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Communication

Preparation of Chlamydomonas Chloroplasts for the *in Vitro* Import of Polypeptide Precursors[1]

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ABSTRACT

To study the import of polypeptide precursors we have adapted and compared two procedures for the isolation of competent chloroplasts from the green unicellular alga, *Chlamydomonas reinhardtii*: silicasol gradient centrifugation and elutriation. The chloroplasts actively import the precursor of the small subunit of ribulose bisphosphate carboxylase-oxygenase *in vitro*.

A majority of the proteins in chloroplasts and mitochondria are encoded by nuclear genes, translated as precursors in the cytoplasm, and imported and processed in the organelles. The precursors contain an amino-terminal extension, the transit peptide, which contains information that is sufficient to promote the import of the attached proteins (10, 20). Transit peptides of chloroplasts and mitochondria share a number of common features such as the abundance of serines and threonines, and an excess of positively charged residues over acidic ones. The primary sequences are overall quite divergent, although chloroplast transit peptides do share short blocks of sequence similarity (14, 17). Some transit peptides are composite and also contain information for the localization of the proteins in suborganellar compartments (9, 13, 21). While the structure and role of the transit peptides have been thoroughly investigated, little is known about the components of the transport machinery presumably located in the organellar envelope (6, 18). The basis for the specific sorting of the chloroplast and mitochondrial precursors to their respective compartments in plant cells is poorly understood (12).

In vitro transport assays have been developed with preparations of vascular plant chloroplasts (5, 11) and have been very useful to elucidate the role of the transit peptide. Here we present a protocol for the preparation of transport competent chloroplasts from the unicellular alga, *Chlamydomonas reinhardtii*. The preparation of intact chloroplasts from *C. reinhardtii* is hindered because the cells are small and contain only a single cup-shaped chloroplast that occupies roughly half of the volume bounded by the cell wall. It is, therefore, convenient to start from a cell-wall deficient mutant (cw15) and to use a gentle lysis procedure, such as the Yeda press at low pressure (16). Intact chloroplasts can thus be recovered which are competent for the *in vitro* import of radiolabeled precursor polypeptides (3, 4). We have compared two approaches for their isolation: silicasol gradient centrifugation and elutriation (19, 24).

MATERIALS AND METHODS

Percoll and Ficoll 400 were obtained from Pharmacia; BSA A7030 from Sigma, polyethylene glycol 4000 from Serva, proteinase K from Merck, linear polyacrylamide from Aldrich, *Escherichia coli* RNA polymerase from Genofit, (^{35}S)methionine from Amersham, and wheat-germ lysate was the gift of Dr. E. C. Hurt.

Chloroplast Isolation

Chlamydomonas reinhardtii cw15(mt−) (7) were inoculated at $4 \cdot 10^4$ cells/mL in 1.5 L high salt minimal medium (23) with magnetic stirring and bubbling with a mixture of 5% CO_2 in air, and grown under cycles of 12 h light (5000 lux) and 12 h darkness at 25°C for 4 d to a density of approximately 10^6 cells/mL. The algae were collected and washed two times with 20 mM Hepes-KOH (pH 7.5) by centrifugation at 4000*g* for 5 min at room temperature. They were resuspended at a concentration of $5 \cdot 10^7$ cells/mL in breaking buffer ([24]; 300 mM sorbitol, 50 mM Hepes-KOH, 2 mM EDTA, 1 mM $MgCl_2$, 1% BSA, pH 7.2) at room temperature. The cells were rapidly chilled in ice/water, loaded in the ice-cold Yeda press, equilibrated for 3 min to a pressure of 4 bar with N_2, and lysed quickly (2, 16). The lysate was loaded on 25 mL linear Percoll gradients (10–80% Percoll, 0.9 × GR buffer [*i.e.* 300 mM sorbitol, 45 mM Hepes-KOH, 0.9 mM $Na_4P_2O_7$, 1.8 mM EDTA, 4.5 mM $MgCl_2$, 4.5 mM $MnCl_2$, pH 6.8], 5 mM isoascorbate, 0.6 mM GSH, 0.3% PEG 6000, 0.1% Ficoll 400, 0.1% BSA) prepared according to Price and Reardon (19). They were centrifuged at 8000*g* for 20 min at 0°C. The chloroplasts (sometimes partly aggregated) at the lower part of the tubes were collected, diluted approximately fourfold in cold breaking buffer and gently pelleted by accelerating the centrifuge to 5000 rpm and immediately stopping. They were resuspended gently in 1 mL of HepKS buffer (50 mM Hepes-KOH [pH 8.0], 0.3 M sorbitol) and the Chl concentration was measured (1) and adjusted to 0.15 mg/mL. The yield from 10^9 cells was 300 to 500 μg Chl.

