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Cryopreservation of Chloroplasts and Thylakoids for Studies of Protein Import and Integration[1]

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ABSTRACT

A method is presented for preservation of isolated intact chloroplasts and isolated thylakoids for use in chloroplast protein import and thylakoid protein integration studies. Chloroplasts of pea (*Pisum sativum*) were preserved by storage in liquid nitrogen in the presence of a cryoprotective agent. Dimethyl sulfoxide was the most effective of several cryoprotectants examined. Approximately 65 to 70% of chloroplasts stored in liquid nitrogen in the presence of dimethyl sulfoxide remained intact upon thawing and were fully functional for the import of precursor proteins. Imported proteins were correctly localized within these chloroplasts, a process that for two of the proteins tested involved transport into the thylakoids. Lysate obtained from preserved chloroplasts was functional for protein integration assays. Preserved chloroplasts retained import and localization capability for up to 6 months of storage. Thylakoids were preserved by a modification of a method previously described (Farkas DL, Malkin S [1979] Plant Physiol 64: 942–947) for preservation of photosynthetic competence. Preserved thylakoids were nearly as active for protein integration studies as freshly prepared thylakoids. The ability to store chloroplasts and subfractions for extended periods will facilitate investigations of plastid protein biogenesis.

Recent advances in our understanding of the mechanisms of chloroplast protein biogenesis derive primarily from the availability of *in vitro* biochemical assays for chloroplast protein import and *in organello* protein synthesis. Active chloroplasts are prepared for these assays by homogenization of fresh tissue followed by a combination of differential and density gradient centrifugation (25). Chloroplasts are then incubated under conditions that either promote synthesis of proteins within the organelle (3, 17) or allow the import of cytoplasmically synthesized plastid proteins into the organelle (16). Isolation of chloroplasts from amenable plant species, such as spinach and peas, is not difficult but requires about two intensive hours from start to finish. This preparation time has become a limiting factor as investigations of transport mechanisms have become more sophisticated and the duration of experiments has increased. A ready source of preserved but active chloroplasts would greatly facilitate such studies. Furthermore, the ability to preserve active chloroplasts for extended periods will obviate the problem of seasonal variation in import competence of chloroplasts and permit investigators to comparatively analyze plastids isolated from different plant tissue at different times.

Recent studies of plastid protein biogenesis have also focused on stromal factors necessary for assembly of thylakoid proteins (7, 8, 14). Purification schemes for the stromal factors require the reconstitution with active thylakoids for activity assays. Such assays are frequently conducted 1 d or several days after the original isolation of stroma and require the isolation of fresh chloroplasts for thylakoid preparation. Accordingly, it would be very helpful if thylakoids from the original chloroplast preparation could be frozen for later use.

Methods for preservation of photosynthetically competent thylakoids have been available for several years (12, 19, 20), but until now, methods for preserving intact chloroplasts have not been described. The present communication describes protocols for the preservation of intact chloroplasts and thylakoids that are active for subsequent studies of protein import and integration, respectively.

MATERIALS AND METHODS

Materials

Tritium-labeled leucine was purchased from Du Pont-New England Nuclear. RNasin and SP6 polymerase were from Promega Biotech. Miracloth was from Behring Diagnostics. Mg-ATP, thermolysin, and Percoll were from Sigma. DMSO was from Aldrich Chemical Company. Ethylene glycol and glycerol were from Fisher Chemical Company. All other chemicals were reagent grade. The *in vitro* expression plasmid for pLHCP[2], psAB80XD/4, is an SP6 derivative of psAB80 (5) and has been described elsewhere (10). The expression plasmid for LHCP, P2HPLC, is a pUC18 plasmid that harbors the coding sequence for the mature form of LHCP from petunia (24), and was generously provided by Dr. Paul Viitanen. The expression plasmid for the precursor to the Rubisco small subunit from pea, pSMS64 (1), was the generous gift of Dr. Steven Smith. The expression plasmid for the precursor to plastocyanin, pSPPC74 (22), was kindly provided by Drs. Thomas Lubben and Kenneth Keegstra.

