bisphosphate carboxylase is assembled (7). In isolated pea chloroplasts, however, newly synthesized large subunits were reported to accumulate in a 600,000- to 700,000-dalton "aggregate" together with a 60,000-dalton polypeptide. This polypeptide has been called the "large subunit binding protein" and no other function has been assigned to it (8). For reasons which will be discussed, we refer to this "aggregate" as a large subunit binding "complex."

Illumination of the chloroplasts for 30 to 60 min resulted in the concomitant decrease of radioactive large subunits in this complex and increase of radioactive large subunits in ribulose bisphosphate carboxylase. Because of the presence of unidentified radioactive material in the samples, however, it could not be concluded that the complex plays a role in ribulose bisphosphate carboxylase assembly (8).

Here we resolve the apparent discrepancy concerning the molecular weight characteristics of unassembled large subunits formed in vivo (6, 7) and in isolated chloroplasts (8). We show that both the 7S and 29S complexes are formed in vivo and in isolated chloroplasts; that the 7S and 29S complexes each contain more than enough large subunits to account for all the assembly of large subunits into ribulose bisphosphate carboxylase in isolated chloroplasts; and that excess large subunits are degraded in isolated chloroplasts.

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MATERIALS AND METHODS

Chloroplast Preparation

Pea seedlings (*Pisum sativum*, var. "Progress #9") were obtained from Agway, Inc., Buffalo, NY and grown in vermiculite on a 12-h light/12-h dark cycle at 25°C. Chloroplasts were isolated from 9- to 13-d-old seedlings after a final 18-h dark period, using the procedure described by Bouthyette and Jagendorf (9), which is adapted from the procedures of Chua and Schmidt (4) and Morgenthaler et al. (10). The buffers are identical to those described by Morgenthaler et al., except that HEPES-KOH (50 mM) pH 8.5 and EGTA (5 mM) are included in the grinding buffer.

In Organello Protein Synthesis

Chloroplasts (90 μg chlorophyll) were illuminated in 0.35 ml of solution containing 330 mM sorbitol-50 mM HEPES-KOH (pH 8.5) in the presence of up to 500 μCi [^{35}S]methionine (New England Nuclear, Waltham, MA; 700–1200 Ci/mmol), at 25°C and 10,000 lux of red light. After a short lag (~2 min), [^{35}S]-methionine incorporation proceeds on a roughly linear time course for ~20 to 30 min after which further increases in acid-insoluble radioactivity do not occur. The final extent of incorporation varied from 100 to 500 pmol methionine per mg chlorophyll.

Polypeptide Analysis

After illumination, the chloroplasts were diluted with the same buffer, centrifuged by bringing the rotor to 5,000 *g* momentarily to pellet the intact chloroplasts, and then lysed with a 5-times excess of a solution containing 5 mM Tris-HCl (pH 8.5)—7 mM 2-mercaptoethanol—1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 12,000 *g* 10 min to pellet membranes, and the supernatant was centrifuged on 5–20% linear sucrose gradients containing 50 mM Tris-HCl (pH 7.6)—7 mM 2-mercaptoethanol—1 mM PMSF. The gradients were fractionated with a motor-driven syringe and a fraction collector, and the individual fractions were analyzed by SDS PAGE two-dimensional electrophoresis (11), or liquid scintillation counting, as previously described (6, 7). For nondenaturing electrophoresis, the methods used previously (6, 7) were employed, except that SDS was omitted from the buffers, and a gradient of 3 to 15% polyacrylamide was used in the running gel. Gels were stained with Coomassie Blue and fluorographed using a commercial solvent-scintillant system (EN^{3}Hance, New England Nuclear).

RESULTS

Chloroplasts were illuminated in the presence of [^{35}S]methionine for 30 min and then lysed. The soluble fraction of the lysate was centrifuged on a sucrose gradient, and the gradient fractions were analyzed by SDS PAGE and fluorography. Radioactive material sedimented predominantly in the 7S and 29S regions of the sucrose gradient. Most of this radioactivity coelectrophoresed with marker large subunits of ribulose bisphosphate carboxylase in the SDS polyacrylamide gel (Fig. 1). A number of minor low molecular weight proteins were present. These were similar in all gradient fractions and are presumed to be degradation products of the labeled 55,000-dalton polypeptides. The radioactive proteins from the 7S region of a comparable gradient were subjected to two-dimensional electrophoresis by the procedure of O'Farrel (11) (Fig. 2). Radioactivity occurred at two positions in the slab gel. Most of the radioactivity coincided with marker large subunit of ribulose bisphosphate carboxylase; a minor radioactive spot had a similar molecular weight but a more acidic isoelectric point than large subunit. Thus, the bulk of the radioactive material in the 7S region of the sucrose gradient corresponded to the large subunit of ribulose bisphosphate carboxylase. The behavior of this large subunit complex, which is labeled *in organello*, is similar to that of the 7S large subunit complex found in vivo (6). The labeling of the 7S large subunit complex *in organello* has not been reported previously, probably because nondenaturing polyacrylamide gel electrophoresis has been the principal experimental technique used to search for large subunit

