2005

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Received 30 March 2005; received in revised form 28 August 2005; accepted 6 September 2005

Abstract

Trehalose has extensively been used to improve the desiccation tolerance of mammalian cells. To test whether trehalose improves desiccation tolerance of mammalian mitochondria, we introduced trehalose into the matrix of isolated rat liver mitochondria by reversibly permeabilizing the inner membrane using the mitochondrial permeability transition pore (MPTP). Measurement of the trehalose concentration inside mitochondria using high perfOrmance liquid chromatography showed that the sugar permeated rapidly into the matrix upon opening the MPTP. The concentration of intra matrix trehalose reached 0.29 mmoVmg protein (~190 mM) in 5 min. Mitochondria, with and without trehalose loaded into the matrix, were desiccated in a buffer containing 0.25 M trehalose by diffusive drying. After re hydration, the inner membrane integrity was assessed by measurement of mitochondrial membrane potential with the fluorescent probe JC 1. The results showed that following drying to similar water contents, the mitochondria loaded with trehalose had significantly higher inner membrane integrity than those without trehalose loading. These findings suggest the presence of trehalose in the mitochondrial matrix affords improved desiccation tolerance to the isolated mitochondria.

Keywords: Trehalose; Desiccation; Mitochondria; Permeabilization; Mitochondrial membrane potential; Mitochondrial permeability transition pore

1. Introduction

There has been an increasing interest in the storage of desiccated mammalian cells at ambient temperatures using sugars such as trehalose and sucrose because the ability to desiccate and preserve cells could have significant applications in many fields including tissue engineering and medicine. Trehalose, a non-reducing disaccharide accumulated in some anhydrobiotic organisms, has been shown to be a key component for increasing desiccation tolerance by preserving the structure and function of membranes and proteins [1,2], presumably through a combination of two mechanisms-water replacement [3,4], and formation of inert glass (vitrification) [1,5,6].

Mammalian cell membranes are impermeable to disaccharides. To date, numerous methods have been developed to introduce trehalose into mammalian cells, including microinjection [7,8], thermal shock [9], genetically engineered pores [4,10,11] and gene expression for trehalose synthesis in cells [11,12]. Intracellular trehalose has been reported to have beneficial effects on cell viability during drying. Recently, human platelets, non-nucleated cytoplasmic bodies, have been successfully freeze-dried with trehalose [13]. However, stabilization of nucleated mammalian cells in the desiccated state has not yet been achieved [12]. One reason for this could be that cytoplasmic trehalose cannot penetrate the membrane-bound organelles, and these organelles are damaged during desiccation. For example, mammalian mitochondria have two membranes under normal physiological conditions. Even though the outer membrane is permeable to molecules of 5000 metabolites and ions, with the exception of those for which there are specific carriers [15,16]. Stabilization of mitochondria by trehalose loading into their matrix could improve the desiccation tolerance of mammalian cells.

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The mitochondrial permeability transition pore (MPTP) can be opened by the addition of excess Ca^{2+}, and the process is accelerated by the presence of inducers such as inorganic phosphate (Pi), Hg^{2+}, the protonophore m-chlorophenylhydrazone, and others [15]. When open, the pore allows molecules of :=1500 Da to cross the inner membrane [15]. In the present study, we introduced trehalose into isolated rat liver mitochondrial matrix by transient opening of the MPTP. This procedure allowed us to test the hypothesis that the presence of trehalose inside the matrix protects against desiccation damage. Here, we report that up to 190 mM trehalose can be loaded into the matrix of isolated mitochondria. We also show that the presence of trehalose in the mitochondrial matrix affords improved desiccation tolerance to isolated mitochondria as assessed by the re-establishment of the mitochondrial membrane potential.

