Protein Synthesis in Plant Leaf Tissue

THE SITES OF SYNTHESIS OF THE MAJOR PROTEINS

Protein synthesis in the leaves of green pea seedlings (Pisum sativum) is examined by short term labeling with [35S]methionine and autoradiography of the labeled proteins after fractionation by sodium dodecyl sulfate-acrylamide gel electrophoresis. The two subunits of ribulose-1,5-diphosphate carboxylase and the chloroplast lamellae proteins are identified as the major proteins being synthesized.

Three protein-chlorophyll complexes are characterized by sodium dodecyl sulfate-acrylamide gel electrophoresis: all three complexes are disrupted by heating to 100° in sodium dodecyl sulfate solution.

Studies with inhibitors of protein synthesis indicate that the large subunit of ribulose-1,5-diphosphate carboxylase is synthesized in the chloroplast, in contrast to the majority of the soluble proteins, including the small subunit of ribulose-1,5-diphosphate carboxylase, which is synthesized in the cytoplasm. PI1 protein, the major lamellar protein associated with photosystem II, is also synthesized on cytoplasmic ribosomes. However, many of the lamellar proteins are synthesized within the chloroplast.

Integration into the lamellar system of at least one of the chloroplast-synthesized proteins is shown to be dependent on cytoplasmic protein synthesis.

One of the first requirements in studies concerned with the biosynthesis of plant proteins is to determine their respective sites of synthesis. A popular approach to this problem has been through the use of inhibitors like cycloheximide and chloramphenicol (1-5), which selectively inhibit synthesis either on the cytoplasmic ribosomes, or on the prokaryotic-like ribosomes found within the plant organelle. Such studies have shown, for Chlamydomonas reinhardi (1, 2) and Vicia faba (3), that ribosomes both in the cytoplasm and the chloroplast are required for the synthesis of chloroplast lamellar proteins. A similar sharing of function seems to exist for the synthesis of ribulose-1,5-diphosphate carboxylase. Isolated pea chloroplasts have been shown to synthesize the large subunit of this protein (6) and inhibitor studies with barley (7) show the cytoplasm to be the likely site of synthesis for the small subunit. Recently, the partial in vitro synthesis of the small subunit on polysomes derived from the cytoplasm of Phaseolus vulgaris has been reported (8). Thus, at least in the case of higher plants, the synthesis of the two subunits of ribulose-1,5-diphosphate carboxylase seems to occur at distinct sites, although the inhibitor studies concerned with the site of synthesis of the small subunit are far from unequivocal. For the green algae, the situation is not clear, the possibility existing that both subunits are synthesized within the chloroplast (1, 9-12).

As a prelude to studies concerning the regulation of protein synthesis in the leaf tissue of pea seedlings, it was of interest to characterize the major proteins being synthesized and their sites of synthesis. In the course of these studies, the role of cytoplasmic ribosomes in the synthesis of chloroplast proteins has been confirmed. In addition, it is shown that cytoplasmic protein synthesis is also required for the integration into the chloroplast lamellae of at least one of the chloroplast-synthesized proteins.

EXPERIMENTAL PROCEDURE

Materials—Pea (Pisum sativum var. Massey) and spinach (Spinacia oleracea, hybrid 102) seeds were obtained from Yates. Radiochemicals were obtained from The Radiochemical Centre, Amersham/Searle. Tris (Sigma), glycine (Ajax Chemicals Ltd., Sydney), and acrylamide (Eastman) solutions were all filtered through a 0.8-μ Millipore filter prior to use in gel electrophoresis. Phenylmethylsulfonylfluoride and Coomassie brilliant blue R were obtained from Sigma, sodium diethyldithiocarbamate and sodium dodecyl sulfate from BDH Chemicals Ltd., Nonidet P-40 from Shell and Miracloth from Calbiochem. Kodak royal blue and Kodirex films were used for autoradiography, and development was with Kodak liquid X-ray developer (type 2) and Kodak liquid fixer.

Labeling and Extraction of Pea Seedlings—Pea seedlings, grown in pumice at 20° with a 12-hour light period, were harvested 9 days after planting by slicing the epicotyl approximately 2.5 cm below the leaves. They were labeled by standing the excised seedling in a small volume of amino acid mixture containing [35S]methionine. During labeling, the seedlings were irradiated with a 250-watt projector lamp, focused, and cooled through a spherical flask containing water. After labeling, the total leaf tissue, including the meristematic tissue, was homogenized in a conical glass tissue grinder (Kontes) with approximately 10 volumes of extraction buffer (10 mM Tris; 10 mM glycine; 1 mM phenylmethylsulfonylfluoride, and 1% 2-mercaptoethanol, pH 8.9). After centrifugation through Miracloth, the filtrate was fractionated into soluble and particulate components by centrifugation for 30 min at 27,000 x g. The supernatant was treated with 1/10 volume of 10% sodium dodecyl sulfate and immediately incubated for 2 min at 100°.
The pellet was resuspended in one-half the original volume of extraction buffer, heated with sodium dodecyl sulfate as above, and the solubilized proteins clarified by centrifugation for 30 min at 27,500 × g.