Alternatively, for centrifugal elutriation (24) the lysate from the Yeda press was diluted twofold in breaking buffer and

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loaded in the elutriation rotor (JE-6B, Beckmann Instruments) at 4000 rpm with a flow rate of 12 mL/min. They were washed with 70 mL breaking buffer and then eluted in 70 mL at a flow rate of 23 mL/min. The chloroplasts were pelleted gently and resuspended in HepKS buffer as above.

Preparation of Labeled Precursor

The cDNA plasmid CS2.1 (8) encodes the precursor of the small subunit of Rubisco[3] (pSS). It contains the entire coding sequence together with 22 bases upstream of the AUG start codon and the 3'noncoding trailer with the polyA tail. This cDNA fragment was inserted, using the *PstI* sites in its linkers (sequence: CTGCAGGA), into the *PstI* site of plasmid pDS6 (22) and was cloned in *E. coli*. The resulting plasmid, called PM1, was transcribed *in vitro* with *E. coli* RNA polymerase; the optimal ratio of enzyme to DNA was determined for each preparation. The resulting mixture containing capped pSS mRNA was directly added to nuclease-treated wheat-germ extract for *in vitro* translation to produce (^{35}S)methionine labeled pSS (22).

Transport Assay

The translation mixture was diluted 10-fold in 100 mM K-acetate, 20 mM Hepes-KOH (pH 7.6), 25 mM L-methionine, 3mM dithiothreitol, 3 mM Mg-acetate. For *in vitro* transport, the assay contained 100 μL diluted labelled pSS and 200 μL of chloroplasts in siliconized glass tubes and was incubated for 20 min at 25°C with rotary agitation under 1500 lux from fluorescent lights, or in the dark wrapped in foil. The samples were chilled on ice, diluted with 1.8 mL HepKS buffer and the chloroplasts were gently pelleted as before, washed with 1.8 mL HepKS buffer, and finally resuspended in 320 μL HepKS buffer. Aliquots (80 μL) were taken for the appropriate proteinase K (6 μg/mL) and Triton X-100 (0.5%) treatments for 30 min on ice and for Chl determination. The reactions were stopped by the addition of 25 μL HepKS buffer containing 10 mM benzamidine-HCl and 50 mM ACA, 2.5 μL of 100 mM PMSF in ethanol (1) and 100 μL of 20% (w/v) TCA. After 30 min on ice, the precipitates were recovered by centrifugation (13,000*g* for 10 min), washed with 80% acetone, briefly dried and resuspended in 75 μL sample buffer (12% sucrose, 1.5% SDS, 50 mM Tris-HCl [pH 6.8], 1% β-mercaptoethanol, 1 mM benzamidine-HCl, 5 mM ACA, 1 mM PMSF). They were heated to 100°C for 1 min, resuspended thoroughly, and heated for 3 min. Aliquots (35 μL) were loaded on 15% polyacrylamide slab gels containing 0.3% linear polyacrylamide with the SDS buffer system of Laemmli (15). After electrophoresis, the gels were fixed in boiling 5% TCA for 15 min, washed with water, neutralized with 50 mM Tris-HCl (pH 7.5), soaked in 1 M Na-salicylate for 45 min, and dried for fluorography using preflashed x-ray film. For scintillation counting, the bands were excised and dissolved for 24 h at 50°C in 0.2 mL perchloric acid (70%) plus 0.4 mL H_2O_2 (30%). For each band the background was subtracted by counting an adjacent area of the same lane.

[3] Abbreviations: Rubisco, ribulose bisphosphate carboxylase/oxygenase; SS, small subunit of Rubisco; pSS, precursor to the SS; ACA, epsilon-amino-*n*-caproic acid; PMSF, phenylmethylsulfonylfluoride.