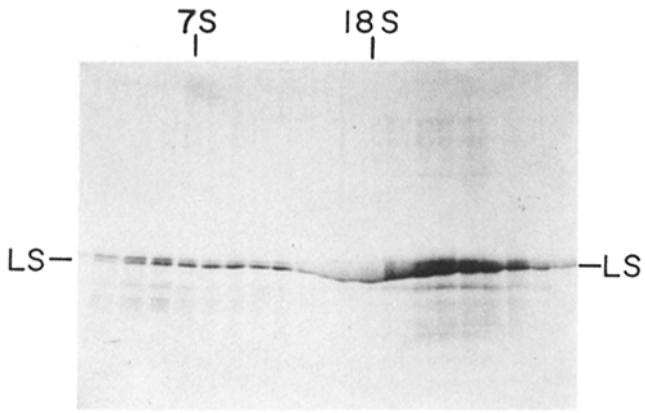

FIGURE 1 Synthesis of 7S and 29S large subunit complexes in isolated chloroplasts. Chloroplasts (357 μg chlorophyll) were illuminated for 30 min in the presence of 208 μCi of [^{35}S]methionine, diluted with resuspension buffer, centrifuged momentarily to pellet the chloroplasts, lysed with 1 ml of lysis buffer, and centrifuged to remove membranes. The supernatant (1 ml) was applied to a 12-ml linear sucrose gradient and centrifuged as described under Materials and Methods. Aliquots (0.09 ml) of each gradient fraction were concentrated by lyophilization and electrophoresed on an SDS polyacrylamide gel. The stained gel was dried and exposed to Kodak AR film for 30 d at −80°C. The position of the 18S RuBPCase peak (*18S*) and the large subunit (*LS*) were determined from the staining pattern. The small subunit was run off the end of the gel in this experiment. In comparable experiments, no radioactivity was detected at the small subunit position. The x-ray film and the dried gel were compared by registering the positions of radioactive ink marks with regions of exposure outside the gel area. Sedimentation: left to right. Electrophoresis: top to bottom.

complexes (8). When alternate sucrose gradient fractions comparable to those shown in Fig. 1 were analyzed by nondenaturing electrophoresis, the radioactive 29S band could be seen clearly, where it trailed behind the position of the 18S ribulose bisphosphate carboxylase marker protein (Fig. 3). (The carboxylase band was not labeled significantly in this short illumination period). In contrast, the radioactive 7S large subunit material, seen clearly on SDS gels, was smeared out in the nondenaturing gel. In many experiments using SDS gels, and using illumination times from 15 s up to 60 min, from one-half to one-third of the total large subunit radioactivity occurred in the 7S region of sucrose gradients, while the remainder was localized in heavier gradient fractions.

The radioactive 29S large subunit-containing complex was isolated and dialyzed against chloroplast lysis buffer. The retentate was then used to lyse a fresh batch of unlabeled chloroplasts. This lysate was centrifuged on a second sucrose gradient. ~10% of the large subunit radioactivity dissociated from the complex and trailed behind the 29S peak (Fig. 4). This dissociated material exhibited a slight peak in the 5 to 7S region but appeared more polydisperse and less abundant than the newly synthesized 7S material seen in other experiments. The binding of large subunits to the 29S complex is not as strong as the binding of large subunits in ribulose bisphosphate carboxylase, which does not dissociate under these conditions.

In previous studies, no radioactive 29S large subunits were detected in hypotonic extracts of pea leaves that had been labeled in vivo with [^{35}S]methionine for periods of 30 min or more (6, 7). In chloroplasts isolated from such leaves, we have detected the 7S large subunit complex and the 3S small subunit pool in addition to fully assembled ribulose bisphosphate

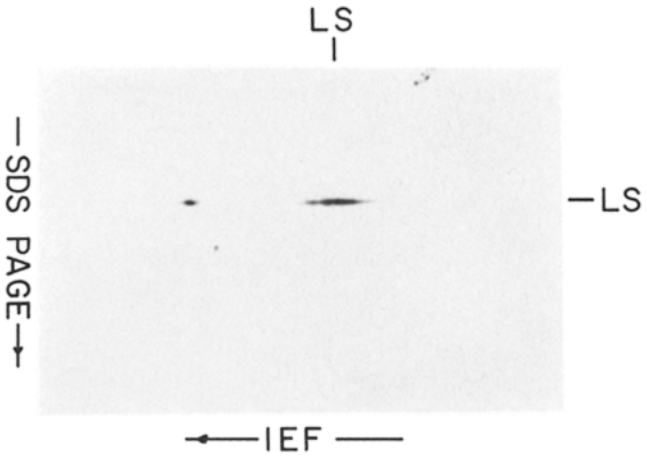

FIGURE 2 Two-dimensional electrophoresis of the 7S large subunit complex. Pooled fractions from the 7S region of a sucrose gradient comparable to that seen in Fig. 1 were lyophilized together with 1 μg of ribulose-1,5-bisphosphate carboxylase, reconstituted in O'Farrel's buffer "0" (11), and subjected to isoelectric focusing (*IEF*) and SDS polyacrylamide slab gel electrophoresis (*SDS PAGE*) as previously described (9). Autofluorography was carried out using a commercial solvent-scintillant (EN^{3}HANCE, New England Nuclear, Boston, MA). The positions of the ribulose bisphosphate carboxylase marker large subunit and small subunit were determined by comparison of the staining pattern with control chloroplast protein patterns in comparable gels lacking carrier ribulose bisphosphate carboxylase.