**Sodium Dodecyl Sulfate-Acrylamide Gel Electrophoresis**—The slab gel apparatus described by Reid and Bieleski (13) was used. Acrylamide gels (15% acrylamide, 0.085% bisacrylamide; 14 × 11 × 0.15 cm) were prepared in 0.1 M Tris, 0.1 M glycine, and 0.1% sodium dodecyl sulfate (pH 8.9). The gels were pre-electrophoresed for 1 hour at 150 volts using an electrode buffer of 0.1 M Tris, 0.1 M glycine, and 0.1% sodium dodecyl sulfate (pH 8.9). The samples (5 to 20 µl; previously made 10% in glycerol) were loaded (15 min, 20 volts) and then electrophoresis was for 3 hours at 150 volts. After staining (3 hours; 0.25% Coomassie blue in acetic acid/methanol/water, 1/5/5), gels were dehydrated by shaking for several days in acetic acid/methanol/water, 1/4/15. The gels were dehydrated onto Whatman 3MM paper according to the procedure described by Maize (14), and autoradiographs were obtained if the seedling is light irradiated for 2 hours while being labeled with [35S]methionine. The proteins were again fractionated by electrophoresis, and in Fig. 2, the large and small subunits of Fraction I protein (17, 18) (ribulose-1,5-diphosphate carboxylase) were expected to be confirmed by comparison with purified labeled Fraction I protein prepared by immunoprecipitation (Fig. 2). The internal lamellar structure of the chloroplast contains a significant portion of plant protein, and consequently, it seemed likely that many of the particulate proteins would be derived from the chloroplast lamellae. This comparison is made in Fig. 2, c and d. Quite clearly, the majority of the particulate proteins are seen to correspond to chloroplast lamellar proteins. Not surprisingly then, the predominant protein synthesis in the leaves of young light-grown pea seedlings is associated with the development of the stromal and lamellar systems of the chloroplast.

**RESULTS AND DISCUSSION**

**Characterization of the Major Proteins**—A fractionation by sodium dodecyl sulfate-acrylamide gel electrophoresis of the peptides derived from the total proteins present in the leaves of 9-day light-grown pea seedlings is shown in Fig. 1. By co-electrophoresis with appropriate standards (16), the molecular weights of the major peptides have been calculated.

In order to determine which proteins were being actively synthesized, excised pea seedlings were light-irradiated for 2 hours while being labeled with [35S]methionine. The proteins were again fractionated by electrophoresis, and in Fig. 2, the autoradiograph shows which proteins are being synthesized in the leaves of 9-day pea seedlings. An essentially identical profile is obtained if the labeling is done with 14C-amino-acid mix (Amersham CFB.25), and similarly, an identical profile is obtained if the seedling is light-irradiated for 2 hours while standing in water prior to the 2-hour labeling with [35S]methionine.

It was of interest to characterize the major peptides depicted in Fig. 2. The large and small subunits of Fraction I protein (17, 18) (ribulose-1,5-diphosphate carboxylase) were expected to form major components of the soluble proteins, and this was confirmed by comparison with purified labeled Fraction I protein prepared by immunoprecipitation (Fig. 2). The internal lamellar structure of the chloroplast contains a significant portion of plant protein, and consequently, it seemed likely that many of the particulate proteins would be derived from the chloroplast lamellae. This comparison is made in Fig. 2, c and d. Quite clearly, the majority of the particulate proteins are seen to correspond to chloroplast lamellar proteins. Not surprisingly then, the predominant protein synthesis in the leaves of young light-grown pea seedlings is associated with the development of the stromal and lamellar systems of the chloroplast.

An attempt was made to characterize further the lamellar proteins PI and PII depicted in the autoradiograph of Fig. 2. In Fig. 3 is shown a stained sodium dodecyl sulfate-acrylamide gel fractionation of the proteins derived from both pea and spinach lamellae, and by comparative electrophoresis, the labeled PI and PII proteins have been shown to correspond to the major lamellar proteins in this diagram. An unusual feature of some chloroplast lamellar proteins is that they run as undissociated protein-chlorophyll complexes on electrophoresis in sodium...
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Figure 2 (left). Sodium dodecyl sulfate-acrylamide gel electrophoresis of [3S]methionine-labeled proteins from pea seedling leaves. Seedlings were labeled by standing in a 60 µl solution of amino acids including [3S]methionine (33.9 Ci/mmol; 25 µM; 19 other amino acids, 166 µM). For the preparation of lamellar proteins, 0.5 ml of leaf extract was layered directly onto a linear sucrose gradient and sedimented as described under “Experimental Procedure.” The green lamellar band was recovered (200 µl) and treated with sodium dodecyl sulfate. The autoradiograph shows a, Fraction I immunoprecipitate (2704 cpm); b, soluble proteins (53,825 cpm); c, particulate proteins (50,466 cpm) and d, chloroplast lamellar proteins (72,350 cpm).