RESULTS AND DISCUSSION

Chloroplast Isolation

In initial experiments, we found that lysis was more reproducible starting from a homogeneous population of cells. The algae were therefore grown under a light-dark cycle in minimal medium supplemented with CO_2, and harvested 1 h after the beginning of the light period. We have not compared cells harvested at other times in the cycle.

The lysis step is critical for the yield of intact chloroplasts. Rapid chilling of the cells and quick lysis with the Yeda press are important in this respect (2). The lysis can be monitored with the light microscope under phase contrast: intact chloroplasts are bright and refringent while broken ones are dull and darker. The proportion of intact chloroplasts varied in the range of one-third to two-thirds. Following silicasol gradient centrifugation (19), a diffuse band of mostly intact chloroplasts was obtained in the lower part of the tubes (roughly 90% intact), while broken material stayed in the upper part. The bands were, however, not as sharp or well separated as with spinach chloroplasts in analogous preparations. The chloroplasts in the lower part of the gradients were collected and washed by centrifugation, and used in the transport assays.

Transport Assay

In these experiments the substrate for transport was the precursor of the small subunit of Rubisco (pSS). A full length cDNA fragment encoding the pSS inserted in plasmid pDS6 downstream of a strong promoter was transcribed with *Escherichia coli* RNA polymerase and the mRNA was translated in a wheat germ extract in the presence of (^{35}S)methionine (8, 22). Chloroplasts and the labeled precursor were incubated for 20 min in moderate light to minimize aggregation of the chloroplasts. Longer incubations or stronger light resulted in more severe clumping. The integrity of the chloroplasts was preserved better after incubation at pH 8.0 than at pH 7.5. Increasing the Mg^{2+} concentration from 1. to 2.5 mM relieved aggregation, but was inhibitory for transport. After the incubation, the chloroplasts were washed to remove unbound precursor, and some aliquots were treated with proteinase in the absence or presence of detergent. They were analyzed by SDS polyacrylamide gel electrophoresis and fluorography (Fig. 1, lanes 7–12). The precursor is actively imported into the chloroplast according to the following criteria: (a) light-dependent uptake (lane 11 *versus* lane 8); (b) processing; (c) protection of the processed form against externally added protease (lane 11); and (d) reversal of this protection by detergent lysis of the chloroplasts (lane 12). Approximately 5 to 15% of the input precursor was recovered inside the chloroplasts (Table I), but the transport efficiency varied with different preparations.

Elutriation

As an alternative method we have also used elutriation to prepare the chloroplast fraction (24). In this procedure, particles are separated in the chamber of an elutriation centrifuge rotor based on the equilibrium between their rate of sedimentation and the counter-flow of buffer in the chamber. This method also yielded chloroplasts that were competent in the

