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Biochemistry

Cell-free synthesis of leaf protein: Identification of an apparent precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase

(immunoprecipitation/tryptic peptides/organelle protein biosynthesis)

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ABSTRACT **Cytoplasmic mRNA has been isolated from the leaves of pea seedlings. Translation of this RNA in the wheat germ cell-free system produces two major products, RI and RII, with molecular weights of 33,000 and 20,000, respectively. Both of these products are considerably larger than the small subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], which is the major product of cytoplasmic protein synthesis *in vivo* and has a molecular weight of 14,000. Antiserum prepared against the small subunit of ribulose-1,5-bisphosphate carboxylase precipitates from the cell-free products, in 2–3% yield, three polypeptides of molecular weights 18,000, 16,000, and 14,000. The smallest of these polypeptides is indistinguishable, by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, from the small subunit of ribulose-1,5-bisphosphate carboxylase. Although the cell-free product RII is not precipitated with antiserum prepared against the small subunit of ribulose-1,5-bisphosphate carboxylase, the two polypeptides do show extensive sequence homology, as indicated by ion exchange chromatography of their tryptic peptides. The production of RII can also be achieved in a polysome-primed cell-free system, where protein synthesis is restricted to the completion of polypeptide chains that have already been initiated *in vivo*. These results indicate that RII is apparently a precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase. We suggest that the selective transport of cytoplasmically synthesized organelle proteins, like animal secretory proteins, may be achieved via the production of precursor polypeptides.**

The synthesis of animal secretory proteins has recently been shown to occur via the production of precursor polypeptides (1). The amino-terminal region of the nascent precursor is thought to be involved, both in the generation of membrane-bound ribosomes and in the vectorial transport through the membrane into the intracisternal space (2–4). By this means proteins destined for secretion are segregated from nonsecretory proteins. The precursors are either very short-lived, as in the case of the biosynthesis of parathyroid hormone (5), or have no finite existence at all, as they may be cleaved at the amino terminus prior to completion of translation of the nascent polypeptide (3). Consequently, these precursors have become apparent only through the use of cell-free protein-synthesizing systems, which in some cases appear to lack the appropriate processing enzymes.

The problem of selective transport of newly synthesized polypeptides also exists in the biosynthesis of organelle proteins. Within the leaf cell, for example, cytoplasmic ribosomes are involved in the synthesis of not only cytoplasmic proteins, but also chloroplast (6) and, presumably, mitochondrial (7) proteins. Thus the small subunit of ribulose-1,5-bisphosphate (Ru-P_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is synthesized on cytoplasmic ribosomes (6, 8, 9) and must be transported through the cellular membrane system into the chloroplast. In this paper we describe the cell-free synthesis of an apparent precursor of the small subunit of Ru-P_2 carboxylase. Our results indicate that the selective transport of organelle proteins, like animal secretory proteins, may be achieved via the production of precursor polypeptides.

EXPERIMENTAL PROCEDURES

Materials. Radioactive compounds were purchased from the Radiochemical Centre, Amersham, at the following specific activities: L-[^{35}S]methionine, 240–570 Ci/mmol; uniformly ^{14}C-labeled protein hydrolysate (CFB.25), 54 mCi/mmol of carbon, and ^{3}H-labeled amino acid mixture (TRK.440), 0.34–60 Ci/mmol. Trypsin was TPCK grade from Worthington and hydroxylapatite was Bio-Gel HTP grade from Bio-Rad. Other chemicals were as previously described (6, 10).

Preparation of Radioactively Labeled Proteins from Pea Leaves. The growth, labeling, and extraction of pea seedlings was as previously described (6). The extracted proteins were separated into soluble and particulate fractions by centrifugation of approximately 1 ml of extract, in a Sorvall HB-4 rotor for 30 min at 13,000 rpm (27,578 × g_{max}). Ru-P_2 carboxylase was pelleted from the soluble fraction by centrifugation in a Beckman 65 rotor for 2 hr at 50,000 rpm (218,000 × g_{max}). The pelleted protein was solubilized in 0.5 ml of 10 mM Tris/10 mM glycine/5% (vol/vol) 2-mercaptoethanol/2% sodium dodecyl sulfate ($NaDodSO_4$)/1 mM phenylmethylsulfonyl fluoride, prior to fractionation by gel electrophoresis.

