

# Cryopreservation of Plant Mitochondria as a Tool for Protein Import or in Organello Protein Synthesis Studies

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Cryopreserved chloroplasts and thylakoids have recently been proven to be suitable for protein import and integration assays. The possibility of recovering intact plant mitochondria after storage would also facilitate a wide range of investigations that are currently underway on the molecular biology of these organelles, e.g. mitochondrial transcription, RNA editing, in organello protein synthesis, and protein or transfer RNA import. Therefore, we addressed the question whether cryopreservation of isolated plant mitochondria was also possible. Tobacco (*Nicotiana tabacum*) or broad bean (*Vicia faba*) mitochondria were quick frozen and stored in liquid nitrogen in the presence of various concentrations of ethylene glycol as a cryoprotectant. After thawing, up to 90% of the mitochondria stored in 5 to 10% ethylene glycol appeared to retain an intact outer membrane and normal oxidative phosphorylation activity. Their ultrastructural aspect, observed by electron microscopy, was similar to that of freshly prepared mitochondria. Furthermore, efficient in organello protein synthesis was carried out with mitochondria stored in the presence of 7.5% ethylene glycol. Finally, the precursor of the  $\beta$  subunit of the mitochondrial  $F_1$ -ATPase from *Nicotiana plumbaginifolia* was successfully translocated into *V. faba* cryopreserved mitochondria and processed. These data demonstrate that plant mitochondria cryopreserved under the conditions described here remain functional and can be used for a variety of physiological and biochemical studies.

Plant mitochondria have not retained all the tRNA genes of their endosymbiotic ancestor (for a review, see Dietrich et al., 1992) and contain tRNAs encoded by the nuclear genome (Maréchal-Drouard et al., 1988). We have demonstrated that these tRNA species, which correspond to about one-third of the mitochondrial tRNA population in the case of potato (Maréchal-Drouard et al., 1990), are imported from the cytosol (Small et al., 1992). Our main interest is to understand how some tRNAs transcribed in the nucleus are selected to be imported into the mitochondria, and how these polynucleotides cross the double organellar envelope. Understanding mitochondrial protein biogenesis first required the development of in vitro protein import and in organello protein synthesis systems. We are interested in setting up a plant in vitro tRNA import system. These studies require the isolation of pure, intact, and active plant mitochondria, which are obtained only after a time-consuming series of differential

and density-gradient centrifugation steps. Therefore, organelle isolation is a limiting factor, and we asked the question whether it was possible to store isolated plant mitochondria. Moreover, the ability to preserve mitochondria over long periods could minimize the problem of seasonal variations. For instance, we observed fluctuations in the respiratory control of mitochondria isolated throughout the year from the same plant species (our unpublished data) and it seems obvious that such variations in the activity of the mitochondria might affect their ability to synthesize proteins and to import proteins or tRNAs. Finally, preservation could also be used as a tool for comparative analyses of mitochondria obtained from different plant tissues or different developmental stages.

Cryopreservation of plant tissues is now established (for a review, see Chen and Li, 1989), and Yuan et al. (1991) recently presented a method for preservation of active chloroplasts. We tested analogous techniques and show here for the first time that isolated plant mitochondria, quick frozen and stored in liquid nitrogen in the presence of a cryoprotective agent, retain protein synthesis and import activities, demonstrating that such a procedure can be used to facilitate in vitro studies on plant mitochondria.

## MATERIALS AND METHODS

### Plant Materials

Tobacco (*Nicotiana tabacum*) leaves used for mitochondria preparation were harvested from 7-week-old plants grown in a greenhouse (temperature, 18°C during the night, 24°C during the day; humidity, 70%). Broad bean (*Vicia faba*) mitochondria were purified from 10-d-old etiolated hypocotyls grown in a dark chamber at 25°C.

### Isolation of Mitochondria

Highly purified mitochondria were prepared from tobacco leaves according to previously described procedures (Neuburger et al., 1982). The same protocol was used for the isolation of broad bean hypocotyl mitochondria, except that Suc was replaced by mannitol in the grinding and washing buffers.