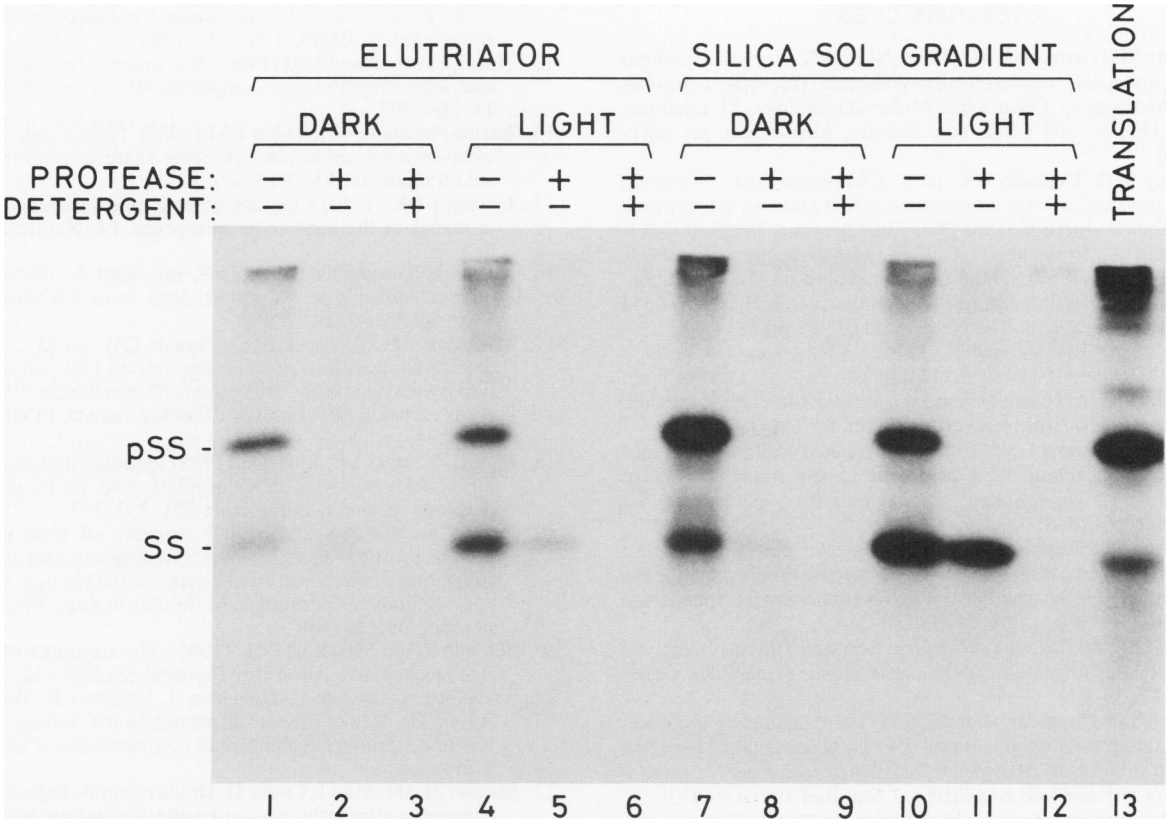

Figure 1. *In vitro* import into isolated *C. reinhardtii* chloroplasts. Chloroplasts were prepared by silicasol gradient centrifugation (lanes 6–12) or centrifugal elutriation (lanes 1–6). They were incubated with radioactive pSS of Rubisco in the dark or in the light as indicated. After washing, aliquots were treated in the presence (+) or absence (−) of protease and detergent as shown, and fractionated by SDS polyacrylamide gel electrophoresis. A fluorogram of the gel is shown. Lane 13, products of the *in vitro* translation (15% of the amount used in the assays, mixed with chloroplasts on ice). The minor lower band migrating slightly faster than the SS and the large mol wt components are unidentified contaminants from the *in vitro* transcription/translation.

Table I. *Quantitation of pSS Import*

Transport assays were performed with chloroplasts prepared by silicasol gradient centrifugation as described in Figure 1. The bands corresponding to input pSS and to imported protease-protected SS were excised from polyacrylamide gels and the radioactivity measured by scintillation counting. Import is expressed as the ratio of imported SS to input pSS corrected for Chl recovery and for the number of methionine residues in pSS (9) and SS(7).

	Input		Recovered		Import
	pSS	Chl	SS	Chl	
	cpm	*μg*	*cpm*	*μg*	%
Experiment 1	5500	29	130	16	5.6
Experiment 2	4300	26	330	18	14

transport assay (Figure 1, lanes 1–6). The preparation contained intact and broken chloroplasts in proportions that apparently reflected those in the initial lysate. The transport activity (relative to total chlorophyll) was thus much less than with the silicasol gradient fraction which is enriched for intact chloroplasts.

CONCLUSION

Our preparations actively sequester the processed form of the SS of Rubisco into a membrane bound compartment, and the reaction is light dependent; this indicates import of the pSS into the chloroplasts and processing. Silicasol gradient centrifugation does not require special equipment and appears as the method of choice; it provides a fraction that is enriched for intact chloroplasts and is more active than the elutriator preparation for *in vitro* import. The assay should be useful for further studies in *Chlamydomonas reinhardtii* of the mechanism of import, of the components that are involved and of how proteins with transit peptides are sorted between the chloroplasts and the mitochondria of plant cells. *C. reinhardtii* offers the advantage of being readily amenable to genetic analysis, in particular because photosynthesis is dispensable for growth on acetate as a source of reduced carbon. It may thus be possible to isolate mutants that are affected in components of the transport machinery. The *in vitro* import assay should also be useful for their characterization.

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