Preparation of Pea Leaf Polysomes and RNA. Pea leaf polysomes, polysomal RNA, poly(A)$^-$ RNA and poly(A)$^+$ mRNA preparations were as previously described (10). Cytoplasmic RNA was prepared by homogenizing, with a Dunmore Type 2B-210 homogenizer, 30 g of leaf tissue in 100 ml of 0.25 M sucrose/0.2 M NaCl/0.1 M Tris·HCl (pH 9.0 at 25°)/10 mM magnesium acetate/14 mM 2-mercaptoethanol. The filtered extract was centrifuged in a Sorvall HB-4 rotor for 10 min at 10,000 rpm (16,318 × g_{max}) and then, after the supernatant had been made 1% in $NaDodSO_4$ and 15 mM in EDTA, the RNA was isolated by extraction with phenol/chloroform/isoamyl alcohol (50:50:1) followed by ethanol precipitation (10).

Cell-Free Protein Synthesis. Conditions were as previously described (10) except that the wheat germ extract was not preincubated. For labeling with the ^{14}C-labeled amino acid mixture, 6.25 μCi of protein hydrolysate was used per 100-μl reaction mix, and unlabeled methionine, asparagine, cysteine, glutamine, and tryptophan were present at 50 μM concentrations.

Chromatography on Hydroxylapatite Columns in the

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Abbreviations: Ru-P_2, ribulose 1,5-bisphosphate; $NaDodSO_4$, sodium dodecyl sulfate.

Presence of $NaDodSO_4$. Large-scale (0.5 ml) cell-free translation mixtures were fractionated, after dissociation with $NaDodSO_4$ and 2-mercaptoethanol, on 20- × 0.6-cm columns of hydroxylapatite. The procedure was as described (11), with 60 ml of a 0.2–0.5 M linear phosphate gradient being used, and 1.5-ml fractions were collected. Fractions were assayed for radioactivity, which showed a single broad peak eluting at approximately 0.35 M phosphate. The four peak fractions, which by electrophoresis of aliquots were shown to contain the major portion of RII, were combined, 100 μg of bovine serum albumin was added, and the sample was made 10% in trichloroacetic acid. After standing at 0° overnight, the protein precipitate was collected by centrifugation and washed with 5% trichloroacetic acid followed by acetone. The precipitate was taken up in 150 μl of 10 mM Tris/10 mM glycine/1% $NaDodSO_4$/5% 2-mercaptoethanol/10% (vol/vol) glycerol and incubated for 2 min at 100° prior to gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was with 15% gels in the presence of $NaDodSO_4$ (6). The gels were fixed, washed, and dried prior to the preparation of autoradiographs (6). The molecular weights of the cell-free translation products were determined by comparison of their mobility with the large and small subunits of Ru-P_2 carboxylase (6).

Immunological Procedures. Ru-P_2 carboxylase was purified from pea leaves (6), dissociated into its subunits by treatment with $NaDodSO_4$ and 2-mercaptoethanol, and then fractionated on Sephadex G-100 in 0.1% $NaDodSO_4$/25 mM Tris·H_2SO_4, pH 7.5/5 mM 2-mercaptoethanol/5 mM EDTA. Fractions containing the small subunit were made 6 M in urea, the $NaDodSO_4$ was removed (12), and the samples were then dialyzed against 0.1 M KCl/50 mM Tris·HCl, pH 8.0/2 mM magnesium acetate/1 M urea. Preparation of antiserum and immunoprecipitation procedures were as previously described (6) except that 400 μg of antigen was used per rabbit for the initial immunization, followed by injections of 240 μg of antigen at two 2-week intervals. In two-dimensional immunodiffusion studies, the antiserum produced precipitin lines with both Ru-P_2 carboxylase and the small subunit of Ru-P_2 carboxylase. A single precipitin line, corresponding to Ru-P_2 carboxylase, was obtained with a leaf protein extract.