The results presented here rely on a high degree of purification of the mitochondria. Using marker enzymes of plastids

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Abbreviation: FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone.

(phosphorylase, EC 2.4.1.1), peroxisomes (catalase, EC 1.11.1.6), and cytosol (pyrophosphate:Fru-6-P phosphotransferase, EC 2.7.1.90), the procedures we used were previously demonstrated to yield mitochondria free of significant plastidial, peroxisomal, or cytosolic contaminants (Neuburger et al., 1982).

### Assessment of Mitochondrial Outer Membrane Integrity

The integrity of the outer membrane was estimated by testing the ability of the fresh or cryopreserved mitochondria to reduce exogenously added Cyt *c* (Douce et al., 1972). Reduction of Cyt *c* (80  $\mu\text{M}$ ) was measured in the presence of 1 mM KCN and 1 mM ATP by following the  $A_{550}$ . Succinate (10 mM) was used as a substrate. Control assays were performed after complete breakage of the outer membrane by a 30-s osmotic shock in 5 mM potassium phosphate (pH 7.5).

### RC Measurement

The mitochondrial RC was estimated by measuring the oxygen uptake ratio between the "active state" (state 3) in the presence of an excess (200  $\mu\text{M}$ ) of ADP and the "resting state" (state 4) in the absence of ADP, using succinate (10 mM) as a substrate (Neuburger et al., 1982; Douce, 1985).

### EM Analysis

For EM analyses, mitochondria were pelleted and then fixed for 2 h at 4°C in washing buffer without BSA (10 mM potassium phosphate, pH 7.5, 0.3 M Suc, 1 mM EDTA, 5 mM Gly) containing 0.5% (w/v) glutaraldehyde and 3% (w/v) paraformaldehyde. The pellets were rinsed two times (for 15 min and one night, respectively) with BSA-free washing buffer. At this step, the organelles were embedded in agarose (1.4% [w/v] agarose in BSA-free washing buffer) and post-fixed for 2 h at 4°C with 1% (w/v)  $\text{OsO}_4$  in the same medium. Agarose samples were washed four times for 20 min at 4°C with 0.1 M sodium phosphate buffer (pH 6.8) and one time for 30 min at 22°C with the same buffer containing 1% (w/v) tannic acid to enhance the contrast, then rinsed two times for 15 min with distilled water. Samples were progressively dehydrated by successive 15-min incubations in 75, 95, and 100% ethanol, followed by two 20-min incubations in propylene oxide, and finally embedding in Epon. Ultrathin sections were prepared on an LKB ultramicrotome and post-stained for 10 min, successively, in 5% (w/v) uranyl acetate and in a saturated solution of lead citrate. Sections were examined with a Philips EM 410 or a Hitachi H 600 electron microscope.

### In Organello Mitochondrial Protein Synthesis

In organello protein synthesis assays with isolated tobacco mitochondria were carried out as described by Kaderbhai et al. (1989) in the presence of sodium succinate (5 mM) and ADP (2.8 mM). Control assays were performed in the presence of 2.5  $\mu\text{M}$  antimycin A (Chaumont et al., 1990) or in the absence of an energy source.

### In Vitro Mitochondrial Import and Maturation of the Precursor of the $F_1$ -ATPase $\beta$ Subunit from *Nicotiana plumbaginifolia*

The plasmid pTZ-ATP<sub>2</sub>, containing the  $F_1$ -ATPase  $\beta$  subunit gene of *N. plumbaginifolia* (obtained from M. Boutry, Catholic University of Louvain, Belgium), was used for in vitro transcription-translation, essentially as previously outlined (Chaumont et al., 1990). Import of the in vitro-synthesized precursor into *V. faba*-isolated mitochondria was carried out under the conditions described by Chaumont et al. (1990). Control assays were performed in the presence of 2.5  $\mu\text{M}$  antimycin A or 1  $\mu\text{M}$  FCCP.