2. Materials and methods

2.1. Preparation of mitochondria

Mitochondria were isolated from the liver of male CD IGS rats (Charles River Laboratories, Wilmington, MA, USA), weighting 250 350 g, by standard differential centrifugation using a mitochondria isolation kit (Sigma-Aldrich Co., St. Louis, MO, USA). The final mitochondrial pellet was suspended in storage buffer (SB, 10 mM HEPES, pH 7.4, containing 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, 1 mM dithiothreitol, and 250 mM sucrose) at a protein concentration of 10 mg/ml. The protein concentration was quantified by Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as the protein standard. Mitochondria isolated by this procedure were highly coupled based on our measurements of respiratory control ratios (RCR), i.e., the maximal ADP-stimulated rate of oxygen consumption (state 3) divided by the rate in the absence of ADP (state 4). RCR values were ;::5 as measured with a Clark-type oxygen electrode-OXYTHERM (Hansatech, Norfolk, UK). All reagents used in this study were purchased from Sigma-Aldrich Co. unless otherwise stated.

2.2. Permeabilization of the inner mitochondrial membrane

Mitochondria (1 mg/ml) were suspended in permeabilization buffer (PB), containing 250 mM trehalose, 10 mM Tris-Mops, 20 AM EGTA, 2 AM rotenone, 3 Ag/ml oligomycin, and 5 mM potassium succinate or 5 mM glutamate plus 5 mM malate, pH 7.4. Mitochondria were permeabilized (i.e., the MPTP opened) by adding 150 AM $CaCl_2$ plus 1 mM P_i at ambient temperature. After incubating mitochondria with the inducer pair for different time periods (0, 1, 5, 15, 30 min), the inner membrane was resealed by the addition of 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM nicotinamide adenine dinucleotide (NAD), which served to close the MPTP [17,18]. The opening of the MPTP caused mitochondrial swelling, which was monitored spectrophotometrically by following the decreased absorbance at k 540 nm. A VERSAmax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) was used for this purpose. The permeabilization and resealing process was also assessed by measurement of the mitochondrial membrane potential (DW) because permeabilization causes the depolarization of inner membrane and collapse of DW. Closing the MPTP allows the re-establishment of DW [18].

2.3. Measurement of DW to assess the integrity of the inner mitochondrial membrane

The lipophilic cationic carbocyanine probe 5, 5¥, 6, 6¥-tetrachloro-1, 1¥, 3, 3¥-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to measure DW. To monitor the kinetics of changes in DW during permeabilization and resealing, mitochondria (1 mg/ml) were incubated with JC-1 (5 Ag/ml) in PB, and fluorescence intensity was recorded at room temperature using an F-max fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). An excitation wavelength of 485 nm was used, and emission by the dye monomers and aggregates was measured at 538 nm (green) and 590 nm (orange-red), respectively. A ratiometric method was used with this dye (590/538 nm) to provide a semi-quantitative measurement of the magnitude of DW. Antimycin A (20 Am) was added to selected mitochondrial suspensions as a positive control to verify the sensitivity of JC-1 to DW.

To determine DW after rehydration of previously desiccated mitochondria, JC-1 (0.2 Ag/ml) was added to mitochondria (10 Ag/ml) suspended in a buffer containing 20 mM Mops, 110 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, and 1 mM EGTA, pH 7.5 at room temperature. Fluorescence readings were recorded 30 min after the addition of JC-1. The ratio of red to green fluorescence for the experimental groups was normalized to that of fresh mitochondria, which was determined by the same method. This normalized value was used as an index of the inner membrane integrity.

2.4. Quantification of mitochondrial matrix trehalose by HPLC

Ten minutes after permeabilization and resealing at room temperature, the loaded mitochondria were washed twice with excess volume of SB to remove extra-mitochondrial trehalose. In preparation for extraction of trehalose from the matrix, mitochondrial pellets were resuspended in 1 ml HPLC grade H_2O. The samples were frozen at −20 -C and thawed to release trehalose. Approximately 50 Al of each lysed mitochondrial sample were used for assays of total protein. The rest of each sample was heated for 20 min at 95 -C and then centrifuged at 15,000×g for 10 min to remove mitochondrial debris. Prior to HPLC analysis, the sugar-containing supernatant was filtered through a 0.2-Am syringe filter (Fisher Scientific, Pittsburgh, PA, USA). An Agilent 1100 Series HPLC with a Hamilton RCX10 column was used to separate carbohydrate peaks. The mobile phase was 100 mM NaOH. Sugars were eluted at a flow rate of 0.75 ml/min and quantified using pulsed amperometric detection (ESA Coulochem II, Cambridge, MA, USA). Peaks were identified by comparison with the retention times of standards.