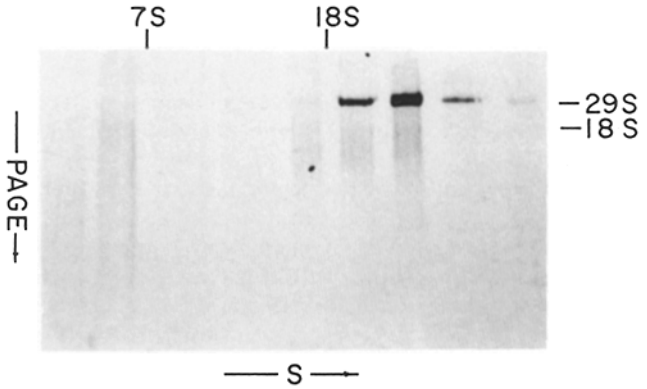

FIGURE 3 Nondenaturing electrophoresis of 7S and 29S large subunit complexes. Fractions from a sucrose gradient comparable to that in Fig. 1 were applied directly to denaturing polyacrylamide gels (*PAGE*) prepared as described under Materials and Methods. The stained gel was examined to determine the position of the RuBPCase peak (*18S*) and the position of a lesser but still prominent stained band (*29S*). Autofluorogram is shown. Sedimentation: left to right. Electrophoresis: top to bottom.

carboxylase (Fig. 5). The identity of these free subunits was confirmed by the procedure of O'Farrel (14), essentially as reported previously (9, 10). The presence of small subunits in these chloroplasts may help explain the fact that assembly of ribulose bisphosphate carboxylase can occur in isolated chloroplasts (8). Recently, we have been able to isolate chloroplasts from pea seedlings which have been labeled for as little as fifteen min. In these chloroplasts, the 7S and 3S large and small subunit pools were detected as before. In this case, however, the bulk of unassembled large subunit radioactivity was localized in the 29S region (Fig. 6). Despite the relative deficiency of large subunit radioactivity in ribulose bisphosphate carboxylase, it is apparent that radioactive small subunits have been incorporated into the fully assembled 18S enzyme. Resolution of the labeled 29S large subunits in sucrose gradients appears to depend on the labeling time before chloroplast isolation. The labeling of the 29S large subunits in vivo has not been demonstrated previously. A number of other proteins, both larger and smaller than large subunit, are labeled in these experiments (Figs. 5 and 6). We attribute most of these

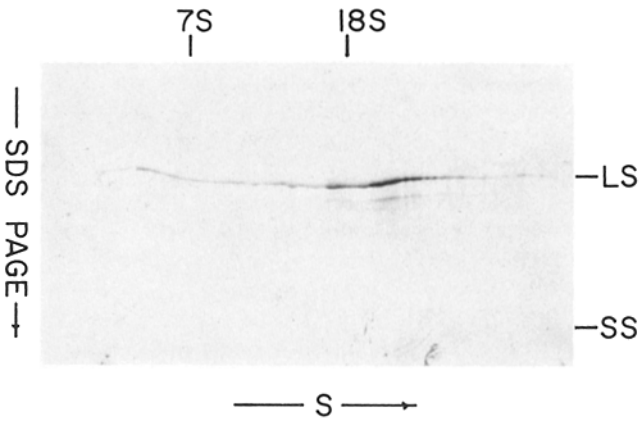

FIGURE 4 Dissociation of the 29S large subunit complex. The [^{35}S]methionine-labeled 29S large subunit complex was isolated from illuminated chloroplasts by sucrose gradient centrifugation, and dialyzed against Tris-mercaptoethanol-PMSF buffer. 1 ml of the dialyzed solution was used to lyse freshly isolated, intact pea chloroplasts. The lysate was centrifuged to remove membranes, and the supernatant was centrifuged on a sucrose gradient. The individual gradient fractions were analysed by SDS PAGE. An autofluorogram of the gel is shown. *Sedimentation*: left to right. Electrophoresis: top to bottom.

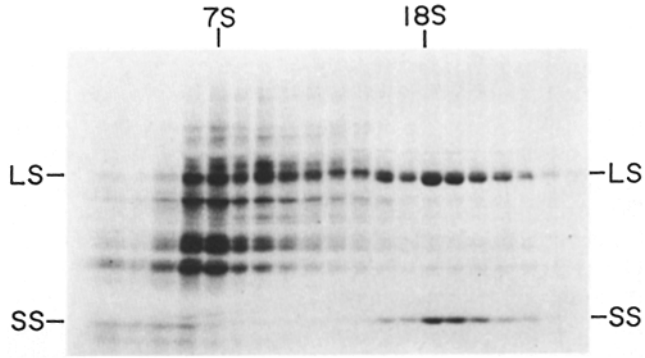

FIGURE 5 Labeling of free subunits of RuBPCase in vivo. A 7-d pea seedling top was labeled for 40 min at 20°C and ~5,000 lux by transpiration of 250 μCi of [^{35}S]methionine. The radioactive leaf was added to 5 g of similar leaves, and intact chloroplasts were isolated on Percoll gradients as described under Materials and Methods. The chloroplasts were resuspended in the sorbitol resuspension buffer and held on ice for 30 min. The chloroplasts were then lysed and centrifuged on sucrose gradients essentially as described in Fig. 1, except that 1% Triton X-100 was included in the lysis buffer and no attempt was made to centrifuge out the membranes (controls later showed that this is without effect on the autoradiographic patterns). An autofluorogram of the stained and dried SDS gel of the gradient fractions is shown. The positions of the large subunit (*LS*) and small subunit (*SS*) of ribulose bisphosphate carboxylase were determined by inspection of the stained gel, which included marker ribulose bisphosphate carboxylase in gel lanes adjacent to both the top and bottom gradient fractions. Sedimentation: left to right. SDS electrophoresis: top to bottom.

to the uptake of cytoplasmically synthesized proteins by the chloroplasts in vivo, since they are not labeled in isolated chloroplasts (Fig. 1).