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[2] Abbreviations: pLHCP, precursor to LHCP; LHCP, light-harvesting Chl *a/b* protein; (p)LHCP = pLHCP or LHCP; PC, plastocyanin; pPC, precursor to PC; SS, small subunit of Rubisco; pSS, precursor to SS.

Preparation of Chloroplasts, Lysates, Thylakoids, and Stroma

Intact chloroplasts were isolated from 10- to 12-d-old pea (*Pisum sativum*, cv Laxton's Progress 9) seedlings as described (8) and were suspended in import buffer, 50 mM Hepes/KOH (pH 8), 0.33 M sorbitol. Chloroplast lysates were obtained from intact chloroplasts by resuspending chloroplast pellets in 10 mM Hepes/KOH (pH 8), 10 mM $MgCl_2$, and after 5 min adjusting to import buffer, 10 mM $MgCl_2$. Thylakoids were prepared from chloroplast lysates by centrifugation at 3,200*g* for 8 min at 4°C. Stromal extract was prepared from the resulting supernatant by further centrifugation at 42,000*g* for 30 min at 4°C. Lysates prepared at a concentration of 0.5 mg Chl/mL are arbitrarily referred to as 1× lysate and the stromal extract resulting from such lysates as 1× stroma. Chl concentrations were determined according to Arnon (2). For cryopreservation, intact chloroplasts were resuspended in import buffer containing varying amounts of cryoprotectant (0, 10, 20, or 30% [v/v] DMSO, ethylene glycol, glycerol, or 0.1, 0.2, or 0.3 M glycine) at final concentrations of 3.0 to 5.0 mg Chl/mL. Thylakoids to be cryopreserved were resuspended in storage buffer (20 mM Hepes/KOH (pH 8), 20 mM sorbitol, and 125 mM KCl) containing either 30% (v/v) DMSO or 30% (v/v) ethylene glycol to 3.0 to 5.0 mg Chl/mL. Aliquots (1.0–2.0 mL) of chloroplasts or thylakoids were placed either in microcentrifuge tubes or thin-walled screw-cap plastic vials and plunged into liquid N_2. Cryopreserved chloroplasts were thawed at room temperature, diluted with import buffer to about 1.0 mg Chl/mL, and repurified on 35% Percoll cushions (8). The percentage of intact chloroplasts was determined from the Chl content of chloroplasts that sedimented through the Percoll relative to the total amount of Chl applied to the cushion. Chloroplasts that sedimented through Percoll were verified to be intact by the ferricyanide reduction assay (25). Repurified chloroplasts were washed twice with import buffer before use. Cryopreserved thylakoids were thawed at room temperature, diluted to about 1.0 mg Chl/mL with storage buffer, and recovered by centrifugation at 3200*g* for 8 min. Thylakoid pellets were subsequently washed twice with import buffer and 10 mM $MgCl_2$, and resuspended in the same buffer to 1.0 mg Chl/mL.

Microscopic Examination of Chloroplast Integrity

Small aliquots of chloroplasts in import buffer were fixed by adding an equal volume of 3% glutaraldehyde in 50 mM potassium phosphate (pH 7.5) and 2 mM $MgCl_2$. After 45 min on ice, chloroplasts were pelleted and sequentially washed with 50 mM Hepes buffer (pH 8), containing decreasing sorbitol concentrations (0.33, 0.16, 0.00 M). Chloroplasts were postfixed with 2% OsO_4 in phosphate buffer (50 mM potassium phosphate, pH 7.5) at room temperature for 2 h and dehydrated in a graded ethanol series, from 12.5, 25, 40, 60, 75, 85, and 95, to 100%, followed by three cycles in 100% acetone. Dehydrated chloroplasts were embedded in Spurr's resin (23) in a graded series of 15, 30, 50, 75, 90, and 100% plastic. Thin sections were prepared on an LKB ultramicrotome and were poststained with uranyl acetate for 20 min followed by lead citrate for 10 min. Sections were examined with a Hitachi HU-11E electron microscope.