2.5. Desiccation of mitochondria

After permeabilization in PB for 5 min (sufficient for maximal loading of trehalose; see Section 3.2) and subsequent resealing, the mitochondria were centrifuged and resuspended at a concentration of 10 mg protein/ml in SB-trehalose. This buffer had the same composition as the SB, except sucrose was replaced with 250 mM trehalose. Non-permeabilized mitochondria were simply resuspended in SB-trehalose. As a non-trehalose control, some mitochondria were resuspended in SB-KCl, which contained 125 mM KCl instead of 250 mM trehalose. Ten microliters of mitochondrial suspension were dispensed onto a pre-weighed coverslip, which was then placed in a small tissue culture dish (35 × 15 mm, Becton Dickinson, Franklin Lakes, NJ, USA). The diameter of the sample solution was approximately 4 mm with an initial thickness of about 2 mm. For diffusive drying, the sample was then transferred to a 4 -C Fisherbrand Unit desiccator cabinet (Fisher Scientific, Pittsburgh, PA, USA), which contained an excess amount of Drierite ($CaSO_4/CoCl_2$) as desiccant (W.A. Drierite Co., Xenia, OH, USA). The relative humidity achieved in the chamber with this approach was approximately 0% RH. Samples were dried to a wide range of final moisture contents, determined gravimetrically using a high sensitivity microbalance (M5, Mettler-Toledo, Switzerland, resolution T1 Ag). Depending on the target moisture content, the time required to reach the desired hydration state was between 3 and 20 h. Upon reaching the target moisture content, the samples were immediately rehydrated by adding 10 times the original volume of SB. DW was measured 1 2 h after rehydration using JC-1 as described above. The water content of the desiccated samples was expressed as grams water per gram of dry mass. The dry mass of the samples was determined by drying in an oven at 70 -C for 2 3 days.

2.6. Statistical analysis

Student's t-test (two-tailed, assuming unequal variances) and analysis of variance (ANOVA) were used to determine statistical significance ($P < 0.05$).

Means, standard deviations, and threshold values for t-test and ANOVA were calculated using Excel (Version 2002 for Windows, Microsoft Corp., Seattle, WA, USA).

3. Results and discussion

3.1. Permeabilization of mitochondria

Light scattering declines with mitochondrial swelling due to the decrease of the refractive index of the mitochondria during swelling. The decreased light scattering is tightly correlated with decreased absorbance at 540 nm, which has been widely used to qualitatively monitor the MPTP [19–21]. Control mitochondria did not show any change in absorbance for the duration of the experiment (Fig. 1A). After the addition of Ca^{2+}/P_i, the mitochondria displayed a rapid decrease in absorbance. Cyclosporine A inhibited the decrease in absorbance induced by Ca^{2+}/P_i, indicating that the swelling of mitochondria was due to the opening of the MPTP. As expected in sucrose-based medium, the addition of EGTA and Mg^{2+} did not cause any absorbance increase; the absence of a transport mechanism for sucrose or trehalose export precludes shrinkage after the MPTP is closed [18].