### Electrophoretic Analysis of Proteins

In organello-synthesized proteins and mitochondrial import products of the  $F_1$ -ATPase  $\beta$  subunit were separated by SDS-PAGE (Laemmli, 1970). Both a constant polyacrylamide concentration (15%, w/v) and a linear polyacrylamide concentration gradient (7.5–15%, w/v) were used. In organello mitochondrial protein synthesis products were also analyzed by two-dimensional PAGE as described previously (Mayer et al., 1987; Guillemaut et al., 1992). The one- and two-dimensional gels were treated with an enhancer (En<sup>3</sup>Hance, New England Nuclear) according to the manufacturer and submitted to fluorography.

## RESULTS AND DISCUSSION

### Cryopreservation of Plant Mitochondria

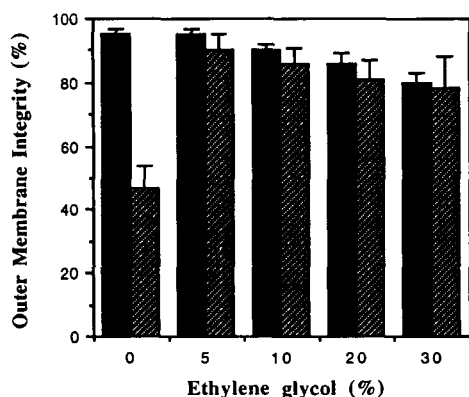
Several compounds, such as DMSO, ethylene glycol, glycerol, or Gly, have been reported to act as cryoprotective agents on plant or animal tissues (for reviews, see Finkle et al., 1985; Chen and Li, 1989). Among these, DMSO and ethylene glycol were recently shown to be the most effective cryoprotectants for pea chloroplasts and thylakoids (Yuan et al., 1991). In our experiments, ethylene glycol proved to be more efficient than DMSO for cryopreserving plant mitochondria (data not shown). Furthermore, DMSO is known to have some relaxing effect on macromolecules and may potentially interfere with tRNA import. For these reasons, we developed our studies mainly with ethylene glycol.

For cryopreservation, purified plant mitochondria were resuspended in washing buffer (10 mM potassium phosphate at pH 7.5, 0.3 M Suc [in the case of tobacco] or mannitol [in the case of broad bean], 1 mM EDTA, 0.1% [w/v] BSA, and 5 mM Gly) containing various amounts of ethylene glycol (0, 5, 10, 20, or 30% [v/v]). The suspensions (70–100  $\mu\text{L}$  with about 5 mg of mitochondrial protein per mL) were placed in microcentrifuge tubes, allowed to equilibrate for 5 min on ice, and plunged into liquid nitrogen. Samples were stored in liquid nitrogen for up to 3 months. Suspensions of cryopreserved mitochondria were quickly thawed by incubation at 42°C for 30 to 40 s. For control assays, fresh mitochondria were incubated for at least 1 h on ice in the presence or absence of ethylene glycol. Before use, all samples of fresh or cryopreserved mitochondria were diluted with washing buffer, centrifuged for 5 min at 9000g, and resuspended in a medium appropriate for the biological test to be performed.

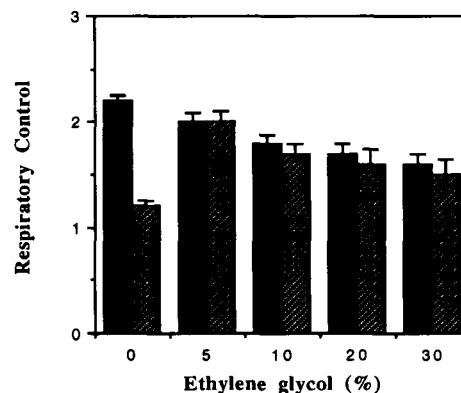
To estimate the proportion of intact mitochondria recovered from samples cryopreserved in the presence of various amounts of ethylene glycol, we analyzed the integrity of the outer membrane by the Cyt *c* reduction assay. As shown in Figure 1, the average proportion of intact organelles, according to this criterion, was 92% for freshly isolated tobacco mitochondria. For cryopreserved mitochondria, the best results were obtained in the presence of 5% (v/v) ethylene glycol and corresponded to about 90% intactness. Without a cryoprotectant, half of the mitochondria appeared to have a disrupted outer membrane after thawing, and when using ethylene glycol concentrations higher than 5%, the proportion of intact mitochondria gradually decreased (Fig. 1 and data not shown).