Preparation of Radiolabeled Precursors

RNA for pLHCP, LHCP, pSS, and pPC was prepared by SP6 polymerase transcription of *Eco*RI-linearized plasmids (9) and translated in the presence of [^{3}H]leucine in a wheat germ system (10). Translations were diluted approximately 6-fold and adjusted to import buffer containing 30 mM leucine before use.

Assays for Import and Integration of Precursor Proteins

Import assays were carried out *in vitro* essentially as described (8). Each assay contained 200 μL of chloroplast preparation (0.5 mg Chl/mL), 50 μL of adjusted translation product, and 50 μL of 60 mM Mg-ATP in import buffer or 50 μL of import buffer alone (light-driven assays). Assays were initiated with addition of precursor and incubated at 25°C either in the presence of white light (~150 $\mu E.m^{-2}s^{-1}$) or in darkness. Assays were for 10 min with manual shaking at 5-min intervals. After the reaction, chloroplasts were treated with thermolysin to remove surface-bound precursors, repurified on Percoll cushions, and washed with import buffer containing 5 mM EDTA as described (11).

Integration assays with chloroplast lysates were performed basically as described (9). Each assay contained 200 μL of lysates (0.5 mg Chl/mL), 50 μL of 60 mM Mg-ATP in import buffer, 20 mM $MgCl_2$ or 50 μL of import buffer, 20 mM $MgCl_2$ alone (light-driven assays), and 50 μL of adjusted translation product. When assays were conducted with reconstituted lysates, reaction mixtures contained 100 μL of thylakoids (1.0 mg Chl/mL) and 100 μL of 2× stromal extract in place of the lysates. Reaction mixtures were incubated at 25°C for 30 min either in darkness or in white light (~150 $\mu E.m^{-2}s^{-1}$) with manual shaking every 5 min. Recovered thylakoids were further treated with thermolysin to remove nonintegrated molecules as described (8).

Analysis of Precursors, Recovered Chloroplasts, and Subfractions

Samples of precursors, recovered chloroplasts, and recovered chloroplast subfractions were subjected to SDS-PAGE (15). For analysis of the assembly of imported SS into Rubisco, stromal extracts of the recovered chloroplasts were

Table I. *Average Percentage of Intact Chloroplasts Preserved*

Values were derived from the average of three experiments conducted under the same conditions. Frozen samples were stored overnight in liquid N_2 (−196°C).

Protective Substances (v/v)	Percentage Preserved
Freshly prepared	85.0
No cryoprotectants	0.0
10% DMSO	45.4
20% DMSO	67.6
30% DMSO	34.5
10% ethylene glycol	62.7
20% ethylene glycol	58.3
30% ethylene glycol	25.3
10% glycerol	<20.0
20% glycerol	21.0
30% glycerol	<20.0

subjected to 6% nondenaturing PAGE (18). After electrophoresis, gels were prepared for fluorography (4) and placed on x-ray film. Radioactive proteins were extracted from the dried gels (26) and quantified as described (8).

RESULTS AND DISCUSSION

Preservation of Isolated Intact Chloroplasts

Various compounds have been reported to act as cryoprotectants and to impart protective qualities to biological tissues during low-temperature storage (6, 13). Among them, DMSO has long been known as one of the most effective cryoprotectants for both plant and animal tissues (13); ethylene glycol, glycerol, and glycine have also been documented as good cryoprotective agents in some cases (12, 13). Here, these cryoprotectants at different concentrations were examined for their ability to preserve intact pea chloroplasts. Immediately after isolation, chloroplasts were resuspended in import buffer containing a cryoprotectant and placed in liquid N_2 for preservation. Samples were thawed at room temperature and the