Preparation of Tryptic Peptides. Polypeptides were fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis. The polypeptides were located by autoradiography, and gel portions were excised, cut into small pieces, and shaken for 16 hr at 25° in 0.05 M ammonium bicarbonate, pH 8.5, containing trypsin at 100 μg/ml (13). For five gel slices, 5 ml of extraction mix was used. The filtered extract was lyophilized and washed with water, and the peptides were dissolved in 0.1 ml of formic acid and oxidized for 2 hr at 0° by the addition of 0.1 ml of performic acid reagent prepared as described (14).

Immunoprecipitates, without prior fractionation, were oxidized with performic acid (14) and then digested in 1 ml of the above trypsin solution for 16 hr at 25°.

Fractionation of Tryptic Peptides. Ion exchange chromatography was performed at 50° on a 0.6- × 33-cm column of Beckman PA35 resin. The sample was loaded in 0.05 M pyridine/30% (vol/vol) acetic acid, pH 2.5 and then, after washing with 20 ml of the same buffer, elution was performed with a gradient prepared from 100 ml of the loading buffer, 100 ml of 0.2 M pyridine/27.8% acetic acid, pH 3.1, and 100 ml of 2.0 M pyridine/14.4% acetic acid, pH 5.0. The flow rate was maintained at 20 ml/hr with a column pressure of approximately 175 pounds/inch^2 (120 kPa) and 2-ml fractions were collected. The fractions were dried, shaken for 5 hr with 1.0 ml of 50 mM Tris/50 mM glycine/0.1% $NaDodSO_4$ and then assayed for radioactivity after the addition of 9 ml of Triton X-100/toluene (1:2) containing 0.27% Omnifluor.

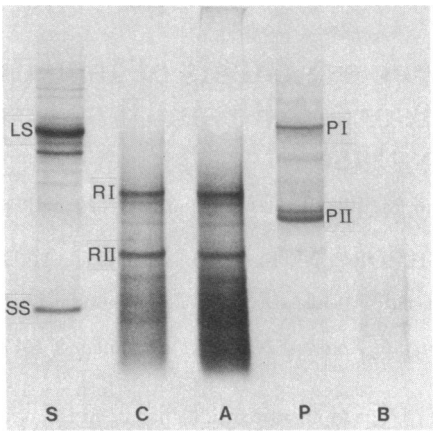

FIG. 1. Autoradiograph of $NaDodSO_4$/polyacrylamide gel electrophoretic separation of [^{35}S]methionine-labeled polypeptides. The soluble (S) and particulate (P) fractions were prepared from pea seedling leaves, and contain the large (LS) and small (SS) subunits of Ru-P_2 carboxylase, and the chloroplast lamellar proteins PI and PII. The cell-free system was primed with a total cytoplasmic RNA preparation (C), poly(A)$^+$ mRNA (A), and no RNA (B). Autoradiography was for 1 day.

[^{35}S]Methionine-labeled tryptic peptides were fractionated by paper electrophoresis on 57-cm strips of Whatman 3 MM paper, at pH 3.5 (5% acetic acid/0.5% pyridine, vol/vol) for 2 hr at 2000 V. The labeled peptides were located by autoradiography.

RESULTS

Cell-Free Protein Synthesis. Cytoplasmic messenger RNA isolated from the green leaves of young pea seedlings exists as two classes, which differ according to the presence or absence of poly(A) sequences (10). Addition of this mRNA to a wheat germ cell-free protein-synthesizing system results in a marked stimulation in amino acid incorporation, with synthesis continuing for at least 90 min (unpublished results). In order to examine the products of cell-free synthesis we have fractionated the polypeptides by $NaDodSO_4$/polyacrylamide gel electrophoresis. The autoradiograph depicted in Fig. 1 shows that two major products, RI and RII, are obtained, with molecular weights of 33,000 and 20,000. These two polypeptides represent the major cell-free products irrespective of whether we prime the system with poly(A)$^+$ mRNA (Fig. 1), poly(A)$^-$ RNA (10), a cytoplasmic RNA preparation (Fig. 1), a total nucleic acid preparation (unpublished results), or polysomes (unpublished results). The predominant cell-free products do not correspond in molecular weights with either the chloroplast lamellar protein, PII, or the small subunit of Ru-P_2 carboxylase, these representing the two major cytoplasmically synthesized polypeptides (6).

