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Protein Transport in Intact, Purified Pea Etioplasts

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We have developed a method to isolate intact, purified pea etioplasts. These etioplasts were capable of recognizing, transporting, and processing the precursor form of the small subunit of the ribulose-1,5-bisphosphate carboxylase, a protein which is not detectable at this developmental stage. Transport of proteins was completely dependent on ATP and could not be substituted for or stimulated by light. The transported precursor protein was processed to its proper molecular weight. The mature form of the small subunit was assembled with the large subunit of the ribulose-1,5-bisphosphate carboxylase already present at this stage to form an oligomer. Protein transport was completely abolished using the phosphatase inhibitor sodium fluoride. This is the first time protein transport has been demonstrated in isolated, purified etioplasts. © 1986 Academic Press, Inc.

The development of chloroplasts requires the coordinated expression of two distinct genetic systems. A great number of chloroplast proteins are coded for in the nucleus while others are coded for on plastid DNA itself (1). Holocomplexes of enzyme proteins exist whose assembly depends on subunits coded for and synthesized in different cell compartments. Ribulose-1,5-bisphosphate carboxylase is an example of such an enzyme, composed of two subunits: the large subunit (LSu),[2] molecular weight of about 52,000, coded for and made within the plastid (2, 3), and the small subunit (SSu), molecular weight of about 14,000, coded for in the nucleus and made on cytoplasmic ribosomes in a precursor form (pSSu) (2, 3). The precursor form of the SSu is post-translationally transported across the chloroplast envelope (4), cleaved by a specific protease into the mature form (5), and assembled into the holoenzyme in the soluble chloroplast matrix. Chloroplast differentiation depends strongly on light in higher plants. Thus if plants are grown in the dark, special forms of plastids are developed, e.g., etioplasts, which can be transformed to chloroplasts by bringing them into the light. This phenomenon is widely used to study the different events which are necessary until a fully competent chloroplast is made. Many reports have clearly demonstrated the effect of light on the transcriptional level (for a review see (6)), e.g., the amount of translatable mRNA for pSSu increases dramatically in the light.

Less is known at the translational or post-translational level. Transport of cytoplasmically synthesized proteins and their assembly into holocomplexes is a post-translational event and a promising target for regulation. In fact Grossmann *et al.* (7) proposed the influence of light on the transport in intact chloroplasts. Intact chloroplasts have been the only tool for studying protein transport until now, where protein transport refurbishes for protein turnover in the organelles. The de-

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[2] Abbreviations used: LSu, large subunit of ribulose-1,5-bisphosphate carboxylase; SSu, small subunit of ribulose-1,5-bisphosphate carboxylase; pSSu, precursor form of SSu; Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; DTT, dithiotreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS or LDS, sodium or lithium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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velopmental steps leading from an etioplast to a chloroplast require massive protein transport from the cytoplasm into the changing plastid. In order to understand the processes occurring during plastid development we have worked out a method to isolate purified, intact etioplasts from peas and studied the etioplasts' capacity for protein transport and assembly as an initial step in etioplast transformation to mature chloroplast.

EXPERIMENTAL PROCEDURES

Isolation of purified intact plastids. Etioplasts or chloroplasts were isolated from pea plants (*Pisum sativum* var. *miranda*) grown on vermiculite for 11 days in the dark or in the light, respectively. Chloroplasts were isolated in a medium containing 330 mM sorbitol, 30 mM 4-morpholinepropanesulfonic acid (Mops), 0.1% bovine serum albumin, and 0.4 mM $MgCl_2$; pH was adjusted to 7.2 by adding solid Tris. A crude chloroplast pellet was further purified by isopycnic centrifugation in Percoll gradients (8). The purified intact chloroplasts were then used in the transport experiments. Etioplasts from dark-grown pea shoots were obtained from the leaves only. The leaves were harvested under dim green safe light into chilled buffer and homogenized with a specially equipped blender (9, 10) in a medium containing 500 mM sucrose, 30 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), 1 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin; pH was adjusted to 7.2 with NaOH. The homogenate was filtered through six layers of cheesecloth and one layer of nylon gauze (30-μm aperture). A crude pellet was obtained after centrifugation for 10 min at 1000*g*. The etioplasts were further purified by centrifugation on a preformed Percoll gradient for 15 min at 6500*g*. The Percoll gradient was made from 5 ml 80% Percoll in isolation medium overlaid by 20 ml 30% Percoll in isolation medium; the gradient was then preformed for 45 min in a vertical rotor at 10,000*g*. Intact, purified etioplasts were recovered from the interface of the linear gradient and 80% cushion and were washed free of Percoll with isolation buffer and subsequently used for transport experiments.