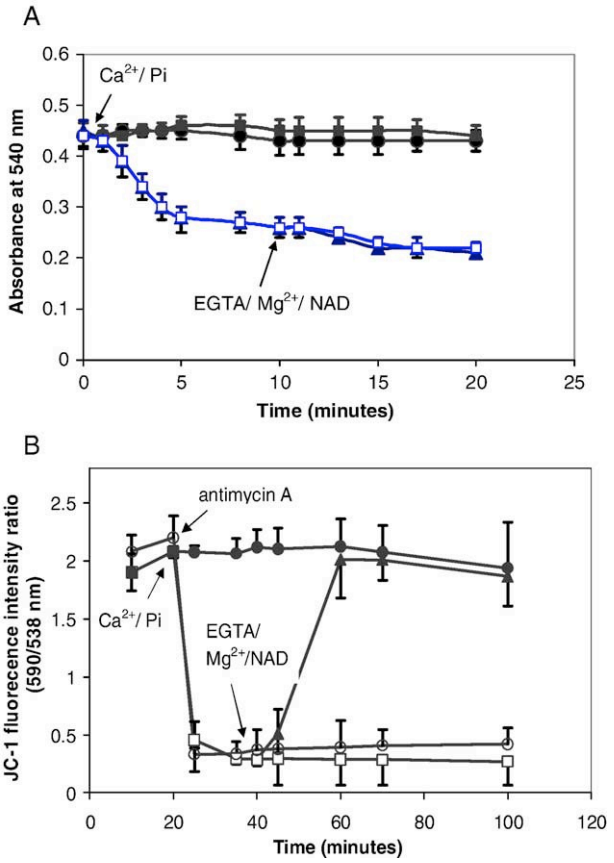

Fig. 1. Reversible permeabilization of the mitochondrial inner membrane. (A) Mitochondrial swelling induced by MPTP opening as monitored by reduced absorbance at 540 nm. (B) Changes in DW as measured with JC-1 during reversible permeabilization of isolated mitochondria. Mitochondria (1 mg/ml) were suspended in PM. Where indicated by arrows, 150 AM $CaCl_2$/1 mM P_i (open squares), 20 AM antimycin A (open circles), 1 mM EGTA/1 mM $MgCl_2$/1 mM NAD (close triangles) were added. Control mitochondria (solid circles) were exposed to PM only. The curve indicated by solid squares is mitochondria incubated with cyclosporine A (1 AM) and with the addition of 150 AM $CaCl_2$/1 mM P_i. Values are means T S.D. (N 3).

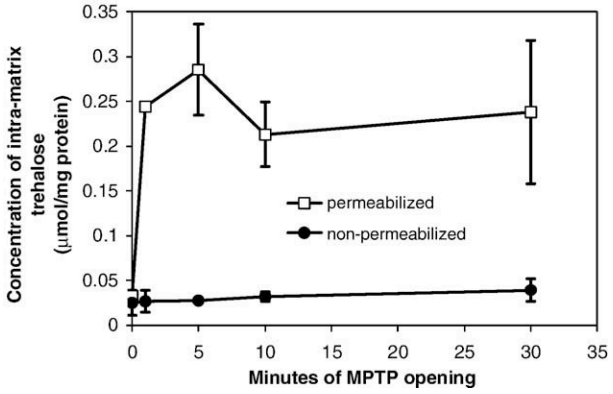

Fig. 2. Quantification of trehalose loaded into the mitochondrial matrix as a function of time after MPTP opening. Mitochondria (1 mg/ml) were suspended in PM. The MPTP was opened for different periods of time (0, 1, 5, 15, 30 min) by the addition of 150 AM $CaCl_2$ plus 1 mM P_i, and then the mitochondria were resealed by the addition of 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM NAD, which promoted MPTP closure. After washing to remove extra-mitochondrial trehalose, the sugar present in the matrix was determined by HPLC and normalized to protein concentration. Values are means T S.D., (N 3).

Intact isolated mitochondria displayed a membrane potential as measured by the JC-1 fluorescence ratio of red to green (Fig. 1B). Antimycin A is an electron transport inhibitor and causes de-energization of the mitochondrion and lowers DW. The dissipation of DW by antimycin A confirmed the reliability of DW measurements using JC-1 as reported by others [22–25]. The addition of the MPTP inducer Ca^{2+}/P_i also caused rapid dissipation of DW, consistent with opening of the MPTP. Mitochondrial membrane repolarization (i.e., recovery of the dissipated DW) by the addition of EGTA, Mg^{2+}, and NAD verified MPTP closure and mitochondrial resealing. This pattern confirmed that the permeabilization of inner mitochondrial membrane was reversible [17,18,20].

