Freezing Damage to Isolated Tomato Fruit Mitochondria as Modified by Cryoprotective Agents and Storage Temperature

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ABSTRACT

Isolated tomato (*Lycopersicon esculentum* var. Kc 146) fruit mitochondria could be stored successfully in the frozen state without a cryoprotective agent if the mitochondria were frozen quickly by immersion in liquid nitrogen and later thawed quickly at 30 C. Criteria of freezing damage were rate of respiration, adenosine diphosphate to oxygen ratio, and respiratory control ratio. Marked reduction in respiration and loss of respiratory control occurred when mitochondria were transferred from liquid nitrogen to −5, −10, or −18 C for 15 minutes prior to thawing at 30 C. Dimethylsulfoxide (5%) prevented freezing damage when mitochondria were incubated at −5 C but did not prevent freezing damage at −10 or −18 C. Isolated tomato mitochondria show promise as a model system for studying the nature of freezing damage and the mode of action of cryoprotective agents.

A complete knowledge of what freezing damage is and how to prevent it might allow the frozen storage of frost-sensitive plants or plant parts such as fleshy fruits. Better understanding of the causes and the nature of freezing damage would probably also aid studies on factors responsible for frost hardiness in plants (26).

Freezing damage to intact plant cells results primarily in damage to membranes and inhibition of enzyme systems associated with membranes (17). Electron microscope studies by Mohr and Stein (29) revealed extensive disruption of cell membranes in frozen and thawed tomato fruit parenchyma cells. Membrane disorganization was minimal when tissue was frozen rapidly on Dry Ice and thawed slowly in air at 2 C. Such treatment caused some disruption of plasmalemma and tonoplast, but plastids and nuclei remained intact. The effect of freezing on function of the cells or organelles was not studied, nor were there any data concerning possible protection of the tissue by cryoprotective agents.

Heber's extensive studies (16–18) reveal that oxidative phosphorylation and photophosphorylation are inhibited in intact leaves damaged by freezing. The ability of isolated spinach chloroplasts and mitochondria to form ATP is damaged by freezing as well as chloroplast permeability and light-dependent proton uptake. Sucrose protects chloroplasts against freezing damage.

There are a number of reports concerning freezing and animal mitochondria (8, 23–25, 33), but relatively little is known about how freezing affects isolated plant mitochondria. Heber and Santarius (18) reported that frozen and thawed spinach mitochondria partially retained phosphorylative and oxidative properties if sucrose was added, but protection was not as great as for photophosphorylation by chloroplasts. The spinach mitochondria were assayed with α-ketoglutarate as substrate, and all P/O ratios were less than 1. Hence, these workers may have observed the effect of freezing on the substrate level phosphorylation only.

Our earlier work (5) showed that isolated tomato fruit mitochondria were severely damaged by freezing. Oxygen uptake decreased 89%, or more after 24 hr of storage at −18 C or in liquid nitrogen, and the damaged mitochondria lost respiratory control. The cryoprotective agent dimethylsulfoxide prevented freezing damage during storage in liquid nitrogen, and mitochondria were stored a month without decrease in ADP/O or loss of respiratory control.

The present study also deals with freezing of isolated tomato mitochondria and is a continuation of the earlier work. Experiments were conducted to learn if DMSO was the only effective cryoprotective agent, whether rate of warming was related to a need for a cryoprotective agent, and the time of freezing damage at several subzero temperatures.

The cause of freezing damage is still not completely known (8, 17, 28), and the mode of action of cryoprotective agents is not clear (3, 22, 27, 31). Isolated tomato mitochondria show promise for studying these phenomena.

MATERIALS AND METHODS

Tomato fruit mitochondria were isolated and assayed with a Clark oxygen electrode as described earlier (5–7). Outer pericarp from mature green tomato fruits (*Lycopersicon esculentum*, var. Kc 146) was ground at 0 C in a food mill with a medium (2 g of medium per g of tissue) containing 0.5 M mannitol, 0.05 M Na barbital (pH 7.8), 4 mM cysteine, 5 mM EDTA, and 1.5 mg/ml bovine serum albumin fraction V powder. The homog- nate was strained through muslin, and mitochondria were collected as the fraction precipitating between 1,500 g for 15 min and 15,000 g for 15 min. Mitochondria were washed once in 0.5 M mannitol, 5 mM Na barbital, 1.5 mg/ml bovine serum albumin and then resuspended in the same medium. Protein nitrogen was determined according to Thompson and Morrison (32). Oxygen uptake was measured at 25 C and pH 7.5 in a standard reaction medium containing 0.5 M mannitol, 5 mM MgCl2, 10 mM tris, 10 mM KH2PO4, 0.5 mM EDTA, 0.2 ml of mitochondria, final volume 2.95 ml. The organic acid substrate was 20 mM succinate or 40 mM malate plus 40 mM pyruvate. When malate and pyruvate were used, the standard reaction medium was supple