Immunoprecipitation. Addition of antiserum, prepared against the small subunit of Ru-P_2 carboxylase, to the soluble cell-free translation products, resulted in the immunoprecipitation of from 2.4 to 3.4% of the [^{35}S]methionine-labeled polypeptides (Table 1). By $NaDodSO_4$/polyacrylamide gel electrophoresis, three polypeptides, of molecular weights 18,000, 16,000, and 14,000, were shown to be present in the immunoprecipitate (Fig. 2). The smallest of these three polypeptides was indistinguishable, by $NaDodSO_4$/polyacrylamide gel electrophoresis, from the small subunit of Ru-P_2 carboxylase. None of these three polypeptides were detected in the minus-RNA immunoprecipitate or in the immunoprecipitate

Table 1. Immunoprecipitation of polypeptides synthesized in the wheat germ cell-free system

Exp.	Primer for cell-free synthesis	% ^{35}S-labeled polypeptides immunoprecipitated
1	Total nucleic acids	2.4
	Cytoplasmic RNA	3.0
	Polysomal RNA	2.6
	Poly(A)$^+$ mRNA	2.6
	Polysomes	2.3
2	Poly(A)$^+$ mRNA	3.4
	Poly(A)$^+$ mRNA	0.26*
	Minus RNA	0.67†

Soluble polypeptides were prepared by centrifugation of the [^{35}S]methionine-labeled cell-free products (100 μl) for 60 min at 40,000 rpm in a Beckman 40 rotor (144,880 × g_{max}). Immunoprecipitation was similar to before (6), but involved an excess of antiserum (500 μl) prepared against the small subunit of Ru-P_2 carboxylase, and included the addition of the small subunit (4 μg) as unlabeled carrier. The immunoprecipitates were washed (6), dissolved in 50 μl of buffer containing 1% NaDodSO$_4$, and trichloroacetic acid-insoluble radioactivity was determined.

* Immunoprecipitation was with 10 μl of bovine γ globulin antiserum and 5 μg of bovine γ globulin.

† In Exp. 2, 5.94 times as much [^{35}S]methionine was incorporated into soluble polypeptides in the poly(A)$^+$ mRNA-primed reaction as in the minus-RNA reaction. Consequently, (3.4 × 5.94)/0.67 = 30.1 times as much [^{35}S]methionine-labeled polypeptide material was present in the poly(A)$^+$ mRNA immunoprecipitate as in the minus-RNA immunoprecipitate.

obtained with antiserum prepared against bovine γ globulin (Table 1 and unpublished gel electrophoresis results).

Characterization of Tryptic Peptides. As an alternative approach to characterizing the polypeptides produced by the cell-free system, we have compared their tryptic peptides with those obtained from the small subunit of Ru-P_2 carboxylase. By electrophoresis at pH 3.5 it is seen that the small subunit has two methionine-containing tryptic peptides (Fig. 3). If the same tryptic peptides are fractionated by ion exchange chromatography, one major and a number of minor peaks are observed (Fig. 4), indicating that one of the peptides observed on electrophoresis is in fact heterogeneous. We have also examined the tryptic peptides of both RII and the immunoprecipitate obtained with the antiserum prepared against the small subunit of Ru-P_2 carboxylase. Both by electrophoresis (Fig. 3) and by ion exchange chromatography (Fig. 4) it is seen that the major methionine-containing tryptic peptides are very similar for these two samples. Furthermore, one of these peptides cochromatographs with one of the methionine-containing tryptic peptides found for the small subunit (Fig. 4).

The above results suggest that RII possesses a sequence relationship with the small subunit of Ru-P_2 carboxylase, even though RII is not immunoprecipitated with antiserum prepared against the small subunit. The structure of RII was examined more critically by labeling the cell-free product with a mixture of 15 ^{14}C-labeled amino acids and preparing the small subunit of Ru-P_2 carboxylase labeled with an analogous mixture of ^{3}H-labeled amino acids. The tryptic peptides were prepared from these labeled samples and they were then fractionated by ion exchange chromatography (Fig. 4). It is observed that extensive sequence homology exists for the two polypeptides, with the majority of the small subunit peptides being present in the cell-free product.