Contamination by mitochondria and peroxisomes was tested using cytochrome-*c* oxidase and hydroxypyruvate reductase, respectively, as marker enzymes (11). Intactness of purified etioplasts was judged using two different methods, (i) phase contrast microscopy and (ii) purified etioplasts were washed free of Percoll, divided into identical aliquots, and recovered by centrifugation. The plastids were resuspended in buffer containing different concentrations of sucrose and left on ice for 15 min to allow breakage in hypotonic buffer solution. The etioplasts were pelleted again and the latency of LSu was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, see below) followed by Coomassie brilliant blue stain and a subsequent scan at 583 nm (Vitatron densitometer, TLD 100). Results were quantified using a Vitatron integrator.

Isolation of poly(A)RNA. Total RNA was isolated from peas which had been grown for 4 days in the dark and then transferred to continuous light for 60 h prior to RNA isolation. RNA was purified by centrifugation through a CsCl gradient containing guanidinium rhodanide as described in (12). Poly(A) RNA was enriched by oligo(dT)-cellulose chromatography and after repeated precipitation with ethanol stored in H_2O at −196°C (12).

In vitro translation and uptake experiments. Poly(A) RNA was translated in a cell-free wheat germ system (prepared as in (13)), containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-KOH (pH 7.6), 100 mM KCl, 3.5 mM Mg acetate, 1 mM ATP, 20 μM GTP, 2 mM dithiothreitol (DTT), 8 mM creatine phosphate, 25 μM each of the amino acids (minus methionine), 4 μg creatine kinase, 100 μCi [^{35}S]methionine (sp act 800 Ci/mmol) and 4 to 5 μg of poly(A) RNA in a final volume of 100 μl. After incubation at 25°C for 90 min the translation mixture was centrifuged at 140,000*g* for 1 h. The postribosomal supernatant was used for uptake experiments. In some cases proteins from the postribosomal supernatant were precipitated by adding solid $(NH_4)_2SO_4$ (52 mg/100 μl); precipitation was allowed to complete for 30 min at 4°C. The pellet recovered after centrifugation at 140,000*g* for 30 min was dissolved in 50 mM Hepes-KOH (pH 8.0) and dialyzed against the same buffer for 3 h. The dialyzed protein solution was then used in the same manner for uptake experiments as above. The uptake mixture (final volume 300 μl) contained 3 mM $MgSO_4$, 10 mM methionine, 26 mM K gluconate, 2% bovine serum albumin, 300 mM sorbitol, 6.6 mM ATP (unless otherwise indicated), 50 mM Hepes-KOH (pH 8.0), 30 μl of postribosomal supernatant derived from the translation mixture, and intact plastids (equivalent to about 1 mg of protein). Experiments were carried out in the dark. All samples were incubated at 25°C for 30 min. Following the incubation, transport was halted by spinning the plastids down for 4 min at 12,000*g*. Unbound proteins were removed by subsequent washing in isolation buffer and centrifuging the plastids as stated above. The etioplasts or chloroplasts, respectively, were then lysed on ice by resuspending them in 10 mM Tricine-KOH (pH 7.9) and 4 mM $MgCl_2$. The broken plastids were separated into a soluble extract fraction and a membrane fraction, containing both thylakoid and envelope membranes, by centrifugation for 5 min at 12,000*g*. The fractions were then analyzed by PAGE.

To verify protease-resistant transport into the organelles, the etioplasts were treated with thermolysin

(100 μg/ml) (14). Etioplasts were resuspended after the washing procedure described above in 50 mM Hepes-KOH (pH 8.0), 300 mM sorbitol, 0.5 mM $CaCl_2$ and treated with the protease for 30 min at 4°C. The reaction was stopped by the addition of 10 mM EDTA and the etioplasts were recovered and washed as above, except that 5 mM EDTA was present throughout. The etioplasts were lysed and prepared for electrophoresis as above.

Transport experiments were quantified by PAGE (see below). Aliquots of post-translational supernatant and from the transport experiments were electrophoresed on the same polyacrylamide gel. The pSSu and SSu were located exactly on the gel by autoradiography, the spots were excised from the gel rehydrated in H_2O, and the radioactivity was recovered by treatment of the gel slices with 0.2 M NH_3 in 30% H_2O_2 for 48 h at 37°C. The gel pieces were by then completely dissolved and scintillation fluid (Rotizint 22, Zinsser, FRG) was added and radioactivity was quantitatively determined using a scintillation counter (Kontron, Betamatic). Specific activity of SSu was calculated from the published sequence in (15). The amount of SSu bound to LSu after transport was determined by two-dimensional gel electrophoresis (see below) of one half of the transport assay, while the second half of the transport assay was analyzed by SDS-PAGE only and taken as 100% transport. The radioactivity was quantified as above.