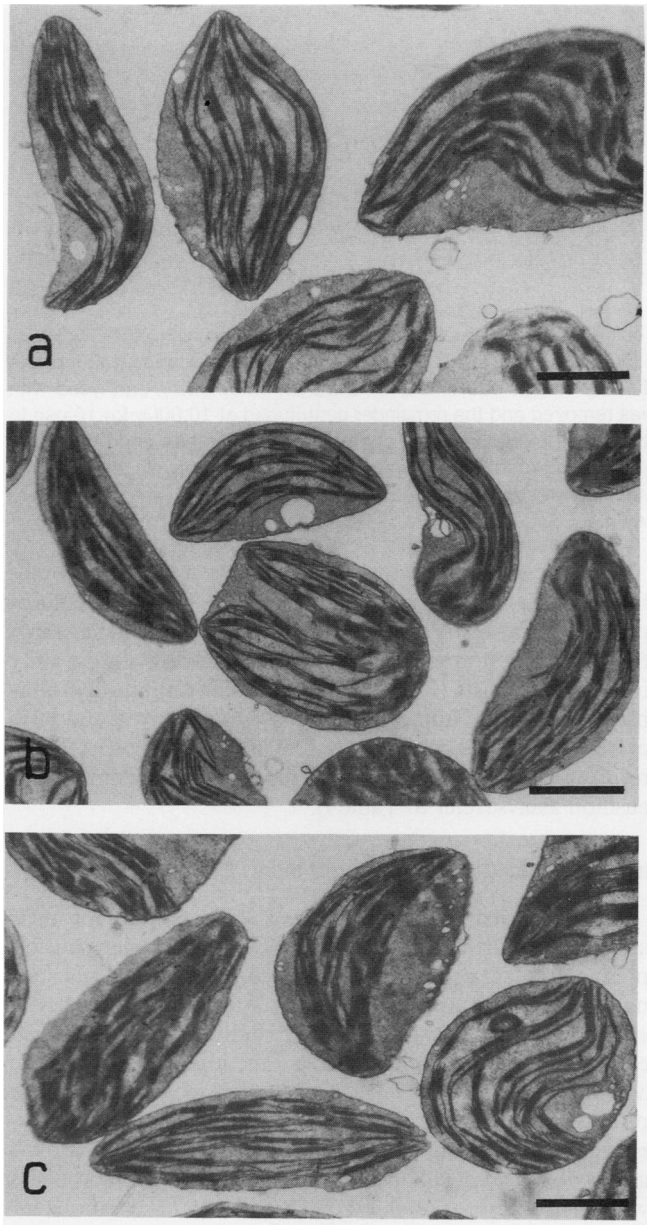

Figure 1. Ultrastructural features of fresh and cryopreserved pea chloroplasts. (a) Freshly prepared chloroplasts; (b) chloroplasts preserved in 20% (v/v) DMSO; (c) chloroplasts preserved in 10% (v/v) ethylene glycol. Cryopreserved samples were stored in liquid N_2 for 2 weeks. Bar, 2 μm.