DISCUSSION

We have shown that priming the wheat germ cell-free protein-synthesizing system with pea leaf cytoplasmic mRNA produces two major products, RI and RII. Neither of these corresponds to the chloroplast lamellar protein, PII, or the small subunit of Ru-P_2 carboxylase; these two chloroplast proteins

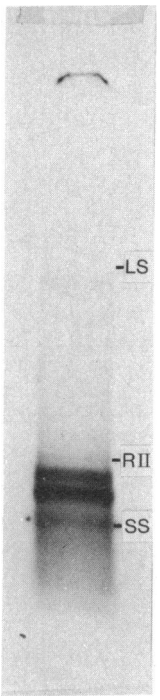

FIG. 2. Autoradiograph of NaDodSO$_4$/polyacrylamide gel electrophoretic separation of [^{35}S]methionine-labeled immunoprecipitate. The immunoprecipitate was prepared from a poly(A)$^+$ mRNA-primed cell-free synthesis (Table 1). The large subunit of Ru-P_2 carboxylase (LS) and RII were run as parallel markers and the small subunit of Ru-P_2 carboxylase (SS) was an internal marker, present as unlabeled carrier, and identified in the stained gel. Autoradiography was for 4 days.

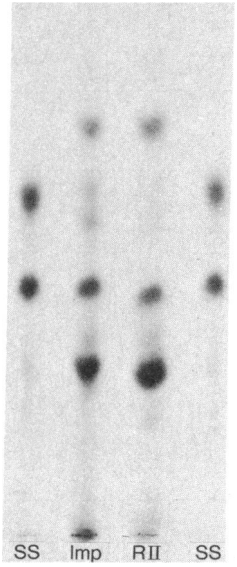

FIG. 3. Autoradiograph of paper electrophoretic separation of [^{35}S]methionine-labeled tryptic peptides. The small subunit of Ru-P_2 carboxylase (SS) and RII were fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis prior to digestion with trypsin. The cell-free product, RII, was subjected to an initial fractionation by chromatography on hydroxylapatite, followed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The immunoprecipitate (Imp) was obtained from poly(A)$^+$ mRNA-primed cell-free synthesis (Table 1 and Fig. 2) and was not fractionated prior to digestion with trypsin. Autoradiography was for 7 days.