Polyacrylamide gel electrophoresis and autoradiography. All samples were analyzed on a SDS-polyacrylamide gel containing a 7.5–15% linear acrylamide gradient and 1% bisacrylamide. Proteins were solubilized in 2.5% lithium dodecyl sulfate (LDS), 12% sucrose, 70 mM DTT, and 60 mM Na_2CO_3. Electrophoresis was done essentially as described by Delepelaire *et al.* (16). Stained polyacrylamide gels were fluorographed according to Bonner and Laskey (17) and autoradiographed using an intensifying screen (Agfa-Gevaert MR 600) at −80°C. Two-dimensional gel electrophoresis was according to Smith and Ellis (5). The first dimension was a nondenaturing gel which was cast as a 5–10% linear gradient gel using 0.15% bisacrylamide. Once the separation gel polymerized it was turned 90° and the stacking gel was poured, thus creating a gel with a horizontal acrylamide gradient, 5% polyacrylamide on the left side to 10% on the right side of the slab gel. The gel was prerun and run as in (5), stained and destained, and the spots containing carboxylase protein were cut out and run in the second dimension under denaturing conditions in the SDS-polyacrylamide gel system described above. The exact position of carboxylase protein in the nondenaturing gel was determined using Western blot analysis and specific antibodies (14). The antibodies were raised in rabbits against the carboxylase holoenzyme from spinach (gift of Professor Oesterhelt, Munich). Autoradiography was as above.

RESULTS

In order to study protein transport into etioplasts a method had to be developed to isolate these organelles in an intact and highly purified state. We choose to work with pea plants because they are easily grown in the dark and many protein transport studies into chloroplasts from pea have been described in the literature (2–4). The isolation was achieved by a combination of a special blending technique (10) and purification of the intact etioplasts on a linear silica sol gradient preformed from 30% Percoll. Intactness of the purified etioplasts was about 95% as judged by phase contrast microscopy and higher than 80% by determining the latency of the LSu in different hypotonic buffer solutions (Fig. 1). The purity as examined by marker enzyme distribution (Table I) and low-magnification electron microscopy (Fig. 2) was high. The yield of intact, purified etioplasts, which are a prerequisite for transport studies, was between 30 and 40 mg protein/100 g fresh wt and is comparable to that of chloroplasts from spinach, 25–45 mg/100 g fresh wt (18). Purified etioplasts contained only protochlorophyllide and no chlorophyllide or chlorophyll was detected.

Addition of poly(A) RNA to a wheat germ cell-free system stimulated the synthesis of a large number of proteins. Of these the pSSu and the precursor of the light harvesting complex apoprotein are

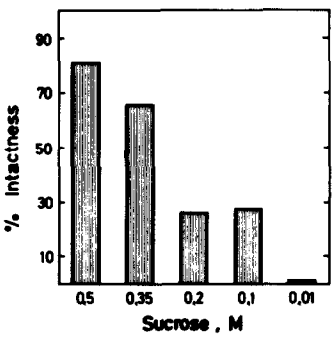


FIG. 1. Latency of stromal proteins as determined for LSu. Etioplasts were resuspended in buffer of different osmolarity as indicated and left on ice. Intactness of etioplasts was then determined as described under Experimental Procedures.

TABLE I

PURIFICATION OF PEA ETIOPLASTS

Fraction	Volume (ml)	Cytochrome-*c* oxidase total activity		Hydroxypyruvate reductase total activity	
		μmol/min	μmol/mg min	μmol/min	μmol/mg min
Crude pellet	10	2745	25.3	2.42	0.022
Top layer Percoll gradient	2	1220	36	0.66	0.0197
Broken etioplasts	4	1028	33.2	0.51	0.0166
Purified, intact etioplasts	1	6.4	0.74	0.005	0.0006

predominant products. In the present study we examined the uptake of pSSu into the etioplasts in an *in vitro* system. Experiments were done in the dark in the presence of ATP. As shown in Fig. 3, lane b/b' pSSu is readily taken up and processed to