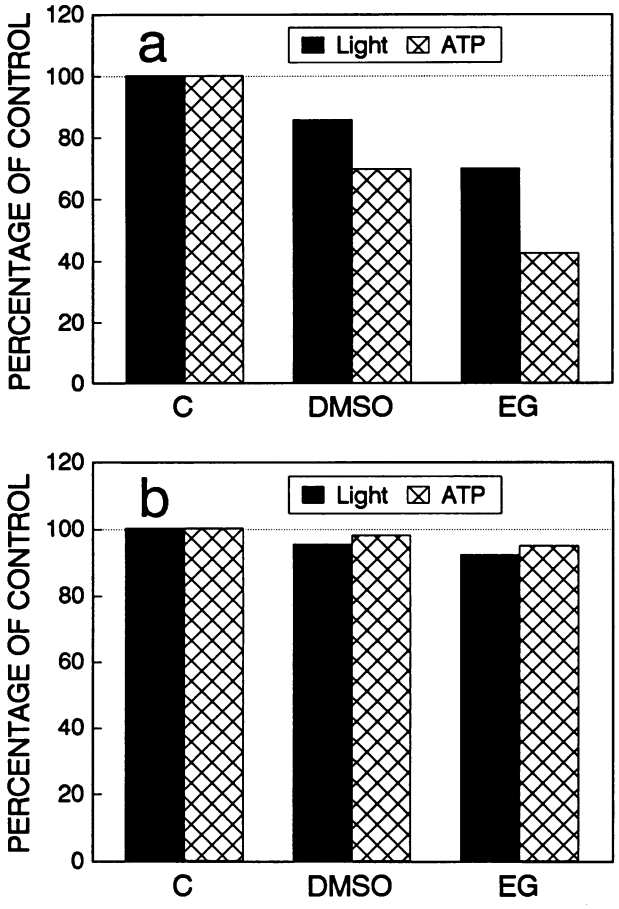

Figure 2. (a) Relative import of pLHCP by cryopreserved chloroplasts. Isolated chloroplasts were stored for 2 d in liquid N_2 in the presence of either 20% (v/v) DMSO or 10% ethylene glycol (EG). Cryopreserved chloroplasts were thawed, repurified, and assayed for their ability to import pLHCP ("Materials and Methods"). Each assay received 2025 molecules of pLHCP per chloroplast. The control (C) was freshly prepared chloroplasts. The absolute number of molecules imported per chloroplast was 323 (light-driven assays) and 227 (ATP-driven assays) for the control. (b) Effects of preincubation with cryoprotectants on import competence. Before use, freshly prepared chloroplasts were incubated either with 20% (v/v) DMSO or 10% EG for 10 min. Incubated chloroplasts were then washed twice with import buffer and assayed for their ability to import pLHCP ("Materials and Methods"). Each assay received 2450 molecules of pLHCP per chloroplast. The control (C) received no cryoprotectants during the 10-min incubation. The absolute number of molecules imported per chloroplast was 398 (light-driven assays) and 302 (ATP-driven assays) for the control.