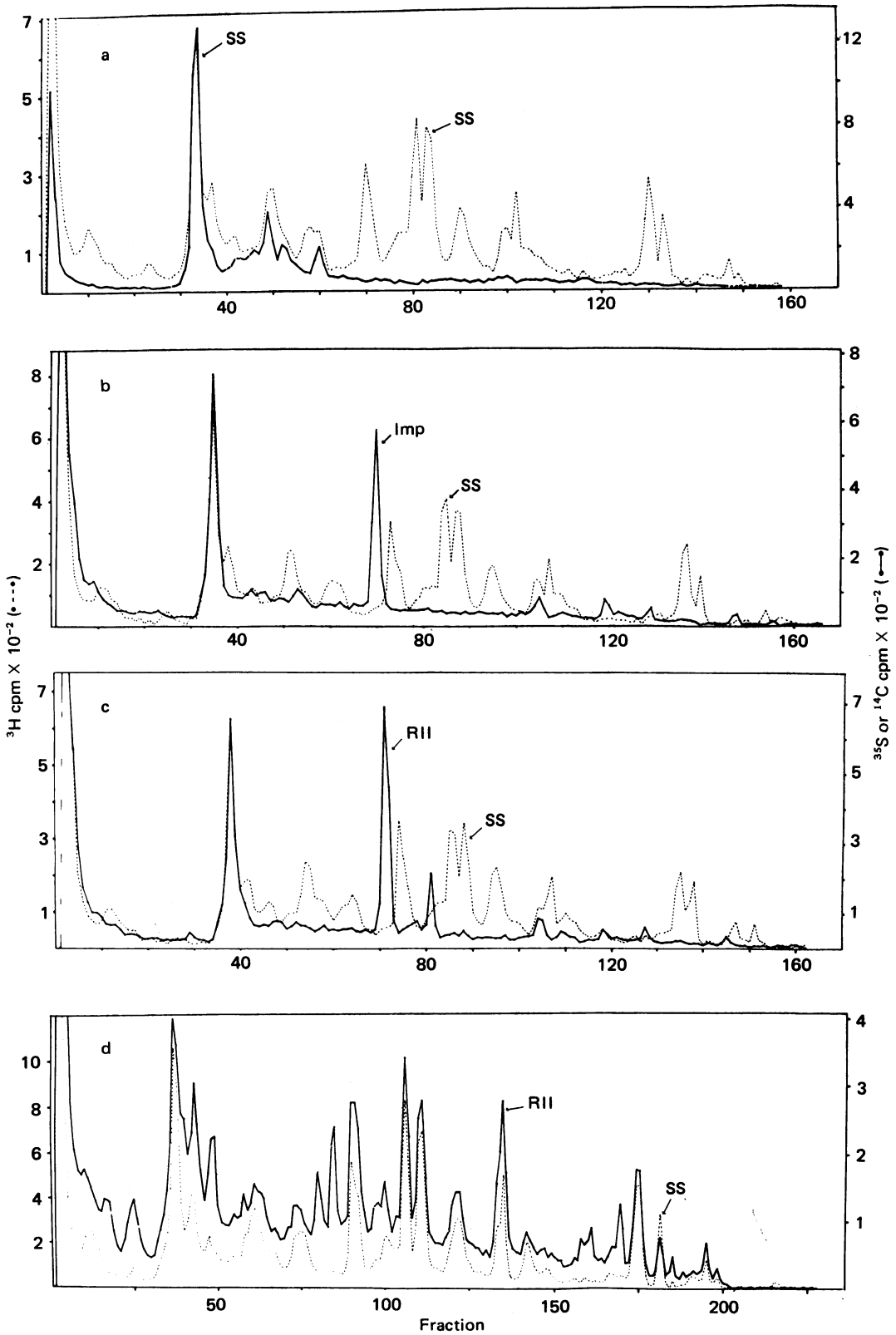

FIG. 4. Ion exchange chromatography of tryptic peptides. The small subunit of Ru-P_2 carboxylase (SS), the cell-free translation product (RII), and the immunoprecipitate (Imp) were prepared for digestion with trypsin as described for Fig. 3. Columns *a* (SS labeled with either a ^{3}H-labeled amino acid mixture or [^{35}S]methionine), *b* (SS labeled with a ^{3}H-labeled amino acid mixture and Imp labeled with [^{35}S]methionine), and *c* (SS labeled with a ^{3}H-labeled amino acid mixture and RII labeled with [^{35}S]methionine) were eluted with a pyridine/acetate gradient of three 100-ml components. Column *d* (SS labeled with a ^{3}H-labeled amino acid mixture and RII labeled with a ^{14}C-labeled amino acid mixture) was eluted with a pyridine/acetate gradient of three 150-ml components. The radioisotopes were counted at efficiencies of ^{3}H, 30% and ^{14}C, 68%; spillovers of ^{3}H into the ^{14}C or ^{35}S channel were 2.7%; ^{14}C spillover into the ^{3}H channel was 11.8%; and ^{35}S spillover into the ^{3}H channel was 11.6%. In the profiles presented the cpm have been corrected for spillover. The following percentages of applied radioactivity were recovered in the first 5 column fractions (^{3}H percentages given first); *a*, 14 and 12; *b*, 14 and 18; *c*, 15 and 35; *d*, 15 and 20. An average of 83% of applied radioactivity was recovered from the columns.

are the major products of leaf cytoplasmic protein synthesis *in vivo* (6). In this paper we have examined the relationship between RII and the small subunit of Ru-P_2 carboxylase. Immunoprecipitation with antiserum prepared against the small subunit characterizes three products from the cell-free system, of molecular weights 18,000, 16,000, and 14,000. The smallest of these products is indistinguishable, by $NaDodSO_4$/polyacrylamide gel electrophoresis, from the small subunit of Ru-P_2 carboxylase. Comparison of the methionine-containing tryptic peptides from the polypeptides obtained by immunoprecipitation with those obtained from RII shows a striking similarity for the two samples, indicating a possible relationship between RII and the small subunit of Ru-P_2 carboxylase. That this is indeed the case is illustrated by the experiment depicted in Fig. 4. Here, the tryptic peptides from RII have been compared with those from the small subunit after labeling the two polypeptides with a mixture of either ^3H- or ^{14}C-labeled amino acids. Clearly, RII contains the majority of the peptides present in the small subunit of Ru-P_2 carboxylase.