The intactness of the mitochondrial outer membrane does not necessarily imply a normal oxidative phosphorylation activity. Therefore, the biochemical intactness of tobacco mitochondria cryopreserved in the presence of various amounts of ethylene glycol was checked by measuring their RC. The results obtained are presented in Figure 2. Typically, an RC of 2.0 to 2.3 was obtained for fresh mitochondria. The best results for cryopreserved samples were 2.0 and 1.8 and these again corresponded to mitochondria stored in 5 or 10% ethylene glycol. In the absence of a cryoprotective agent, the RC drops to a value of approximately 1.0, indicating the loss of potential for oxidative phosphorylation. The RC of mitochondria stored in 20 or 30% of ethylene glycol also decreased significantly (Fig. 2).

As an average, the estimation of the outer membrane integrity and the measurement of the RC gave the best results for mitochondria cryopreserved in the presence of 5% ethylene glycol. However, in some of the experiments a concentration of 10% appeared to be the most efficient (data not shown), indicating that optimum cryoprotection is obtained in the range of 5 to 10% ethylene glycol. Therefore, a mean value of 7.5% was used for further analyses.



**Figure 1.** Intactness of purified tobacco mitochondria preincubated (black boxes) or cryopreserved (hatched boxes) in the presence of 0, 5, 10, 20, and 30% ethylene glycol. The proportion of intact mitochondria was determined by testing outer membrane integrity using the Cyt *c* reduction assay. Values are the means of five to seven independent experiments (in the case of 0, 5, 10, and 20% ethylene glycol) or three independent experiments (in the case of 30% ethylene glycol). Bars represent  $\pm$ SE.



**Figure 2.** RC of purified tobacco mitochondria preincubated (black boxes) or cryopreserved (hatched boxes) in the presence of 0, 5, 10, 20, and 30% of ethylene glycol. RC was estimated by measuring the oxygen uptake at 25°C with a Clark-type O<sub>2</sub> electrode. Succinate (10 mM) was used as a substrate in the presence of 200  $\mu$ M ADP (for the "active state," state 3) and in the absence of ADP (for the "resting state," state 4). Values are the means of five to seven independent experiments (in the case of 0, 5, 10, and 20% ethylene glycol) or three independent experiments (in the case of 30% ethylene glycol). Bars represent  $\pm$ SE.

