Uptake and Processing of the Precursor to the Small Subunit of Ribulose 1,5-Bisphosphate Carboxylase by Leucoplasts from the Endosperm of Developing Castor Oil Seeds

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ABSTRACT

Intact leucoplasts from the endosperm of developing castor oil seed were isolated by Percoll density gradient centrifugation. The precursor to the small subunit of ribulose 1,5-bisphosphate carboxylase from pea was synthesized in vitro from hybrid-selected mRNA. Leucoplasts imported this precursor by an ATP-requiring mechanism similar to that described in chloroplasts (AR Grossman et al. 1980 Nature 285: 625-628). The small subunit precursor was processed to a molecular weight that was identical with that of the mature pea small subunit. These results show that leucoplasts, though specialized for fatty acid biosynthesis and not photosynthesis, have a mechanism of protein import similar to that of chloroplasts.

The majority of chloroplast proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and then transported into the organelle (15). Several features of this transport have been identified. Proteins are synthesized on free cytosolic ribosomes and are imported into the chloroplast posttranslationally by a process that is dependent on photosynthesis or ATP (18). The initial translation product is a precursor polypeptide of higher relative molecular mass than the mature protein (15). The precursors are processed to the mature size by a soluble stromal protease (26).

Of the many imported soluble proteins, only the uptake of the SSU2 of RuBPc has been studied in detail. The SSU, with a mature mol wt of 14,000, is synthesized as a precursor (p20) with an Mr of 21,000 (10, 20). The precursor binds to the chloroplast envelope in an energy-independent step. The subsequent import of the p20 into the chloroplast requires energy (11). The N-terminal peptide extension is removed during or very soon after import by a stromal protease that has a tightly bound metal ion (26).

The endosperm of developing castor oil seeds contains many small, nonphotosynthetic plastids called leucoplasts. The fatty acids required for synthesis of the high levels of triglycerides which are stored in this tissue are synthesized in these organelles from hexose imported from the cytosol. Leucoplasts have been shown to contain high concentrations of the enzymes required for conversion of hexose to fatty acids whereas enzymes involved in photosynthesis and starch synthesis are generally reduced or not detectable (13).

We are studying the development and regulation of fatty acid synthesis in oil seeds. The compartmentation of nuclear encoded leucoplast proteins is one aspect of this study. To determine the conditions for uptake of in vitro synthesized polypeptides into isolated leucoplasts we have exploited the fact that leucoplasts contain RuBPc although its function is unknown (5). Here we report the isolation of intact leucoplasts from developing endosperm and demonstrate the uptake and processing of the in vitro synthesized p20 polypeptide of *Pisum sativum* by them.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L. cv Little Marvel) were grown under a 23 h photoperiod of 100 μE m-2 s-1 of cool white fluorescent light at 20°C and 80% RH. They were harvested just prior to the first leaf unfolding. Castor oil plants (*Ricinus communis* L. cv Baker 296) were glasshouse grown under natural light supplemented with 16 h fluorescent light.

Materials. Sephacryl S-1000 superfine was obtained from Pharmacia; [35S]methionine (>1000 Ci/mmol) was from New England Nuclear and nuclease-treated rabbit reticulocyte lysate and oligo-dT-cellulose were purchased from Bethesda Research Laboratories.

Isolation of Leucoplasts. All procedures were carried out at 4°C. Endosperm was dissected from castor oil seeds (stages 5-6) (17) and ground in a mortar and pestle in homogenizing buffer (50 mM Hepes-KOH, pH 7.5, containing 0.4 mM sorbitol, 2 mM EDTA, 1 mM MgCl2, 1 mM DTT, 1% BSA, and 1% Ficoll) for 1 min (2 ml buffer/g fresh weight tissue). The homogenate was filtered through four layers of cheesecloth and centrifuged at 500g for 5 min. The resulting supernatant was centrifuged at 10,000g for 10 min. The pellet was resuspended in approximately 5 ml of homogenizing buffer and layered over a discontinuous Percoll density gradient modified from the method of Bartlett et al (3). The gradient was composed of steps of a solution (PBF-Percoll) containing PEG-4000 (3% w/v), BSA (1% w/v), and Ficoll (1% w/v) in Percoll (all from Sigma). It consisted of 5 ml of 80% (v/v), PBF-Percoll, 7.5 ml of 35% PBF-Percoll, 7.5 ml of 22% PBF-Percoll and 7.5 ml of 10% PBF-Percoll all in 50 mM Hepes-KOH, pH 7.5, containing 0.4 mM sorbitol, 2 mM EDTA, and 1 mM MgCl2.