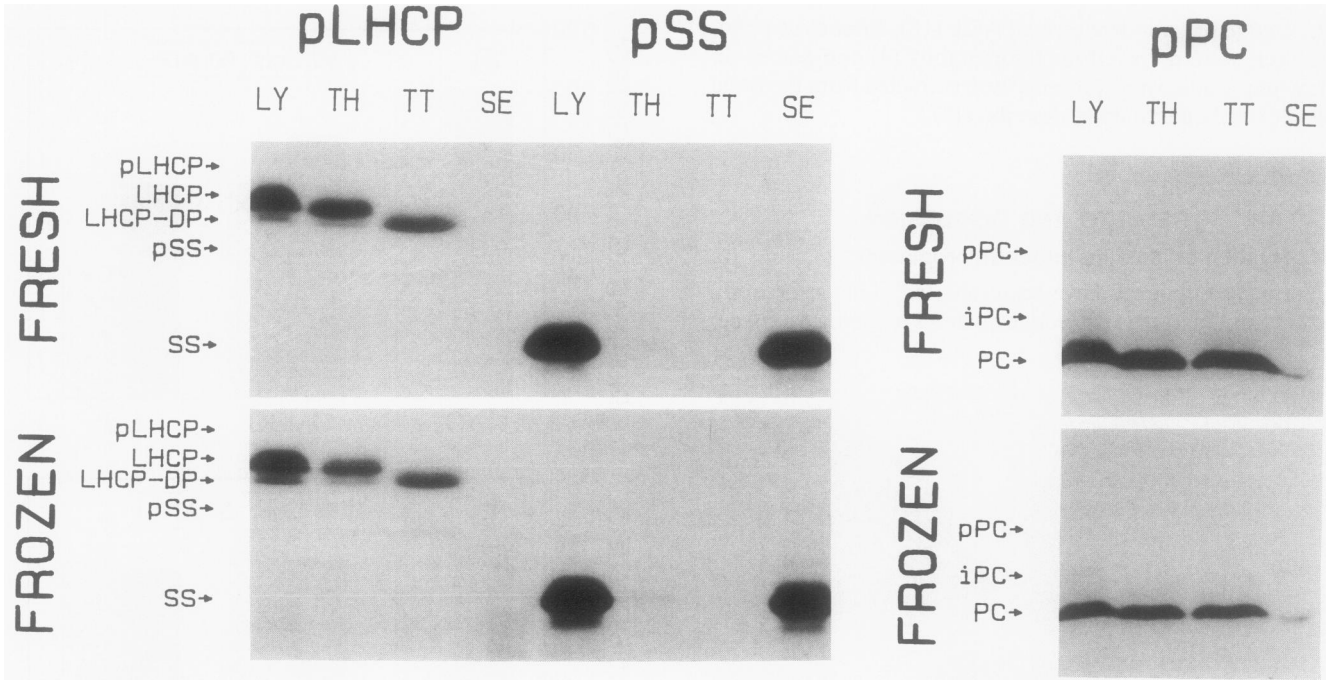

Figure 3. Targeting and assembly of proteins imported into cryopreserved chloroplasts. Chloroplasts were cryopreserved with 20% DMSO for 6 months. Chloroplasts were thawed, repurified, and used for import assays (600 μL) with pLHCP, pSS, and pPC. Following import, chloroplasts were treated with thermolysin to remove surface-bound molecules, repurified on Percoll cushions ("Materials and Methods"), and lysed with 75 μL of 10 mM Hepes/KOH (pH 8). An aliquot (25 μL) of each lysate (LY) was removed and the remainder centrifuged at 10,000*g* for 10 min to separate stroma from thylakoids. Stromal extract (SE) was obtained from the supernatant by further centrifugation ("Materials and Methods"). The pelleted thylakoids were resuspended in 800 μL of import buffer. Aliquots (400 μL) of thylakoids were treated with thermolysin at 50 μg/mL. Thermolysin-treated thylakoids (TT) as well as untreated thylakoids (TH) were then resuspended in 25 μL of 20 mM EDTA. All samples were analyzed by SDS-PAGE/fluorography. A photograph of the fluorogram is shown.

percentage of intact chloroplasts was determined with a Percoll cushion assay ("Materials and Methods"). The average percentage of intact chloroplasts preserved under different conditions is shown in Table I. Typically, the average percentage of intactness was 85% for freshly prepared chloroplasts. With preserved materials, best results were found to be approximately 65% intact for samples stored in 10% ethylene glycol or in 20% DMSO. For those stored in glycerol, less than 30% intact chloroplasts were preserved at several concentrations, and nearly all chloroplasts were broken when stored in glycine at 0.1 to 0.3 M. Because of the poor preservation of chloroplasts stored in glycerol and glycine, only DMSO- (specifically, 20% [v/v]) and ethylene glycol (10% [v/v])-preserved chloroplasts were used in subsequent experiments.

Microscopic Examination of Chloroplast Integrity

Cryopreserved chloroplasts were examined by thin section transmission electron microscopy. Low-magnification micrographs gave a good impression of the intactness of chloroplasts (Fig. 1). Examination of high-magnification photographs revealed no significant differences between freshly prepared chloroplasts and those stored at low temperature in the presence of cryoprotectants (not shown). Outer and inner envelope membranes were present and seemingly unchanged in cryopreserved chloroplasts. Thylakoid structure appeared to be the same in both newly prepared and preserved chloroplasts, with a nearly identical shape and degree of stacking. Percoll cushion repurified samples contained almost only intact chloroplasts (Fig. 1). Examination of unpurified samples revealed that approximately 70% of preserved and 85% of freshly prepared chloroplasts were intact (not shown). These results were fairly consistent with those obtained from Percoll cushion assays (refer to Table I).

Import of pLHCP by Preserved Intact Chloroplasts

Cryopreserved intact chloroplasts were assayed for their ability to import proteins. Both DMSO- and ethylene glycol-preserved intact chloroplasts imported pLHCP into an external protease-resistant state (Fig. 2a). Import could be driven either by light or by exogenous ATP, indicating that preserved chloroplasts had retained both a functional photosynthetic apparatus and a functional adenylate translocator. Almost no pLHCP was imported when assays were carried out in darkness in the absence of ATP (not shown). Furthermore, import ability was virtually abolished by pretreatment of chloroplasts with thermolysin (not shown). These latter observations indicate that import occurred by the physiological mechanism. As far as import activity is concerned, cryopreserved chloroplasts were up to 85% as active in import assay as freshly prepared chloroplasts. Of the two kinds of cryoprotectants used, DMSO preservation resulted in chloroplasts that were