What is the biological relationship between RII and the small subunit of Ru-P_2 carboxylase? The cell-free product does not result from incorrect initiation because it is also produced in a polysome-primed system under conditions where protein synthesis is restricted to the completion of nascent polypeptide chains that have already been initiated *in vivo* (unpublished results). In view of this, the most likely explanation for the cell-free product RII is that it represents a precursor of the small subunit of Ru-P_2 carboxylase. The amino-terminal amino acid for the small subunit isolated from pea leaves is methionine (15). By both ion exchange chromatography and paper electrophoresis it is observed that one of the methionine-containing tryptic peptides found for the small subunit is absent in the cell-free product, and therefore it seems likely that RII, in common with other precursors (1), differs from the small subunit in that it has additional amino acid residues at the amino terminus. In comparison with other precursors (1), RII is unusually large relative to the small subunit in that it possesses approximately 60 additional amino acid residues. At this stage we do not know whether all these additional residues are located at the amino terminus or whether the carboxyl terminus is also extended.

RII is not precipitated by antiserum that we have prepared against the small subunit of Ru-P_2 carboxylase. This presumably reflects the relatively large size difference of the two polypeptides, which might allow RII to adopt a conformation in which the antigenic sites of the small subunit are either altered or not exposed. That immunological reactivity may be influenced by the conformation of protein antigens is not unexpected, and is well documented in the case of plant viral proteins (16). Indeed, the apparent inability to completely immunoprecipitate preproinsulin with insulin antiserum has been ascribed to conformational differences (17). The polypeptides of molecular weights 18,000 and 16,000, which have been characterized by immunoprecipitation with the small subunit antiserum, may represent translation products resulting from premature termination (18, 19). Alternatively, they may result from proteolytic cleavage of RII, and this could reflect an *in vivo* processing system. These two polypeptides apparently do not result from incorrect initiation, for, as with RII, they are produced in a polysome-primed translation system where protein synthesis is primarily restricted to the completion of nascent polypeptides (unpublished results).

Two recent publications confirm the general findings we have presented. Roy *et al.* (9) have described a polypeptide, of 20,000 daltons, which is synthesized on wheat seedling polysomes and is apparently immunologically related to the small subunit of Ru-P_2 carboxylase. Similarly, Dobberstein *et al.* (20) have described a product of cell-free translation of mRNA from *Chlamydomonas reinhardtii*, which is also 20,000 daltons and is immunologically related to the small subunit of Ru-P_2 carboxylase. In contrast to our observations, the putative precursor from *Chlamydomonas* was not produced in a polysome-primed cell-free system, where it was processed to yield the small subunit (20). This proteolytic activity was also present in a postribosomal supernatant. In agreement with the findings of these authors (20), we also find that the small subunit-precursor is synthesized on ribosomes that have been isolated under conditions expected to yield only ribosomes that exist unattached to membranes (unpublished results). In this respect the precursors for the small subunit of Ru-P_2 carboxylase appear to differ significantly from precursors for animal secretory proteins, which are apparently synthesized, primarily, on membrane-bound ribosomes (1).

The observations presented in this paper indicate that the small subunit of Ru-P_2 carboxylase is synthesized via a precursor. We suggest that the function of this precursor is the selective transport of the cytoplasmically synthesized polypeptide, through the cellular membrane system, into the chloroplast. Indeed, it seems likely that the selective transport of cytoplasmically synthesized organelle proteins, in general, may be achieved via the production of precursors. We expect that PII protein, the other major cytoplasmically synthesized chloroplast protein (6), is also synthesized as a precursor, and a likely candidate for this is the cell-free product RI, although this is yet to be confirmed by peptide analyses.

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