The gradient was centrifuged at 9,200g for 6 min in a Beckman JS-13 rotor. The band of plastids on the 35 to 22% Percoll interface was removed and diluted with 20 ml of resuspension buffer (50 mM Hepes-KOH, pH 8.0, containing 0.4 mM sorbitol, 1 mM MgCl2, and 1 mM DTT). The plastids were recovered by centrifugation at 10,000g for 10 min and resuspended in 0.50

1 Supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

2 Abbreviations: SSU, small subunit; RuBPc, ribulose 1,5-bisphosphate carboxylase/oxygenase; ADH, alcohol:NAD+ oxidoreductase; PMSF, phenylmethylsulfonyl fluoride.
ml of resuspension buffer.

**Isolation of Pea Chloroplasts.** Chloroplasts were isolated from pea seedlings by the method of Fish and Jagendorf (16) except that gradient tubes were centrifuged at 8,260g in a Beckman JS-13 rotor for 9 min. Total Chl was determined by the method of Arnon (1).

**Enzyme Assays.** The following enzymes were measured by previously described methods: RuBPC (25); fumarase (EC 4.2.1.2) (19); catalase (EC 1.11.1.6) (22); ADH (14); enolase (EC 4.2.1.11) (23). Protein was measured according to Bradford (7) with a Biorad assay kit using γ-globulins as standards.

**RNA Extraction and Enrichment for PolyA+ RNA.** All glassware used in extracting RNA was rinsed in 1 N NaOH, then in water that had been treated with 0.1% diethylpyrocarbonate and autoclaved. It was then baked at 180°C for at least 2 h. All media used to isolate RNA were treated with 0.1% diethylpyrocarbonate and autoclaved unless one of its components had a primary amine group. In these cases, the media were simply autoclaved.

Pea shoots were excised, immediately frozen in liquid N2 and ground to a powder with a mortar and pestle. The frozen powder was allowed to thaw after suspension in an emulsion of equal parts of extraction buffer (100 mM Tris-HCl (pH 8.0), containing 0.5% SDS, 25 mM EDTA, and 75 mM NaCl) and distilled phenol. The suspension was homogenized three times (30 s each) with a Polytron homogenizer and the phases were separated by centrifugation at 5000g for 5 min. The phenol phase was removed and re-extracted with an equal volume of extraction buffer. The aqueous phases were combined and extracted four times with equal volumes of phenol-chloroform (1:1). The aqueous phase was made 0.5 mg/ml in proteinase K and incubated at 37°C for 1 h after which the proteinase K was removed by phenol:chloroform (1:1) extraction. The RNA was then precipitated by adding 2 volumes of 95% ethanol and storing at −20°C overnight.

The precipitated material was recovered by centrifugation, dried under vacuum and dissolved in sterile deionized H2O. Sufficient 4 mM sodium acetate, pH 5.5, was added to bring the final concentration to 3 M. After incubation at −20°C for 30 min the precipitated RNA was collected by centrifugation. The sodium acetate precipitations were repeated four times. The final precipitate was dissolved in 1 ml of 0.1 M sodium acetate (pH 5.0) and the RNA precipitated overnight at −20°C after adding 2 volumes of 95% ethanol.

The RNA was dissolved in sterile water to which was then added 4.4 mM sodium acetate and 1 mM Tris-HCl, pH 7.5, to final concentrations of 0.4 M and 0.1 M, respectively. PolyA+ RNA was isolated from the total RNA by chromatography on oligo(dT)-cellulose according to Aviv and Leder (2) except that sodium acetate was used instead of KCl for washing the oligo(dT)-cellulose.