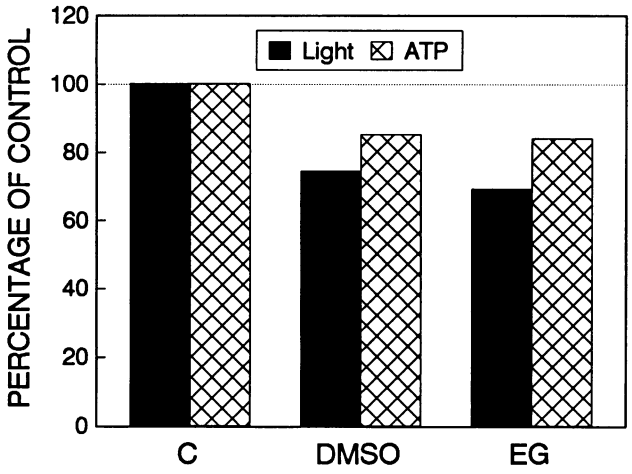

Figure 4. Relative integration of (p)LHCP by lysates from cryopreserved chloroplasts. Chloroplasts were cryopreserved overnight in the presence of either 20% (v/v) DMSO or 10% ethylene glycol (EG). Chloroplasts were thawed, repurified, and lysed ("Materials and Methods"). Lysates were assayed for the ability to integrate (p)LHCP into their thylakoid membranes ("Materials and Methods"). Each assay received 5380 molecules of pLHCP per chloroplast equivalent. Control experiments (C) were conducted with lysates prepared from fresh chloroplasts. The absolute number of molecules integrated per chloroplast equivalent was 249 (light-driven assays) and 296 (ATP-driven assays) for the control.

moderately more active. DMSO and ethylene glycol on their own did not significantly affect the ability of freshly prepared chloroplasts to import pLHCP (Fig. 2b).

Correct Assembly of Imported Proteins

Chloroplasts, cryopreserved for 6 months, were used to import several different precursor proteins, including pLHCP, the precursor to the small subunit (precursor to SS) of Rubisco, and the precursor to plastocyanin. Following import, chloroplasts were recovered and subfractionated into thylakoids and stroma to assess the localization and assembly status of the imported proteins (Fig. 3). LHCP was correctly processed to mature size, and properly assembled into the thylakoids as determined by characteristic partial resistance to protease (8). Only a trace amount of LHCP was detected in the stromal fraction. Similarly, mature-sized plastocyanin was recovered with the thylakoid fraction in a protease-protected state. The intermediate-sized plastocyanin precursor (trace amount), which has previously been described (21), was present in the stromal fraction. Mature-sized SS was recovered with the stromal fraction. Analysis of the stroma by 6% nondenaturing PAGE demonstrated that SS was assembled into the Rubisco holoenzyme (not shown). These results are virtually identical to those obtained with fresh chloroplasts and demonstrate that the assembly apparatus within chloroplasts has remained functional in the cryopreserved chloroplasts.

Membrane Insertion of (p)LHCP by Chloroplast Lysates

Many of our studies focus on the transport of proteins into and across the thylakoid membranes. These studies utilize an assay in which thylakoid protein transport is reconstituted in chloroplast lysates. Thus, it was important to determine if lysates from preserved chloroplasts were functional in these assays. Cryopreserved intact chloroplasts were lysed and assayed for their ability to integrate (p)LHCP. The results showed that lysates from both DMSO- and ethylene glycol-preserved chloroplasts were capable of integrating (p)LHCP into their thylakoid membranes (Fig. 4). Relative integration of (p)LHCP by lysates from preserved chloroplasts was approximately 85% of that by lysates from freshly prepared chloroplasts for ATP-driven assays, and approximately 70% for light-driven assays. As has previously been shown for lysates from fresh chloroplasts (8), virtually no integration occurred when assays were carried out in darkness in the absence of ATP or when stroma was omitted from the assay mixture (not shown). DMSO preservation resulted in lysates that were slightly more active in integration assays than ethylene glycol preservation (Fig. 4).