When extracts of such in vivo pulse-labeled chloroplasts were electrophoresed on nondenaturing polyacrylamide gels, a major radioactive band trailed behind ribulose bisphosphate carboxylase and coincided exactly with a prominent stained band. Upon second-dimension electrophoresis in an SDS polyacrylamide gel, this radioactive material migrated as a 55,000-dalton polypeptide, while the stained band behaved as a 60,000-dalton protein (Fig. 7). When the chloroplasts, labeled in vivo, were incubated further in the light under conditions supporting protein synthesis, radioactive material declined at the position of the prominent stained band while it increased in the ribulose bisphosphate carboxylase band (Fig. 8). This behavior is similar to that observed for the high molecular weight large subunit complex which is labeled *in organello* (8, and M. Bloom, unpublished data). It is clear that the 29S large subunit complex observed here corresponds to the high molecular weight large subunit containing "aggregate" or complex reported by Barraclough and Ellis (8). It may be noted that radioactive small subunits are not detected in the 29S complex (Fig. 7).

The increase in labeling of ribulose bisphosphate carboxylase in Fig. 8 is attributed to increases in in vivo synthesized large and small subunits in the 18S carboxylase band (P. Milos, unpublished data). When isolated chloroplasts are labeled *in organello*, however, no small subunits are labeled. Under these conditions, the increases in labeling of the carboxylase are attributable to large subunits alone. These increases are detectable only after prolonged illumination of the chloroplasts and are believed to take place after protein synthesis has virtually stopped in isolated chloroplasts (8). There is a possibility, however, that protein synthesis continues at a low rate during this period. To demonstrate whether the late labeling of the enzyme must be attributed to large subunits formed earlier, a pulse-chase experiment was carried out.

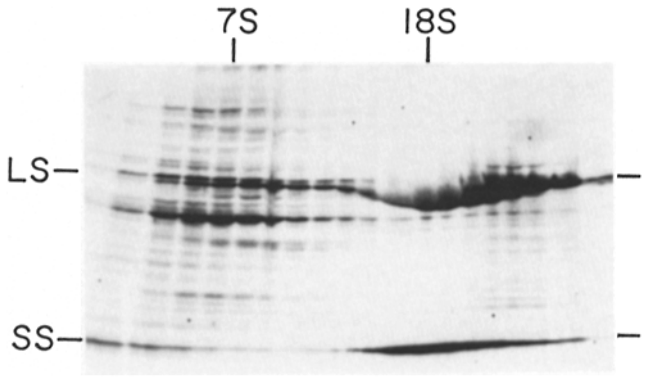

FIGURE 6 Labeling of 7S and 29S large subunit complexes in vivo. A 10-d pea seedling top was illuminated with 5,000 lux of white light and allowed to take up 385 μC of [^{35}S]methionine by transpiration for 15 min. The labeled plant was then mixed with 5 g of carrier leaves, and chloroplasts were isolated on Percoll gradients as described in Materials and Methods. The chloroplasts were lysed and membranes removed by centrifugation, and the lysate was centrifuged on sucrose gradient and analysed by SDS gel electrophoresis and autoradiography. The positions of the ribulose bisphosphate carboxylase large and small subunits and of the 18S peak were determined by visual inspection of the stained gel. The 29S region is a few fractions to the right of the 18S peak. Sedimentation: left to right. SDS electrophoresis: top to bottom.

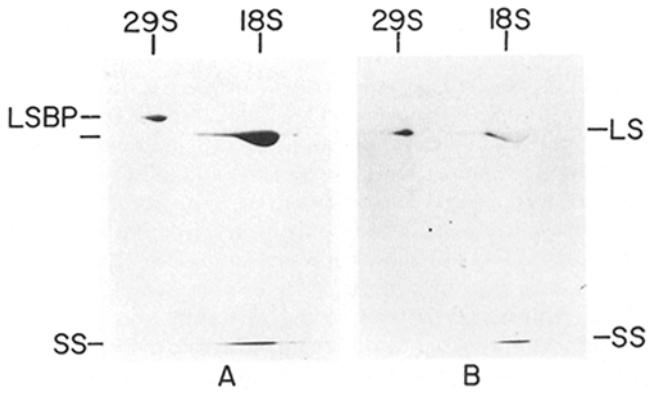

FIGURE 7 Two-dimensional electrophoresis of in vivo labeled large subunits. Chloroplasts from in vivo labeled plants were lysed in hypotonic buffer and centrifuged to remove membranes. An aliquot of the supernatant was electrophoresed on a nondenaturing polyacrylamide gel (left to right). The gel strip was equilibrated with SDS (11) and electrophoresed in an SDS polyacrylamide gel (top to bottom), which was then stained and autoradiographed. The position of the ribulose bisphosphate carboxylase is indicated by *18S*. The position of the large subunit of this enzyme is indicated by *LS*. The position of the slowly migrating complex of large subunits with the 60,000-dalton protein is marked *29S*, and the position of the 60,000-dalton protein is indicated by *LSBP*. Panel *A*: Stained Gel. Panel *B*: Autoradiogram.