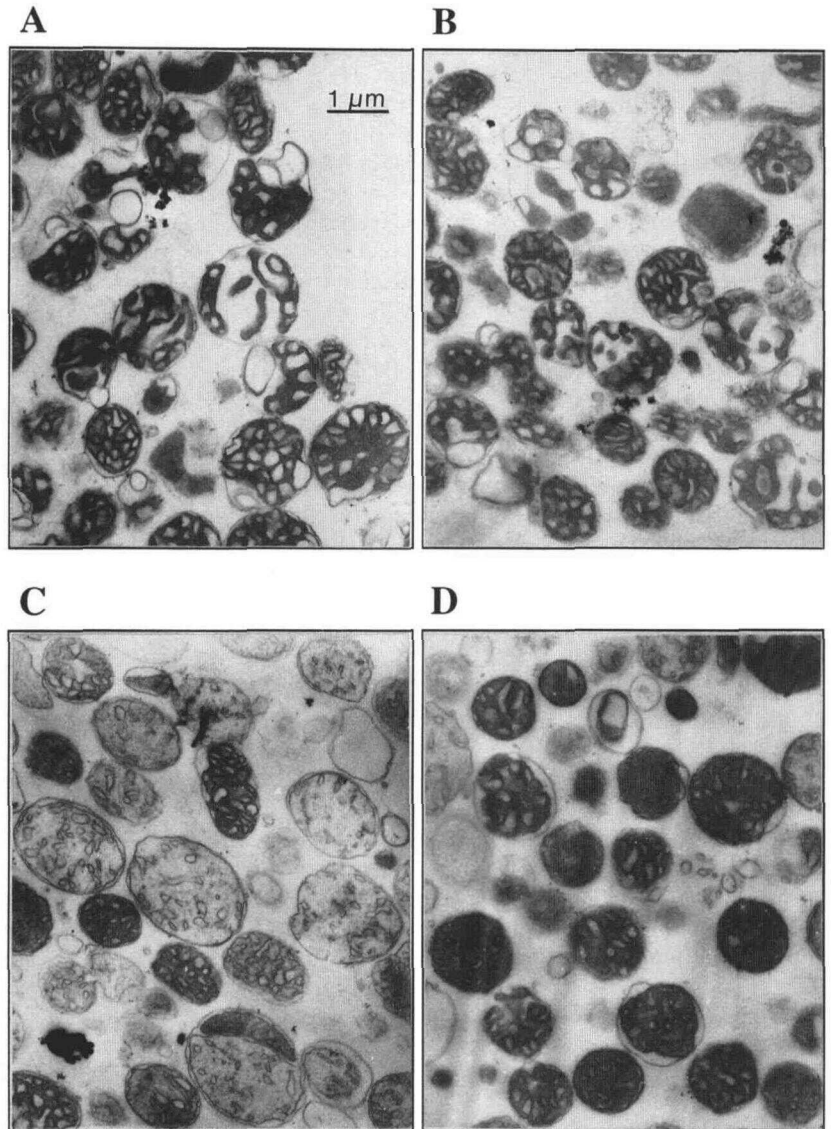
All samples of tobacco mitochondria observed by thin-section transmission EM at low magnification ( $\times 27,000$ ) appeared to be free of contamination with intact plastids or microbodies. Up to 95% of the freshly prepared mitochondria (whether incubated or not with 7.5% ethylene glycol), as well as of the mitochondria cryopreserved with 7.5% ethylene glycol, exhibited normal ultrastructural features with intact membranes and a dense matrix (Fig. 3, A, B, and D). A few of the cryopreserved mitochondria showed some separation between the outer and inner membranes, but on average no significant differences were detected at higher magnification ( $\times 70,000$ ) between freshly isolated mitochondria and mitochondria cryopreserved in the presence of 7.5% ethylene glycol (data not shown), although in some instances the latter appeared to be more dense (Fig. 3).

Up to 60% of the mitochondria preserved in the absence of cryoprotectant appeared to be "empty" and larger in size than intact mitochondria (Fig. 3C), although only some of these empty mitochondria showed a disrupted outer membrane at high magnification ( $\times 70,000$ ). The percentage of organelles still showing a dense matrix (about 40%) under EM examination roughly correlated with the percentage of intactness estimated for the same samples by the Cyt *c* reduction assay (about 45%, see Fig. 1), suggesting that the large, empty mitochondria have a leaky envelope.

#### In Organello Protein Synthesis in Cryopreserved Plant Mitochondria

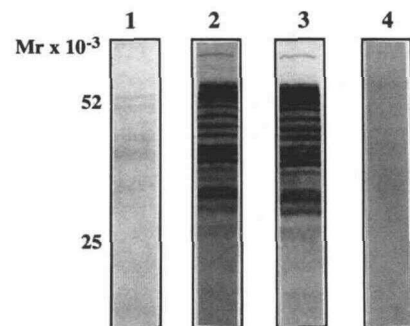
Freshly prepared tobacco mitochondria and mitochondria cryopreserved with 7.5% ethylene glycol were tested for their ability to perform protein synthesis in the presence of [<sup>35</sup>S]-Met. Figure 4 shows typical patterns of the newly synthesized polypeptides. The observed protein synthesis activity is more

**Figure 3.** Electron micrographs of tobacco mitochondria. A, Freshly prepared mitochondria; B, freshly prepared mitochondria preincubated in the presence of 7.5% ethylene glycol; C, mitochondria cryopreserved without a protectant; and D, mitochondria cryopreserved in the presence of 7.5% ethylene glycol. Cryopreserved samples were stored in liquid nitrogen for up to 10 weeks.