**Isolation by Hybrid Selection of mRNA Coding for the Precursor to Pea SSU.** The p20 cDNA cloned in pBR322 was a kind gift from Dr. R. J Ellis, University of Warwick, U.K. Its isolation and sequence have been described (4). Plasmid DNA was isolated (26), cleaved at its sole Eco R1 restriction endonuclease site and covalently bound to Sepharcl S-1000 via a diazobenzoyloxyethyl derivative (8). The Sephadcl was then preincubated in 2.4 M tetraethylammonium chloride, pH 8.0 for 72 h at 45°C (9). The column was prehybridized and mRNA was hybridized and eluted from the column according to Benemann and Westhoff (8) except that the scale was increased from 0.2 g to 3 g of column material. The volumes of wash buffers were increased accordingly. The purified p20 mRNA was dissolved in 40 μl sterile distilled H2O.

**In Vitro Translation of p20 mRNA.** Hybrid selected p20 mRNA was translated in a rabbit reticulocyte lysate in the presence of [35S]methionine according to the manufacturer's directions. The translation products were passed through a 7 by 1 cm Sephadex G-25 column equilibrated with either chloroplast or leucoplast resuspension buffer. This column was required to remove unincorporated [35S]methionine and low mol wt components of the translation system.

**Uptake of in Vitro Synthesized p20 into Isolated Plastids.** Products of in vitro protein synthesis (106 cpm per incubation) were incubated with leucoplasts (2.0 mg total protein) or chloroplasts (200 μg total Chl) for 20 min at room temperature with periodic gentle agitation. Where indicated, the suspensions were illuminated with 100 μE m−2 s−1 of incandescent light with periodic gentle agitation. Plastids were recovered by centrifugation, resuspended in 320 μl of resuspension buffer containing 62.5 μg/ml proteinase K and incubated on ice for 15 min. Plastids were pelleted, resuspended in 500 μl resuspension buffer contain-
Transport of Proteins into Castor Bean Leucoplasts

Isolation of intact, functional leucoplasts from endosperm of developing seeds. We have developed an iso-osmotic discontinuous gradient of Percoll for leucoplast isolation. Figure 1 shows the profiles of marker enzyme activities obtained from such a gradient. Leucoplasts were concentrated at the 22 to 35% Percoll interface as determined by the activity of the marker enzyme RuBPC. The major peak of fumarase activity (a mitochondrial marker enzyme) was well separated from the leucoplasts with less than 8% of the total fumarase activity recovered from the gradient co-sedimenting with the leucoplasts. The majority of the peroxisomal marker, catalase, migrated to a position slightly higher in the gradient than the mitochondria with less than 5% of the total catalase activity recovered from the gradient coincident with the leucoplast fractions. The minor peaks of contaminating organelles in the leucoplast fractions are probably due to aggregates of organelles.

These results show that Percoll density gradient centrifugation of castor bean endosperm homogenates yields a fraction that is highly enriched in leucoplasts.

Uptake of in Vitro Synthesized Pea p20 into Isolated Plastids. The mRNA coding for the precursor to pea SSU was purified by hybrid selection and translated in a rabbit reticulocyte lysate system in the presence of [35S]methionine. The major radioactive product was the p20 (Fig. 2, lane 1). An additional protein with an Mr of 45,000 was labeled to a varying degree in different translations but it was also synthesized in the reticulocyte translation system in the absence of added RNA (results not shown).

The radioactive p20 was taken up and processed to its mature size (Mr of 14,000) by intact pea chloroplasts in the light (Fig. 2, lane 2) and in the dark if 2 mM ATP was supplied (Fig. 2, lane 4). No p20 was taken up in the absence of ATP (Fig. 2, lane 3). It has been reported that some p20 can be taken up into chloroplasts in the dark without ATP (18, 27) but in these cases, the