Integration of (p)LHCP by Cryopreserved Thylakoids

Previous studies have identified optimal conditions for the cryopreservation of the photosynthetic competence of isolated thylakoids (12). Similar conditions were examined for their ability to preserve the integration competence of isolated pea thylakoids. Preserved thylakoids were supplemented with fresh stroma and assayed for their ability to integrate (p)LHCP. The results showed that both DMSO- and ethylene glycol-preserved thylakoids were able to integrate (p)LHCP into their membranes (Fig. 5). Relative integration by cryo-

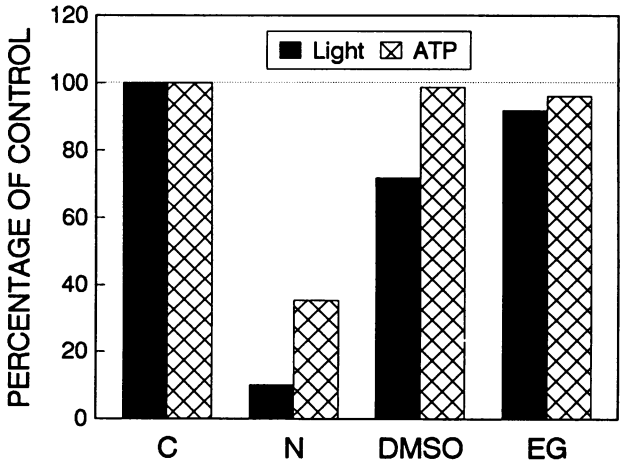

Figure 5. Relative integration of (p)LHCP by cryopreserved thylakoids. Isolated thylakoids were cryopreserved for 4 weeks in storage buffer containing either 30% (v/v) DMSO or 30% ethylene glycol (EG). Preserved thylakoids were thawed, washed, and resuspended in import buffer plus 10 mM $MgCl_2$ to a Chl concentration of 1.0 mg/mL. Purified thylakoids were furnished with fresh stroma in integration assays. Each assay received 4800 molecules of pLHCP per chloroplast equivalent. The positive control (C) was freshly prepared thylakoids. The negative control (N) was frozen thylakoids with no protective substances. The absolute number of molecules integrated per chloroplast equivalent was 226 (light-driven assays) and 274 (ATP-driven assays) for the positive control.

preserved thylakoids was from 95 to 98% of that by freshly prepared thylakoids (ATP-driven assays). For light-driven assays, ethylene glycol-preserved thylakoids were slightly more active than DMSO-preserved thylakoids (Fig. 5), suggesting that ethylene glycol may be marginally better than DMSO for stabilizing the photosynthetic apparatus. This result is consistent with previous observations by Farkas and Malkin (12).

CONCLUSIONS

In this study, we have described for the first time a simple and very convenient protocol for cryopreservation of isolated intact chloroplasts. By following this protocol, we were able to preserve chloroplasts fully functional for protein import and assembly for 6 months (Fig. 3). We found that DMSO is the superior preserving medium for intact chloroplasts and recommend the following protocol. Immediately after isolation of the chloroplasts, the final pellet should be resuspended in import buffer containing 20% DMSO (v/v) at a Chl concentration of 3 to 5 mg/mL. Chloroplasts should then be allowed to equilibrate for about 10 min on ice before freezing. Freezing should be achieved rapidly by submerging small vials in liquid N_2, and thawing should be allowed to occur at room temperature. For best results, chloroplasts should be repurified on Percoll cushions before use.

Chloroplasts stored under the above conditions retained their ability to import a variety of different precursor proteins and to correctly localize and assemble them within the chloroplast. These latter processes frequently require transport into or across the thylakoid membrane. The proper localization of plastocyanin and LHCP by cryopreserved plastids, as well as the ability of lysates from these plastids to assemble LHCP demonstrate that the thylakoid transport apparatus has been preserved during low-temperature storage. Preserved chloroplasts were also active for *in organello* protein synthesis and produced a similar pattern of labeled polypeptides although in a somewhat reduced amount (not shown). For preservation of isolated thylakoids, a modified method of that reported by Farkas and Malkin (12) was an effective means of preserving thylakoids for use in protein integration analysis.

We hope that this method will facilitate future studies of chloroplast protein biogenesis. We recommend that investigators test the efficacy of these preservation methods when different precursors or a different source of plastids is to be employed.

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