than 95% sensitive to antimycin A, an inhibitor of complex III of the mitochondrial respiratory chain, thus demonstrating the absence of significant contaminating cytosolic or chloroplastic activity (Fig. 4, lane 1). It appeared from several independent in organello protein synthesis experiments that neither the level of [<sup>35</sup>S]Met incorporation nor the pattern of the synthesized proteins differed significantly between freshly isolated mitochondria (whether incubated or not with 7.5% ethylene glycol) and mitochondria cryopreserved in the presence of 7.5% ethylene glycol (Fig. 4, lanes 2 and 3). On the other hand, the polypeptide pattern observed was very similar to those previously described for in organello protein synthesis in isolated maize, tobacco, or broad bean mitochondria (e.g. Boutry et al., 1984). Mitochondria preserved in the absence of a cryoprotectant were not able to perform protein synthesis, and almost no labeled polypeptides were detected (Fig. 4, lane 4).

These results were further confirmed by analysis of the in organello mitochondrial protein synthesis products on two-

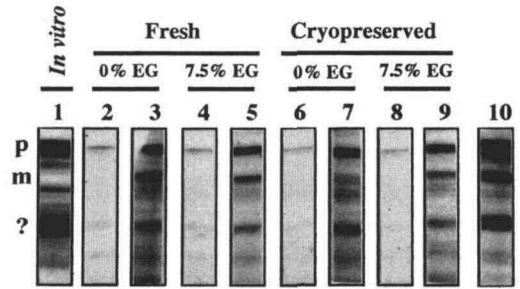


**Figure 4.** Polypeptides synthesized in isolated tobacco mitochondria. In organello protein synthesis in freshly isolated tobacco mitochondria in the presence (lane 1) or in the absence (lane 2) of 2.5  $\mu$ M antimycin A and in mitochondria cryopreserved in the presence (lane 3) or in the absence (lane 4) of 7.5% ethylene glycol. The in organello protein synthesis products were separated by SDS-PAGE and analyzed through autoradiography.

dimensional polyacrylamide gels. Figure 5, which corresponds to a short autoradiography time, clearly shows that a protein localized at the putative position of the F<sub>1</sub>-ATPase  $\alpha$  subunit (Kaderbhai et al., 1989), a typical mitochondrion-encoded protein (Hack and Leaver, 1983), was synthesized at a high level both in freshly isolated mitochondria (Fig. 5B) and in mitochondria cryopreserved in the presence of 7.5% ethylene glycol (Fig. 5D). This protein was not detected when the mitochondria were incubated without an energy source (Fig. 5A) or preserved without a cryoprotectant (Fig. 5C). The labeled polypeptides *a* and *b* found in all four panels of Figure 5 correspond to bacterial contamination.

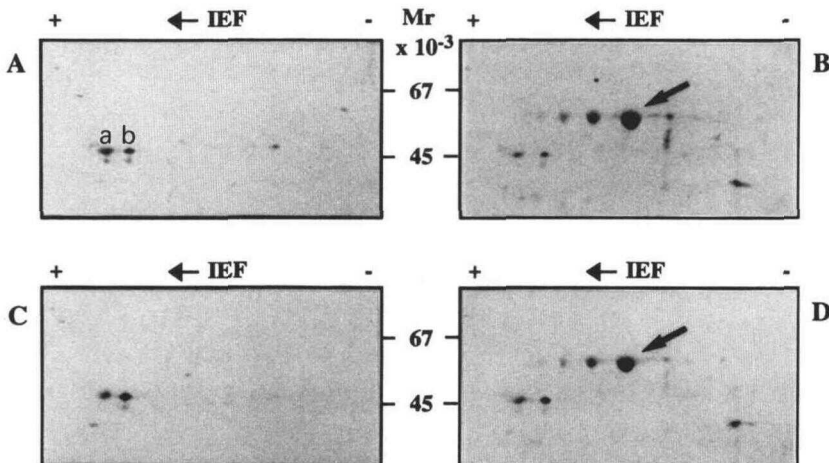
**Ability of Cryopreserved Plant Mitochondria to Import and Process the Precursor of the F<sub>1</sub>-ATPase  $\beta$  Subunit**