Figure 3 (right). Sodium dodecyl sulfate-acrylamide gel electrophoresis of lamellar proteins from pea and spinach chloroplasts. Chloroplast lamellae were purified by sedimentation through sucrose according to the method described under “Experimental Procedure.” The green chloroplast lamellar bands (250 µl; 1.16 g/ml) were recovered from the gradient and made 1% in sodium dodecyl sulfate and 2-mercaptoethanol. One-half of each sample was kept on ice and the rest incubated for 2 min at 100°. Twenty-microliter samples were fractionated by acrylamide gel electrophoresis as described under “Experimental Procedure” except that electrophoresis was for 6 hours at 4°. After electrophoresis, the chlorophyll bands were marked (PSI, PSIIa, PSII, and Chl) prior to staining in Coomassie blue. a, pea lamellar proteins, not heated in sodium dodecyl sulfate; b, pea lamellar proteins, heated in sodium dodecyl sulfate; c, spinach lamellar proteins, not heated in sodium dodecyl sulfate; d, spinach lamellar proteins, heated in sodium dodecyl sulfate.

dodecyl sulfate (3). In the present investigation, four chlorophyll-containing bands (PSI, PSIIa, PSII, and Chl) were observed if lamellar preparations were fractionated by electrophoresis at 4° and the prior dissociation in hot sodium dodecyl sulfate was omitted. However, if the lamellar preparations were heated at 100° prior to electrophoresis, which was standard procedure throughout this investigation, then only one chlorophyll band was found (presumably free chlorophyll). In the heated sample, in contrast to previous claims (19), no protein band was found with electrophoretic properties similar to the photosystem I protein-chlorophyll complex (PSI). When the lamellae are labeled with [3S]methionine and treated according to the procedure in Fig. 3, then, by autoradiography, the complexes PSI, PSIIa, and PSII are shown to be labeled, indicating that they are indeed protein complexes. In the case of the PSII protein-chlorophyll complex, it seems likely that dissociation here gives rise to protein PII, of molecular weight 29 kilodaltons (19-22). Often two proteins are observed in this region (29 and 31 kilodaltons). It has been shown that PSI contains mainly chlorophyll a, whereas PSII contains both chlorophyll a and b (3). The nature of the chlorophyll associated with the minor PSIIa complex has not been determined, although from the color of the band, both before and after staining with Coomassie blue, it is similar to PSII and distinct from PSI.

From Fig. 3 then, it is concluded that the lamellar system of the pea chloroplast contains two major proteins, PI and PII, of respective molecular weights 58 and 29 kilodaltons (obtained according to Fig. 1). PII appears to be strongly associated with chlorophyll within the chloroplast, and from the work of others (19-22), it forms the major protein of photosystem II. In contrast, the author finds no evidence that PI forms a protein-chlorophyll complex, although it does appear to form the major photosystem I protein (19-22). The nature of the protein moieties associated with the complexes PSI and PSIIa, of apparent molecular weights 96 and 70 kilodaltons, has not been clarified.

Sites of Synthesis of Major Proteins—The sites of protein synthesis have been examined with the aid of the inhibitors cycloheximide and chloramphenicol. In Table I, the effect of these inhibitors on the incorporation of [3S]methionine is shown. Cycloheximide is seen to decrease total protein synthesis significantly, and this effect is most pronounced in the case of the soluble proteins. The effect of cycloheximide and chloramphenicol on the synthesis of specific proteins is illustrated by the autoradiographs in Figs. 4 and 5. It is observed that the synthesis of the large subunit of Fraction I protein is unique among the major soluble proteins in that it is sensitive to chloramphenicol and relatively insensitive to cycloheximide. The small subunit of Fraction I protein, along with all of the other major soluble proteins, is synthesized on ribosomes sensitive to cycloheximide. These observations are consistent
TABLE I
Effect of chloramphenicol and cycloheximide on protein synthesis in pea seedling leaves

Pea seedlings were light irradiated for 15 min while standing in water (50 µl), chloramphenicol (200 µg/ml), or cycloheximide (20 µg/ml). To each sample were then added 10 µl of [35S]methionine (34.2 Ci/mmol, 0.15 mM; 19 other amino acids, 1.0 mM) and the irradiation continued for a further 2 hours. The leaves were then homogenized in 1 ml of extraction buffer, fractionated into soluble and particulate components, and treated with sodium dodecyl sulfate as described under "Experimental Procedure." Protein synthesis was determined by trichloroacetic acid precipitation, collection of precipitates on Whatman GFC filters, and liquid scintillation counting.