Fig. 2. Uptake of in vitro synthesized pea small subunit precursor by isolated pea chloroplasts and castor bean endosperm leucoplasts. Pea p20 mRNA was translated in vitro in the presence of [35S]methionine and incubated with isolated pea chloroplasts (lanes 2, 3, 4) or castor bean endosperm leucoplasts (lanes 5, 6, 7) and analyzed by SDS-PAGE and fluorography. Incubation was for 20 min in the light (+) or dark (−) and presence (+) or absence (−) of 2 mM exogenous ATP as indicated. Lane 1 shows the total radioactive products of in vitro protein synthesis. The positions of the radioactive p20 and SSU polypeptides are indicated on the fluorogram.
Fig. 3. ATP requirement for uptake of *in vitro* synthesized pea small subunit precursor by isolated castor bean leucoplasts. Experimental conditions were as described in Figure 2, except that the exogenous ATP concentration was varied as indicated. A, Fluorogram of SDS-PAGE of total *in vitro* translation products (lane 1) and soluble extracts of leucoplasts after incubation with various concentrations of exogenous ATP. B, [³⁵S]Methionine activity in mature SSU as determined by excision of the appropriate bands from the gel (fluorogram of which is shown in (A)) and liquid scintillation counting.
ATP from the translation mixture was not removed prior to uptake and there may have been sufficient ATP remaining for some import of p20. In addition, chloroplasts may vary in their level of endogenous ATP, depending on the growth conditions of the pea seedlings from which they were isolated.

Leucoplasts also took up and processed the p20 when ATP was present (Fig. 2, lane 7). However, no p20 was taken up or processed in the absence of ATP in the light or dark (Fig. 2, lanes 5 and 6, respectively). This result indicates that protein uptake into plastids requires energy. It also suggests that leucoplasts are capable of transporting ATP. This is supported by the observation that exogenous ATP stimulates the incorporation of acetate into lipids by isolated leucoplasts (24).

The ATP concentration that saturated uptake of p20 into leucoplasts was determined. The products of uptake of p20 in various ATP concentrations were analyzed by SDS-PAGE and the amount of p20 that was taken up and processed was determined both by visually inspecting the fluorogram (Fig. 3A) and by determining the radioactivity in the excised gel band that contained the mature SSU (Fig. 3B). Increasing the ATP concentration up to 1.0 mM resulted in increased amounts of p20 being taken up and processed by the leucoplasts. The ATP concentration that was sufficient to saturate the uptake mechanism varied somewhat between experiments. In Figure 3, 1.0 mM ATP was not saturating but in other experiments, 0.5 mM ATP was saturating (results not shown).

Uptake and processing of p20 by leucoplasts stopped after 20 min (Figs. 4, A and B). This result is in contrast with that of Grossman et al. (18) who found the uptake of a mixture of pea precursor proteins into pea chloroplasts was linear for at least 1 h. In a separate experiment, after a 30 min incubation, 1.1% of the total radioactivity was recovered as imported mature SSU in intact, protease treated leucoplasts. We estimate that approximately 75% of the total radioactive material present in the incubation was p20. Thus the imported mature SSU recovered represented 1.5% of total p20 available. Since 50% of the methionine residues in p20 from pea are present in the transit peptide and are not recovered in the mature SSU (12), this represents import of 3.0% of available p20. The recovery of total leucoplast endolase activity was 9% after a mock incubation and protease treatment as described in "Materials and Methods." Using this value to correct for the recovery of intact leucoplasts we estimate that in excess of 33% of the available labeled p20 was imported. This value is lower than the 70% efficiency reported for homologous uptake into isolated chloroplasts (11). The use of heterologous p20 to study uptake by leucoplasts may contribute to the difference. It is also clear that isolated leucoplasts are more labile than isolated chloroplasts under the conditions reported here.

These results show that leucoplasts from developing castor bean endosperm are capable of transporting and processing the precursor to the SSU of RuBPC from pea leaves supplied with exogenous ATP. The mechanism of uptake appears to be similar in both leucoplasts and chloroplasts except that leucoplasts have an absolute requirement for exogenous ATP whereas chloroplasts can use either ATP or light. The uptake of a protein synthesized from castor bean RNA is now being examined to determine if proteins other than SSU use the same import pathway.

**LITERATURE CITED**


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