The most important test for our future work was to assay cryopreserved mitochondria for their ability to import proteins. For this purpose, we used the in vitro protein targeting system described by Chaumont et al. (1990). But, unexpectedly, the in vitro import of the tobacco F<sub>1</sub>-ATPase  $\beta$  subunit precursor into isolated tobacco mitochondria happened to be almost undetectable, as described in the original paper by Chaumont et al. (1990). Therefore, our protein import studies were performed with broad bean mitochondria, as were those of Chaumont et al. (1990). Broad bean mitochondria were cryopreserved in the presence of various concentrations of ethylene glycol in the same manner as were tobacco mitochondria. The intactness of fresh and cryopreserved organelles was estimated by the Cyt *c* reduction assay and the RC was measured (data not shown). The results obtained were comparable to those described in Figures 1 and 2 for tobacco mitochondria, and therefore, broad bean mitochondria cryopreserved with 7.5% ethylene glycol were further tested for their ability to import and process the *N. plumbaginifolia* F<sub>1</sub>-ATPase  $\beta$  subunit. As shown in Figure 6, the 59-kD F<sub>1</sub>-ATPase  $\beta$  subunit precursor was correctly translocated into freshly isolated *V. faba* mitochondria (whether incubated or not with 7.5% ethylene glycol) and partly processed into the 52-kD mature protein, yielding an electrophoretic pattern similar to that previously observed by Chaumont et al. (1990). When *V. faba* mitochondria were cryopreserved in the pres-



**Figure 6.** Ability of fresh or cryopreserved *V. faba* mitochondria to import and process the precursor of the F<sub>1</sub>-ATPase  $\beta$  subunit. The precursor of the F<sub>1</sub>-ATPase  $\beta$  subunit was synthesized in vitro in a reticulocyte lysate cell-free system (lane 1) and was incubated with freshly isolated *V. faba* mitochondria (lanes 2–5 and 10) or with cryopreserved mitochondria (lanes 6–9) as described in “Materials and Methods.” 0% EG, Freshly isolated mitochondria (lanes 2 and 3) or mitochondria cryopreserved in the absence of a protectant (lanes 6 and 7); 7.5% EG, freshly isolated mitochondria preincubated for 30 min in the presence of 7.5% ethylene glycol (lanes 4 and 5) or mitochondria cryopreserved in the presence of 7.5% ethylene glycol (lanes 8 and 9). Lanes 2, 4, 6, and 8 correspond to import assays performed in the presence of 1  $\mu$ M FCCP. After the import step, samples were treated with proteinase K (2.5  $\mu$ g/mL) for 15 min on ice (lanes 2–9). The sample presented in lane 10 was not treated with proteinase K. Import and processing products were resolved by SDS-PAGE on a 7.5 to 15% gel and analyzed through autoradiography. The positions of the 59-kD precursor (p) and of the 52-kD mature (m) forms of the F<sub>1</sub>-ATPase  $\beta$  subunit are indicated. The presence of the major additional radiolabeled polypeptide synthesized in the reticulocyte lysate system and previously described by Chaumont et al. (1990) is indicated by a question mark.

ence of 7.5% ethylene glycol prior to the import assay, only a slight reduction (about 5–10%, as estimated by densitometric scanning of the autoradiograms) in the import and processing capacity was noted compared to that of fresh mitochondria (Fig. 6). The F<sub>1</sub>-ATPase  $\beta$  subunit precursor was also found at a level comparable to that of mitochondria preserved in the absence of cryoprotectant, but in this case low levels of full-size mature protein were detected (Fig. 6).



**Figure 5.** Polypeptides synthesized in isolated tobacco mitochondria. In organello protein synthesis in freshly isolated tobacco mitochondria without (A) or with (B) an energy source and in mitochondria cryopreserved in the absence (C) or in the presence (D) of 7.5% ethylene glycol. The in organello protein synthesis products were separated by high-resolution two-dimensional PAGE and analyzed through autoradiography. The arrows indicate the putative position of the F<sub>1</sub>-ATPase  $\alpha$  subunit according to Kaderbhai et al. (1989). Polypeptides *a* and *b* result from bacterial contamination.