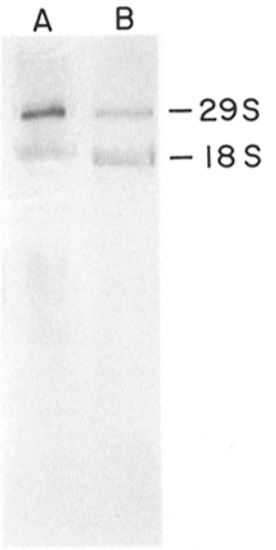

FIGURE 8 Nondenaturing electrophoresis of in vivo labeled 29S and 18S material. Chloroplasts from in vivo labeled plants were lysed in hypotonic buffer, freed of membranes, and the supernatants were electrophoresed on a nondenaturing polyacrylamide gel slab (top to bottom). The positions of RuBPCase (*18S*) and the lesser but prominent large subunit binding protein (*29S*) were determined by visual inspection of the stained gel and are indicated in the figure. (*A*) Chloroplasts lysed immediately upon resuspension in sorbitol following isolation. (*B*) Chloroplasts lysed after a 60-min illumination at 10,000 lux, 25°C.

Chloroplasts were pulse-labeled *in organello* for 30 min followed by a 30-min chase in excess unlabeled methionine. The concentration of methionine used had been shown to be sufficient to abolish incorporation of [^{35}S]methionine into acid-insoluble material immediately upon addition to illuminated chloroplasts (data not shown). The soluble fractions of the pulse and pulse-chase chloroplasts were then centrifuged on sucrose gradients. Aliquots of the upper fractions of each gradient were concentrated by lyophilization to increase the amount of radioactivity that could be loaded, and then solubilized and electrophoresed on SDS polyacrylamide gels to resolve the 7S large subunit polypeptides. The lower gradient fractions were analyzed directly on nondenaturing polyacrylamide gels to separate the 18S ribulose bisphosphate carboxylase from the 29S large subunit binding complex. The SDS

gel and the nondenaturing gel from both the pulse and pulse-chase sample were then fluorographed on the same piece of x-ray film. The loading of the gels was arranged so that the film density for the 7S samples would be similar to the film density for the 29S samples. The reason for this procedure was to optimize the comparison between pulse and pulse-chase samples. However, any comparisons between radioactivity in upper gradient fractions with radioactivity in lower fractions must take the loading differences into account.

The data are shown in Fig. 9. In the pulse sample (Fig. 9*A*, Top), radioactivity appears in the 7S region, and most of it corresponds with the large subunit of ribulose bisphosphate carboxylase, as in Fig. 1. In the pulse-chase sample, the large subunit complexes are also seen, but it is evident from visual inspection that less radioactivity is present (Fig. 9*A*, Bottom). In the pulse sample, the 29S radioactivity migrates as a discrete band in the nondenaturing gel and trails behind the position of the 18S ribulose bisphosphate carboxylase, which contains detectable radioactivity (Fig. 9 *B*, Top). The pulse-chase sample also contains these complexes, but it is apparent that much less radioactivity is present in the 29S large subunit complex, while the radioactivity in the 18S ribulose bisphosphate carboxylase band has increased (Fig. 9 *B*, Bottom). It should be emphasized that all the radioactivity in these complexes has been shown to be due to large subunits and that no radioactive small subunits are formed in isolated chloroplasts. A densitometric analysis of the data on these x-ray films showed that both the 7S and 29S large subunit complexes lost >50% of their radioactivity, while the amount of radioactivity in ribulose bisphosphate carboxylase doubled. Further, it appeared that the loss of radioactivity either from the 7S material or the 29S material alone could account for the entire increase in radioactivity of ribulose