|                  | [35S]Methionine incorporated/seedling (cpm × 10^-4) | % distribution | % of control |
|------------------|--------------------------------------------------|----------------|--------------|
|                  | Soluble | Particulate | Soluble | Particulate | Soluble + Particulate |
| Control          | 16.02   | 9.25       | 68.4   | 36.6       |                     |
| Cycloheximide    | 3.37    | 5.62       | 37.5   | 62.5       | 35.6                |
| Chloramphenicol  | 14.11   | 7.53       | 65.2   | 34.8       | 88.6                |

with the finding that the large subunit of Fraction I protein is synthesized in isolated pea chloroplasts (6), and the small subunit is synthesized on polysomes derived from the cytoplasm of bean leaves (P. vulgaris) (8). It is noted that whereas both inhibitors used show some selectivity, this is not complete, particularly in the case of cycloheximide. This presumably reflects a rather tight coupling between protein synthesis in the cytoplasm and the chloroplast. These inhibitor studies are extended by the results shown in Fig. 6. Here, Fraction I protein has been purified with the aid of specific antiserum. Clearly, the synthesis of the small subunit of Fraction I protein continues to some extent in the absence of large subunit synthesis. However, in the presence of cycloheximide, where the synthesis of the small subunit is severely restricted, so also is the synthesis of the large subunit, or at least its incorporation into the native molecule. Similar results to those depicted in Fig. 6 have been obtained by purifying Fraction I protein by sucrose gradient centrifugation (1).

The particulate proteins present a very different picture (Figs. 4 and 5), as in this case, the synthesis of many of the proteins is sensitive to chloramphenicol. In the case of the chloroplast lamellar proteins (Fig. 5), this sensitivity to chloramphenicol is almost certainly due to their synthesis on chloroplast ribosomes. PII protein is exceptional in that its synthesis is sensitive to cycloheximide and insensitive to chloramphenicol. Similar results regarding the sites of synthesis of bean (V. faba) chloroplast lamellar proteins have been reported (3).

All of the results obtained with chloramphenicol (Fig. 4) have been duplicated using lincomycin.

Cytoplasmic Protein Synthesis Is Required for Chloroplast Lamellar Assembly—A further interesting piece of information is depicted in Fig. 4. This concerns protein PI, which is quite clearly a chloroplast lamellar protein (Fig. 2), and consequently, in Fig. 4, it occurs in the particulate fraction. However, in the presence of cycloheximide, an inhibitor which does not appear to influence its synthesis, approximately 50% of what appears to be PI is found in the soluble fraction. Confirmation of the relationship between this soluble protein...
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FIG. 5. Effect of chloramphenicol and cycloheximide on the synthesis of chloroplast lamellar proteins. Pea seedlings were labeled with [35S]methionine according to the procedure described in Table I. Leaf proteins were extracted with 1 ml of extraction buffer per seedling and 0.5 ml of filtered extract was layered directly onto a linear sucrose gradient. After centrifugation, the lamellar band was recovered, treated with sodium dodecyl sulfate, and fractionated by electrophoresis. The autoradiograph shows proteins synthesized in a, control (28,876 cpm); b, chloramphenicol (21,448 cpm); and c, cycloheximide (17,986 cpm).

and PI protein has been attained by comparative isoelectric focusing. 1 It appears that a continuous supply of a cytoplasmically synthesized component is required for the incorporation of PI into the chloroplast lamellae. A possible identity of such a component is PI1 protein, as it represents the major lamellar protein and its synthesis is sensitive to cycloheximide. Similar results pertaining to PI have been obtained with anisomycin, indicating that the phenomenon is indeed a reflection of the inhibition of cytoplasmic protein synthesis.

In this investigation, it has been shown in pea seedlings that the small subunit of Fraction I protein is apparently synthesized within the cytoplasm and this finding is in agreement with the work of others (7, 8). In Nicotians, the small subunit of Fraction I protein is coded by the nuclear genome (23), and this presumably holds for the pea Fraction I subunit. It has also been shown that the cytoplasm is the likely site of synthesis for the major protein of photosystem II, and this protein is also coded for by the nucleus (24). The cytoplasmic origin of the major photosystem II protein is also found in bean (V. faba) (3) and in a unicellular green alga (C. reinhardii) (25). These conclusions regarding the site of synthesis of the small subunit of Fraction I protein and PI1 protein have recently been confirmed by in vitro studies. 2

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1 A. R. Cashmore, unpublished results.
2 A. R. Cashmore, M. K. Broadhurst and R. E. Gray, manuscript in preparation.

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