For all mitochondria samples tested, the ability to import and process the F<sub>1</sub>-ATPase  $\beta$  subunit precursor was abolished in the presence of 2.5  $\mu$ M antimycin A (data not shown) or 1  $\mu$ M FCCP (Fig. 6). The latter result demonstrates that the electrochemical gradient required for protein import is present in our freshly prepared *V. faba* mitochondria and is maintained after cryopreservation in the presence of 7.5% ethylene glycol.

### CONCLUSION

We show here that it is possible to cryopreserve plant mitochondria. The method described (quick freezing in the presence of 7.5% ethylene glycol, storage in liquid nitrogen, and quick thawing at 42°C) should be of general use because it is simple, reliable, and efficient. Cryopreserved plant mitochondria appear to remain almost fully functional and are suitable for assays as sensitive as in organello protein synthesis and in vitro protein import.

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### LITERATURE CITED

- Boutry M, Faber AM, Charbonnier M, Briquet M (1984) Microanalysis of plant mitochondrial protein synthesis products. *Plant Mol Biol* 3: 445-452
- Chaumont F, O'Riordan V, Boutry M (1990) Protein transport into mitochondria is conserved between plant and yeast species. *J Biol Chem* 265: 16856-16862
- Chen THH, Li PH (1989) Cryopreservation of plant cells and organs. In PH Li, ed, *Low Temperature Stress Physiology in Crops*. CRC Press, Boca Raton, FL, pp 139-152
- Dietrich A, Weil JH, Maréchal-Drouard L (1992) Nuclear-encoded transfer RNAs in plant mitochondria. *Annu Rev Cell Biol* 8: 115-131
- Douce R (1985) Mitochondria in Higher Plants. Structure, Function and Biogenesis. Academic Press, Orlando, FL
- Douce R, Christensen EL, Bonner WD (1972) Preparation of intact plant mitochondria. *Biochim Biophys Acta* 275: 148-160
- Finkle BJ, Zavala ME, Ulrich JM (1985) Cryoprotective compounds in the viable freezing of plant tissues. In KK Kartha, ed, *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton, FL, pp 75-113
- Guillemaut P, Weber-Lotfi F, Blache D, Prost M, Rether B, Dietrich A (1992) Conifer decline in the north-east of France: characteristic changes in chloroplast protein pattern and absence of anti-oxidative defense capability point to an involvement of ozone. *Physiol Plant* 85: 215-222
- Hack E, Leaver CJ (1983) The alpha subunit of the maize F<sub>1</sub>-ATPase is synthesized in the mitochondria. *EMBO J* 2: 1783-1789
- Kaderbhai N, Beechey RB, Kaderbhai MA (1989) Protein synthesis by isolated castor bean mitochondria. *Plant Physiol Biochem* 27: 227-234
- Laemmli UK (1970) Cleavage and structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685
- Maréchal-Drouard L, Guillemaut P, Cosset A, Arbogast M, Weber F, Weil JH, Dietrich A (1990) Transfer RNAs of potato (*Solanum tuberosum*) mitochondria have different genetic origins. *Nucleic Acids Res* 18: 3689-3696
- Maréchal-Drouard L, Weil JH, Guillemaut P (1988) Import of several tRNAs from the cytoplasm into the mitochondria in bean *Phaseolus vulgaris*. *Nucleic Acids Res* 16: 4777-4788
- Mayer JE, Hahne G, Palme K, Schell J (1987) A simple and general plant tissue extraction procedure for two-dimensional gel electrophoresis. *Plant Cell Rep* 6: 77-81
- Neuburger M, Journet EP, Bligny R, Carde JP, Douce R (1982) Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. *Arch Biochem Biophys* 217: 312-323
- Small I, Maréchal-Drouard L, Masson J, Pelletier G, Cosset A, Weil JH, Dietrich A (1992) In vivo import of a normal or mutagenized heterologous transfer RNA into the mitochondria of transgenic plants: towards novel ways of influencing mitochondrial gene expression? *EMBO J* 11: 1291-1296
- Yuan J, Cline K, Theg SM (1991) Cryopreservation of chloroplasts and thylakoids for studies of protein import and integration. *Plant Physiol* 95: 1259-1264