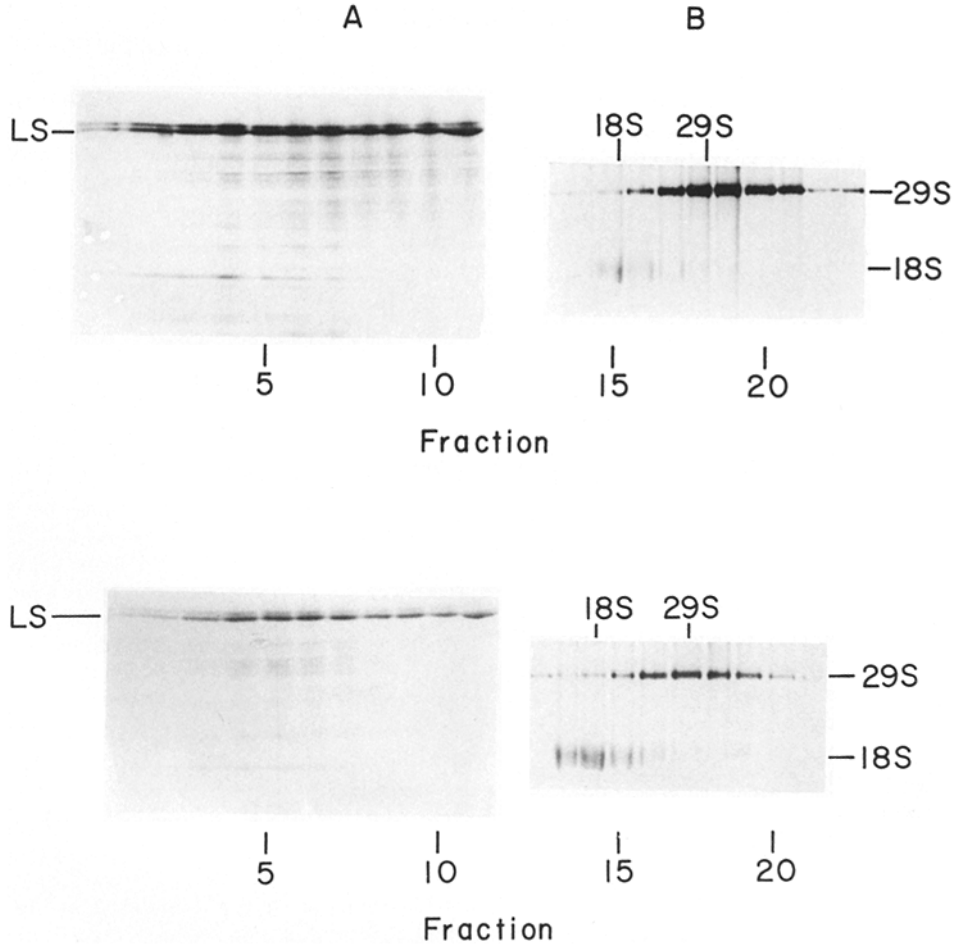

FIGURE 9 Pulse-chase analysis of large subunit complexes. Chloroplasts were labeled with [^{35}S]methionine in vitro as described in Fig. 1, except that labeling was continued for 30 min in one sample ("pulse"; 350 μg chlorophyll) while for the other ("pulse-chased"; 350 μg chlorophyll) the chloroplast suspension was rendered 0.03 mg/ml in unlabeled methionine after 30-min illumination and then illumination was continued for another 30 min. Immediately after each illumination, the chloroplasts were lysed and centrifuged to remove membranes. The supernatants were layered on sucrose gradients and centrifuged as described in Materials and Methods. Aliquots (100 μl) of gradient fractions 1–12 from each sample were lyophilized and electrophoresed on a single SDS slab gel which was stained to show the position of the large and small subunits of ribulose bisphosphate carboxylase, traces of which were present in gradient fractions 11 and 12. Aliquots (40 μl) of gradient fractions 13–24 were electrophoresed on a single nondenaturing gel which also was stained to reveal the positions of the 18S ribulose bisphosphate carboxylase band and the 29S band containing the large subunit binding protein. The gels were prepared for fluorography, dried down on the same piece of filter paper, and exposed to the same piece of x-ray film, which was developed after 2 wk at −80°C. *Top*: fluorogram of pulse-labeled samples. *Bottom*: fluorogram of pulse-chase samples. (*A*), SDS gel, (*B*), nondenaturing gel. Left to right: sedimentation. Gradient fraction numbers are indicated. Top to bottom is the direction of electrophoresis. To get all relevant detail exposed to a single piece of film, the gels were trimmed before drying.

bisphosphate carboxylase (densitometric data available on request). Two repeats of this experiment were carried out, and in each of the three experiments the decline in radioactivity in the 7S and 29S material occurred concomitant with an increase in radioactivity in ribulose bisphosphate carboxylase. The loss of radioactivity in each case exceeded the gains in ribulose bisphosphate carboxylase. The densitometric data also confirmed that about one-third of the radioactivity in large subunits resides in the 7S region, with the remainder localized in the heavier fractions, in the pulse-labeled sample. The loss of radioactivity during the chase was confirmed by liquid scintillation counting of the acid-insoluble material applied to the sucrose gradients. This loss varied from 25% to 75% and occurred despite the fact that the total protein applied to gradients was the same in each sample.

DISCUSSION

Newly synthesized large subunits of ribulose bisphosphate carboxylase labeled in vivo are recovered as low-molecular-weight complexes. These sediment at 7S in sucrose gradients and exhibit an apparent molecular weight of ~117 kdaltons upon gel filtration (6, 7). They may exist as large subunit dimers (7) or as heterodimers containing one large subunit and one other protein of 50 to 60 kdaltons. The presence of a minority of monomers and trimers or tetramers cannot be ruled out. These 7S complexes turn over during periods of ribulose bisphosphate carboxylase synthesis in vivo. The data presented in this paper establish that the 7S complexes are also formed rapidly in isolated intact chloroplasts.