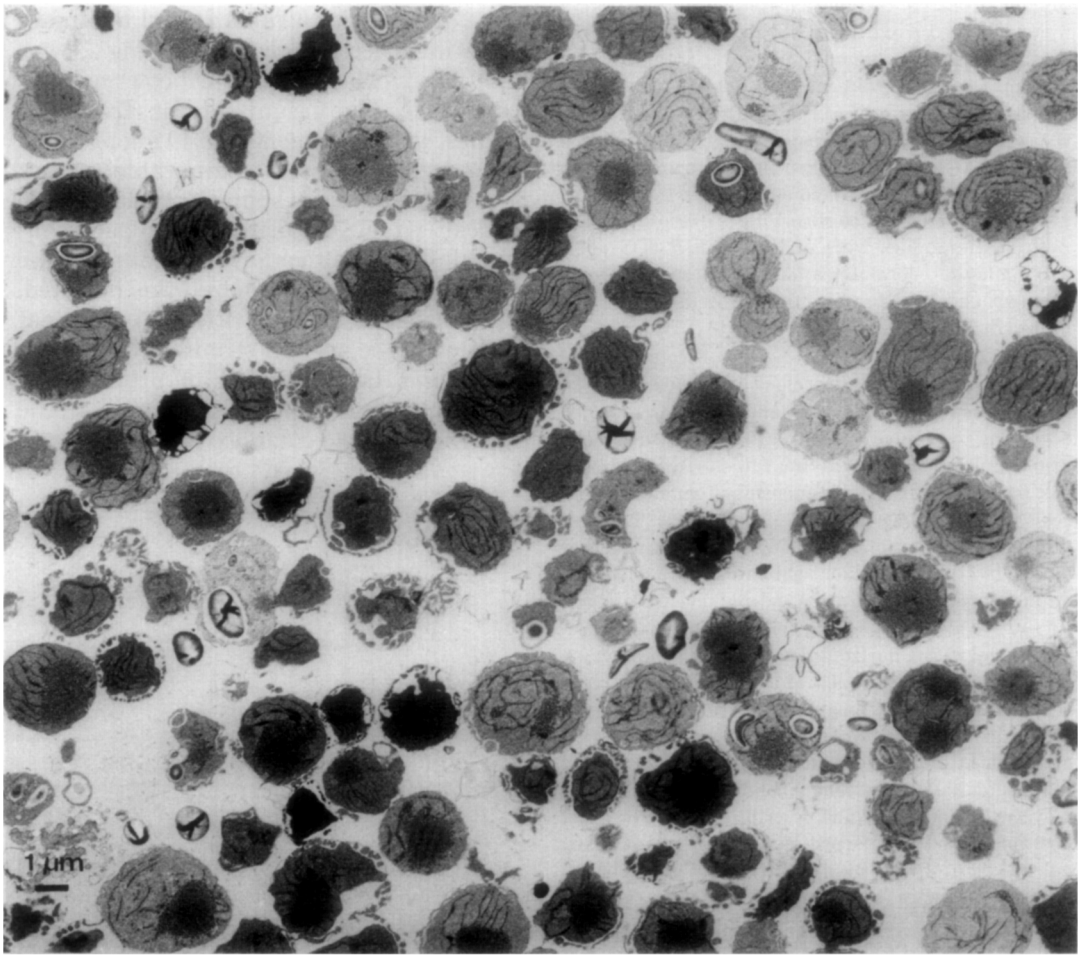


FIG. 2. Electron microscope view of purified, intact pea etioplasts (×3600).

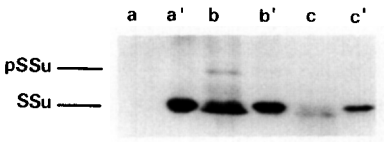


FIG. 3. Autoradiograph of transport and processing of pSSu in chloroplasts and etioplasts. Intact chloroplasts (equivalent to 105 μg chlorophyll, lanes a and a') and intact etioplasts (equivalent to 960 μg protein, lanes b and b') were assayed for protein transport using in vitro translation products of poly(A)RNA from peas. After completion of the experiments plastids were separated into a membrane fraction (lanes a–c) and a soluble fraction (lanes a'–c'). Etioplasts were treated with thermolysin to assay for protease resistent transport (lanes c and c').

the apparent size of the mature form. Identical results are obtained with pea chloroplasts (Fig. 3, lane a/a'). In both cases the processed form has the same molecular weight of about 14,000 while pSSu has a molecular weight of 20,000. To facilitate our analysis the plastids were separated, after the transport experiments had been completed, into a membrane fraction and a soluble fraction. The membranes contained the prolamellar bodies, prothylakoids, and almost all the envelope membranes on which the pSSu binds to specific receptor proteins (4, 19). The soluble supernatant contained the plastid extract and the processed form of the SSu. These initial data demonstrate that etioplasts are capable to bind, transport, and process pSSu. pSSu was selectively bound to the envelope membrane and transported into etioplasts; only very few other proteins were bound or transported and SSu always represented more than 80% of the label in the soluble phase (compare Fig. 7C). Throughout all experiments when protein transport was compared in chloroplasts and etioplasts a higher amount of pSSu was bound to the envelope membranes of etioplasts (compare Fig. 3, data not shown).