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1 Abbreviation: DMSO: dimethyl sulfoxide.
mented with 0.2 mg of thiamine pyrophosphate, 0.4 mg of NAD, and 0.1 mg of coenzyme A. ADP (0.1 mM) was added to initiate the rapid state 3 rate of respiration; second and third additions of ADP were made after respiration rates returned to state 4. Respiratory control ratios (state 3 rate/state 4 rate) and ADP/O ratios were calculated according to Chance and Williams (4). The data below are averages from three additions of ADP to a single reaction mixture. Q02(N) values were calculated from state 3 rates unless otherwise specified.

Freezing experiments were conducted as described earlier (5). Cryoprotective agents were added to mitochondria in the suspension medium at 0 C. Final concentration of the agents is expressed as percentage, v/v.

Mitochondria (0.5-ml samples in 1-dram screw top glass vials) were frozen by immersion in liquid nitrogen. Rapid warming was done by transferring vials from liquid nitrogen to a 30 C water bath with removal to crushed ice just as thawing was complete. Slow warming was done by transferring vials from liquid nitrogen to crushed ice for 10 min followed by rapid thawing at 30 C. Sample temperature reached −20 C after 3 to 4 min in crushed ice and −2 to −4 C after 10 min in crushed ice, as measured with a thermometer (model 143) from Yellow Springs Instrument Co.

In some experiments mitochondria were frozen by immersion in liquid nitrogen and then transferred to an alcohol bath kept at −18, −10 or −5 ± 0.5 C. After incubation in the alcohol bath these samples were thawed rapidly at 30 C as described above.

Table I. Use of DMSO and Glycerol to Prevent Freezing Damage to Tomato Fruit Mitochondria

The protective agent was added to mitochondrial suspension to give a final concentration of 5 or 10% (v/v). At the end of the incubation period at 0 C, a portion was taken for assay, and the rest was immediately frozen under liquid nitrogen. Samples were thawed slowly after storage overnight in liquid nitrogen. Each sample was assayed immediately after being thawed.

Table II. Respiratory Properties of Tomato Mitochondria after Rapid Freezing and Rapid or Slow Thawing in Presence or Absence of DMSO

RESULTS

Glycerol protected mitochondria from freezing damage, but, unlike DMSO, the ability of glycerol to protect was partly dependent on the time between its addition to mitochondria and immersion in liquid nitrogen. Relevant data are given in Table I. In agreement with earlier results (5), respiratory control was abolished and O2 uptake reduced 89% when isolated mitochondria were frozen without a protective agent. The ADP/O, Q02(N), and respiratory control values were not significantly altered when mitochondria were frozen with 5% DMSO. Preliminary experiments established that incubation at 0 C with 5% DMSO did not affect mitochondrial activity, and so this control was omitted to provide mitochondria for the glycerol treatments. Incubation at 0 C with glycerol (5 or 10% glycerol, 5 or 120 min) did not markedly affect mitochondrial activity, and all four glycerol treatments gave complete freeze protection with respect to the ADP/O ratio. However, O2 uptake was reduced in the thawed sample given 5% glycerol 5 min before freezing. State 3 rates in the other glycerol treatments (5% for 120 min, 10% for 5 min, 10% for 120 min) were not reduced appreciably by freezing and thawing. Hence, the lower glycerol concentration conferred freeze protection given enough time, but the higher glycerol concentration protected in a short time. State 4 rates seemed slightly higher in frozen-thawed mitochondria preincubated for 2 hr with glycerol. This indicates possible freezing damage due to release of latent ATPase activity which might be caused by the glycerol itself or by some time-dependent change which reduced the ability of mitochondria to be protected by glycerol.

In the above work and all previous freezing experiments, mitochondria were transferred from liquid nitrogen to crushed ice for 10 min before thawing at 30 C (see "Materials and Methods"

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1 These control samples (held at 0 C) were assayed immediately after isolation (initial assay) and about 2 hours later (final assay) after glycerol treated samples were assayed and frozen. Succinate was the substrate for assays in Table I (see Materials and Methods for details).

2 Not calculable.

3 No respiratory control.
ods”). Subsequently, experiments were conducted to learn whether more rapid warming affected the amount of freezing damage to mitochondria lacking a cryoprotective agent.