Barraclough and Ellis (8) reported that large subunits synthesized by isolated intact pea chloroplasts accumulate in an "aggregate" or complex together with a 60-kdalton polypeptide. The molecular weight of this complex, based on electrophoretic mobility measurements, was reported to be 600 to 700 kdaltons. Most of the protein mass (>90%) in the complex is contributed by the 60-kdalton polypeptide, which was termed the "large subunit binding protein." This suggests that, on average, no more than one large subunit is bound per 29S complex. Conceivably, some complexes could bind more than one if others bind less. Assuming that the 60-kdalton proteins do not dissociate from the complex, probably no more than four radioactive large subunits could be bound to any individual 29S complex without leading to a detectable difference in the electrophoretic mobility of the radioactivity and that of the Coomassie Blue stainable component. Thus, the stoichiometry of the 29S large subunit binding complex would correspond to ~10 or 12 60-kdalton subunits and 0 to, at most, 4 large subunits. Ellis cited unpublished data (3) indicating that large subunits bind to this complex in vivo. The data presented in this paper establish that this complex is indeed labeled with large subunits synthesized in vivo. The electrophoretic behavior of the in vivo labeled complex is similar to that reported by Barraclough and Ellis. Additionally, the data presented here explain the fact that the 7S large subunit complexes were not detected in the experiments of Barraclough and Ellis. Nondenaturing gel electrophoresis apparently leads to smearing of the 7S complexes. Since Barraclough and Ellis relied upon a similar nondenaturing gel procedure, it seems likely that they would not have detected the 7S complexes. The combination of sucrose gradient centrifugation and SDS gel electrophoresis allows the visualization of both the 7S complex and the high molecular weight 29S complex in a single experiment. From one-third to one-half of the newly synthesized large subunits are observed in the 7S region, with the remainder in heavier fractions, with a sharp peak at 29S.

Barraclough and Ellis reported that prolonged illumination of isolated intact chloroplasts led eventually to a decline in large subunit radioactivity in the high molecular weight complex, and to the appearance of large subunit radioactivity in ribulose bisphosphate carboxylase. A considerable amount of insoluble radioactive material at the start of the nondenaturing gel lanes was present. Therefore, they refrained from drawing a conclusion about the role of the high molecular weight complex in ribulose bisphosphate carboxylase assembly. In our work, no insoluble radioactive material has been detected by sucrose gradient analysis. When in vivo labeled chloroplasts were illuminated, the 29S large subunit radioactivity declined as radioactivity increased in ribulose bisphosphate carboxylase. Thus, the kinetic behavior of the large subunits in the in vivo labeled 29S complex is similar to the kinetic behavior of the large subunits in the *in organello* labeled 29S complex.

It is known that large subunits derived by denaturation of ribulose bisphosphate carboxylase are insoluble. Newly synthesized large subunits described here and previously (6, 7, 8) appear to have more favorable solubility characteristics. The 29S high molecular weight aggregate represents the second most abundant protein in the chloroplast stroma as judged by staining intensities in two-dimensional gels (8, and P. Milos, unpublished observation). Nevertheless, there are other proteins of comparable abundance in the stroma, and it is significant that newly synthesized large subunits of ribulose bisphosphate carboxylase are not associated with these abundant proteins. Thus, it cannot be supposed that newly synthesized large subunits are merely "sticky" and adhere to other proteins at random. Similarly, except for the newly synthesized large subunits of ribulose bisphosphate carboxylase, few if any other proteins appear to coelectrophorese or to cosediment with the 29S high molecular weight aggregate. These observations suggest that the interactions between newly synthesized large subunits and the 29S complex are specific and that they occur both in vivo and in isolated chloroplasts. We have observed labeling of the 29S complex in hypotonic extracts of etiolated seedlings and plants grown under a variety of illumination conditions (P. Milos, unpublished data). We therefore consider it unlikely that the association of large subunits with the 29S complex is a consequence of the chloroplast isolation procedure. We therefore suggest that the term "aggregate" used by Barraclough and Ellis (8) in this context be replaced by the term "large subunit binding complex" which more accurately characterizes this monodisperse species.

Theoretically, large subunits should be released from ribosomes as monomers. Despite an earlier report (12), however, we have been unable to determine which of the large subunit-containing complexes—the 7S or the 29S complex—is synthesized first during illumination of isolated intact chloroplasts. Even after very short labeling periods (15–60 s long), both the 7S and the 29S complexes have been detected in at least ten repeat experiments. The relative amount of 7S and 29S radioactivity varies from one batch of chloroplasts to another. This may account for our earlier failure to detect the 29S complex at low labeling times (12). The fact that the large subunits dissociate from the 29S complex in vitro suggests that a similar instability of the complex exists within the chloroplast. This, and the similar labeling behavior of the 7S and 29S complexes, suggests that large subunits may equilibrate between the 7S and 29S complexes. This possibility is further supported by the

results of the pulse-chase experiments described here.