Protease treatment of etioplasts with thermolysin showed that the SSu became protease-resistant after completion of the transport experiment (Fig. 3, lane c'), meaning that SSu had completely entered the etioplasts, while pSSu disappeared from the membrane fraction (Fig. 3, lane c), indicating that the majority of pSSu was bound to the membrane. Between 20 and 35% of the total label present in pSSu in the post-translational supernatant was transported into etioplasts and processed. If the results are compared on a protein basis transport in etioplasts was about twice as efficient as in chloroplasts; e.g., 5.6 $\times 10^6$ molecules SSu/μg protein h in comparison to 3.0 $\times 10^6$ molecules SSu/μg protein h were transported in etioplasts and chloroplasts, respectively.

Transport is rapid and good amounts of mature SSu can already be detected after 3 min (Fig. 4). The initial velocity continues almost for 30 min as can be seen in Fig. 4; longer time courses showed that protein transport continues beyond this time at rates not quite as fast as in the initial phase (data not shown). So 30 min was chosen as a general time for transport experiments.

Others (7, 19) have demonstrated that exogenously added ATP can promote

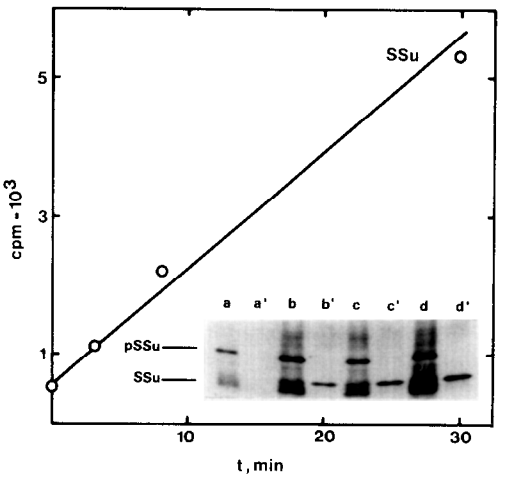
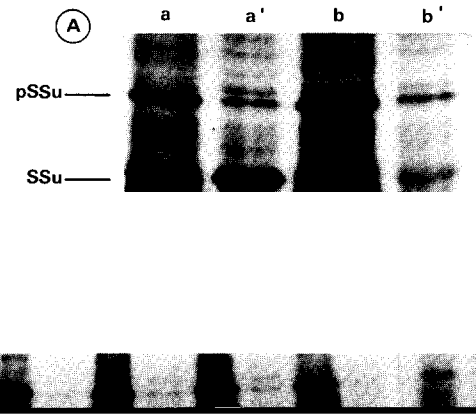


FIG. 4. Time-course study of protein transfer in purified, intact pea etioplasts. Etioplasts (equivalent to 880 μg protein) were used to study the rate of transport at different times (0, 3, 8, 30 min; insert lanes a–d, respectively). Etioplasts were separated into a membrane fraction (lanes a–d) and a soluble fraction (lane a'–d') to analyze for binding and processing, respectively. Samples were analyzed by SDS-PAGE with subsequent autoradiography and quantitative determination of radioactivity by liquid scintillation counting.



GTP concentrations in lanes b and b' are about 0.1 mM resulting from the *in vitro* translation mixture. (B) $(NH_4)_2SO_4$-precipitated translation products were used to fully elucidate the ATP concentration dependence. Etioplasts (equivalent to 1.1 mg protein) were used in the dark with various amounts of ATP: lanes a–e contained 0, 0.1, 0.3, 0.6, and 2 mM ATP, respectively. Lanes f and f', protein transport was assayed in the light in the absence of ATP.

elucidate the ATP requirement *in vitro* translation products were precipitated with $(NH_4)_2SO_4$ in order to remove ATP

salt prior to the assay. Various amounts of ATP were then exogenously added to the

pSSu transport (Fig. 5B, lane f') while illuminated chloroplasts provide enough ATP via photosynthesis to maintain pro-

plied.

While ATP is necessary for protein transport, sodium fluoride is inhibitory

smaller, probably because of conforma-

under the conditions used (see Experimen-