No protective agent was needed if mitochondria were frozen rapidly by plunging into liquid nitrogen and thawed quickly in a 30 C water bath (Table II). Samples frozen without DMSO and warmed rapidly exhibited $Q_0(N)$, ADP/O, and respiratory control values similar to those of unfrozen samples whether the substrate was succinate or malate-pyruvate. As expected, mitochondria warmed slowly without DMSO were severely damaged, so DMSO protected during the slow warming. All samples were assayed 4 hr after thawing as well as immediately afterward to learn whether damage to mitochondria thawed rapidly without DMSO might be expressed as a pronounced decline in ability to withstand subsequent storage at 0 C. Such was not the case, and there was little change in any of the thawed samples during 4 hr in crushed ice.

Further experiments were conducted to learn the relationship between temperature of frozen samples and the time course of freezing damage. Data are given in Figure 1 for samples frozen by immersion in liquid nitrogen, then incubated various times at $-5$, $-10$, or $-18$ C, and thawed rapidly in a 30 C water bath. The effect on state 3 respiration, calculated as $Q_0(N)$, is presented in Figure 1 (top). Respiration declined more rapidly with time as incubation temperature decreased, and 5% DMSO gave at least partial protection at all three temperatures. DMSO protection was greatest at the highest temperature ($-5$ C).

Interestingly, loss of respiratory activity proceeds continuously but relatively slowly at $-5$ C without DMSO and at $-10$ C with 5% DMSO. These treatments might prove useful in future studies on the causes and nature of freezing damage. The time course of possible changes in physical properties of the medium (such as ice crystal growth) and changes in mitochondrial morphology or biochemistry could be compared to the time course in loss of respiratory activity.

Respiratory control (state 3 rate/state 4 rate) and ADP/O ratios for the same samples are also given in Figure 1. In the absence of DMSO, respiratory control was lost within 15 min at each incubation temperature. DMSO prevented a decrease in respiratory control at $-5$ C but not at $-10$ or $-18$ C. DMSO also prevented changes in ADP/O ratios when samples were incubated 5 hr at $-5$ C and up to 30 min at $-10$ C. The $-10$ and $-18$ C experiments indicate that the ADP/O ratio is not decreased as readily as $Q_0(N)$ by freezing damage. Oxidative phosphorylation may have continued in samples lacking respiratory control (state 3/state 4 = 1) but the ADP/O ratio could not be calculated.

**DISCUSSION**

Isolated tomato mitochondria can be stored frozen without a cryoprotective agent if sample temperature is lowered and raised quickly and if a sufficiently low temperature is maintained during storage. Rat liver mitochondria can also be frozen and thawed without damage to respiration or oxidative phosphorylation if glycerol or DMSO is present (14, 15). However, the effect of freezing on respiratory control was not established in the rat liver experiments.

It seems quite likely that mitochondria isolated from plant tissues other than tomato can be stored frozen. Previously reported freezing damage to isolated spinach mitochondria (18) might be due to the high storage temperature ($-25$ C). Frozen storage of isolated mitochondria may be useful when the tissue, such as fleshy fruit, is available seasonally or when assays are not completed on the day of isolation. However, the effects of freezing on rates of respiration, respiratory control, and ADP/O were the only parameters studied here. It remains to be seen whether morphology or mitochondrial functions such as osmotic properties, ion uptake, or energy-mediated volume changes are affected by freezing.
The sites of freezing damage and the cause of freezing damage also remain to be established. Decreased state 3 respiration may reflect membrane damage which disrupts the electron transport chain at one or more points. Solubilization of cytochrome c could also decrease respiration. Sites of damage might be pinpointed by assaying various spans of the electron transport chain, as was done with mitochondria isolated from senescing leaves (9). Such damage might result from mechanical disruption by ice crystals (30) or from denaturation of membrane lipoproteins due to withdrawal of water (20, 21, 28) or oxidation of sulfhydryl groups. (19). Damaging concentrations of electrolytes may also occur in the small volume of water remaining unfrozen (2). Furthermore, it is known that several hydrolytic and oxidative reactions proceed more rapidly in ice than in liquid water at 0 C (1, 10–13).

With the conditions employed in the present study, respiration seemed more easily damaged than efficiency of oxidative phosphorylation (ADP/O). This differs from spinach chloroplasts in which phosphorylation, but not electron transport, is sensitive to freezing (17). Lusena (23) found that freezing of rat liver mitochondria released ATPase activity. If loss of respiratory control results from activation of latent ATPase in tomato mitochondria, then perhaps the sites of freezing damage could be located histochemically.

LITERATURE CITED