The kinetics of large subunit labeling in ribulose bisphosphate carboxylase holoenzyme are slow. Radioactivity in the 18S fraction of the gradient is barely detectable after a 30-min illumination of the chloroplasts. Since the accumulation of [^{35}S]methionine into acid-insoluble form has stopped by this time, it would appear that protein synthesis has stopped (see also 8). However, in the experiments reported here, radioactivity in acid-insoluble form actually declined after a 30-min illumination. Thus, the possibility has to be considered that protein synthesis is still occurring but that it has been overtaken by increased rates of proteolysis. The presence of chase levels of methionine therefore provides assurance that increases in labeling of ribulose bisphosphate carboxylase are not due to residual synthesis of large subunit during a chase. This effect of unlabeled methionine is due to oversaturation of the methionine pool in the chloroplast. Control experiments showed that excess methionine was without effect on protein synthesis itself, since labeled leucine was incorporated into acid-insoluble material at unabated rates in the presence of chase levels of methionine. The fact that labeled large subunits continue to accumulate in the 18S ribulose bisphosphate carboxylase band during the chase therefore demonstrates that these subunits were synthesized before the onset of the chase period. Since the only newly synthesized large subunits present are in the 7S and 29S complexes, one or the other or both of these complexes must have provided the large subunits used to assemble the enzyme. The visual appearance of the autoradiograms shows that in fact each of these large subunit complexes experienced a drastic decline in radioactivity during the chase. A densitometric analysis showed that the 7S and the 29S complex each lost more than enough labeled large subunits to account for all the increased radioactivity in the 18S ribulose bisphosphate carboxylase. No consistent preferential loss of radioactivity by the 7S or by the 29S complex was observed in the three pulse-chase experiments. In other words, the relative amount of radioactivity in the 7S vs. the 29S complex remained about the same. This parallel behavior of the two large subunit complexes during the chase therefore represents an additional reason for believing that large subunits may exchange between these two complexes.

Another conclusion emerging from the pulse-chase experiment is that radioactive large subunits appear to be present in excess in the isolated chloroplasts after *in organello* protein synthesis. During the chase, those excess subunits not used for assembly of the 18S ribulose bisphosphate carboxylase are degraded. The degradation of excess large subunits is not a consequence of general chloroplast lysis, since the protein content of the chloroplasts is not reduced significantly during the chase. The degradation appears to be specific for the labeled proteins, as the patterns of stained protein do not seem to be affected. The overall extent of degradation is variable. In a recent study, Bennett (13) proposed that preferential turnover of the chlorophyll a/b protein occurred in chloroplasts of pea leaves transferred to darkness. This interesting hypothesis holds that chlorophyll may be required to bind to the chlorophyll a/b protein to protect it from degradation. It is not clear whether that hypothesis is correct, or whether the situation described here can be compared directly to it.

It is clear that the isolated chloroplasts contain 7S and 29S large subunits and 3S small subunits at the start of *in organello* protein synthesis. Both isolated chloroplasts and in vivo labeled chloroplasts accumulate labeled 7S and 29S large subunits before significant labeling of large subunits in the 18S carboxylase occurs. The striking similarity in the observed sequence of events in vivo and *in organello* demonstrates that the accumulation of radioactivity in the 7S and 29S complexes cannot be attributed to depletion of small subunits. (Clearly, small subunits are being synthesized in vivo, yet the 7S and 29S complexes are still labeled well before the incorporation of large subunits into holoenzyme reaches significant levels). The delay in labeling of the large subunit in the 18S enzyme in isolated chloroplasts therefore must be a consequence of normal properties of the carboxylase assembly system.

In previous studies, the 7S large subunits appeared to be equimolar with the 3S small subunit pool (6). This conclusion is based on the characteristic excess of large subunit radioactivity over small subunit radioactivity in SDS gel analyses both of sucrose gradient fractions and of immune precipitates of the gradient fractions. The discovery that 29S large subunit complexes contain several times as much large subunit radioactivity as the 7S complexes indicates that the physical large subunit pool is much bigger than previously thought. This property of the large subunit pool may be related to the apparent requirement for accumulation of radioactivity in the 7S and 29S complexes before labeling of the large subunits in the 18S carboxylase. This must remain uncertain, since the absolute pool sizes cannot be determined from the data.

Since the 7S and 29S large subunits show similar labeling and chase characteristics, it is clear that pulse-chase experiments with intact chloroplasts cannot tell us which complex (if either) donates large subunits in the final steps of carboxylase assembly. We have prepared a chloroplast extract in which the assembly of prelabeled large subunits occurs. We are continuing to characterize carboxylase assembly using this soluble in vitro system.

The data presented here have resolved the discrepancy concerning the molecular characteristics of unassembled large subunits which had arisen between work reported by our lab (6, 7) and the work of Barraclough and Ellis (8). The apparent nonoccurrence of the 29S complex in vivo reported by us (6, 7) was due to excessive labeling times in our experiments. The apparent nonoccurrence of the 7S large subunits *in organello* reported by them (8) was probably due to a limitation of the nondenaturing gel electrophoresis technique used by them, and possibly the relatively smaller amount of label found in this complex. Using the appropriate procedures, we have found that the 7S and 29S large subunit complexes are formed both in vivo and *in organello*, that decreases in each large subunit pool can account for all the large subunits assembled into carboxylase *in organello*, and that excess large subunits are degraded *in organello*. The data additionally suggest strongly that there is an asymmetry in large and small subunit pool sizes. Since few labeled large subunits are bound to thylakoid membranes, it appears that the 7S and 29S pools are the only reasonable candidates so far for the role of intermediates in assembly of large subunits into ribulose bisphosphate carboxylase.

In several of the experiments reported here, and in our previous work (6), a labeled protein which trails behind the 7S large subunits both in sucrose gradients and in SDS gels was detected. This protein may resemble a putative large subunit precursor described by Langridge (14). The question of the existence of such a precursor is very interesting, but it is not the subject of this paper, nor is it affected significantly by data we have presented here.

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