3.2. Trehalose loading into mitochondrial matrix

The amount of trehalose in each sample of mitochondrial lysate was quantified by integrating the HPLC peak for trehalose and then normalizing the value to the protein concentration of each sample. Fig. 2 shows that trehalose accumulated rapidly in the mitochondrial matrix. The matrix concentration of trehalose reached approximately 0.24 mmol/mg protein in just 1 min after MPTP opening. The maximum concentration of 0.29 mmol/mg protein was reached after 5 min, and extending the time of MPTP opening beyond this point did not promote any further loading. Considering that these rat liver mitochondria should have a matrix space of approximately 1.5 Al/mg protein in this swollen, permeabilized state [17], the intra-matrix concentration would be equivalent to 190 mM, which was 76% of the theoretical maximum (250 mM) if full equilibration had been achieved. One possible reason for the lack of full equilibration could be the loss of loaded trehalose during washing of mitochondria in preparation

for HPLC. Al-Nasser and Crompton [17] found that about 26% of the sucrose originally entrapped in the mitochondrial matrix was lost during washing procedures. Therefore, it is reasonable to assume that trehalose reached equilibrium after 5 min of MPTP opening. It was interesting to find that non-permeabilized mitochondria also retained some trehalose after washing (about one tenth of that in permeabilized mitochondria). The possibility of contamination from residual extra-matrix trehalose was not likely since the amount of trehalose in the final washing solution was negligible (data not shown). One possible explanation is that trehalose could bind to the surface of mitochondrial membranes, which might be refractory to the washing procedures. The interaction of trehalose with lipid membranes in dilute trehalose solution has been reported recently [26,27]. Another possibility is entry of trehalose across the inner membrane occurred due to the gravitational field applied during centrifugation [28].

3.3. Beneficial effect of external and internal trehalose

Because DW is required for normal mitochondrial functions including ATP synthesis, import of mitochondrial protein, and metabolite transport, DW was used to estimate the integrity of desiccated mitochondria upon rehydration. Fig. 3 shows the DW of rehydrated mitochondria which were dried to a water content of 0.15 T 0.05 g water/g solids in SB-trehalose after permeabilization or in SB-KCl solution. The trehalose-loaded mitochondria displayed a high DW after rehydration. The DW is sensitive to the electron transport inhibitor, antimycin, verifying the integrity of the rehydrated mitochondria. On the other hand, the non-trehalose-loaded mitochondria previously desiccated in SB-KCl solution without trehalose did not display a reasonable DW.

Fig. 4 shows the effect of the presence of external and/or internal trehalose on the normalized DW of rehydrated

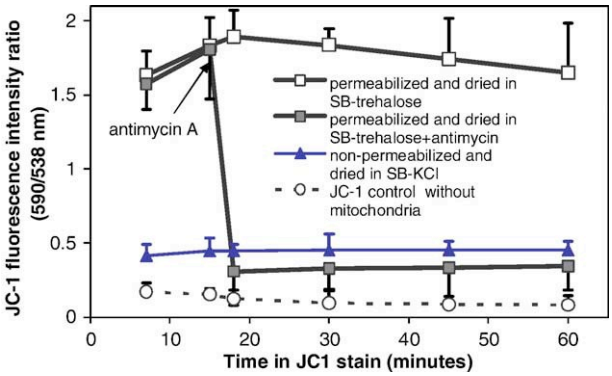

Fig. 3. DW of rehydrated mitochondria and its sensitivity to antimycin. Mitochondria were previously desiccated to a water content of 0.15 T 0.05 g water/g solids in SB-trehalose after permeabilization or in SB-KCl solution. JC-1 (0.2 Ag/ml) was added to the rehydrated mitochondria (10 Ag/ml) suspended in a buffer containing 20 mM Mops, 110 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, and 1 mM EGTA, pH 7.5 at room temperature. Antimycin A (20 AM) was added to the trehalose-loaded mitochondria 15 min after the addition of JC-1. The dash curve is the assay buffer with JC-1 only without mitochondria. Values are means T S.D., (N 3 5).

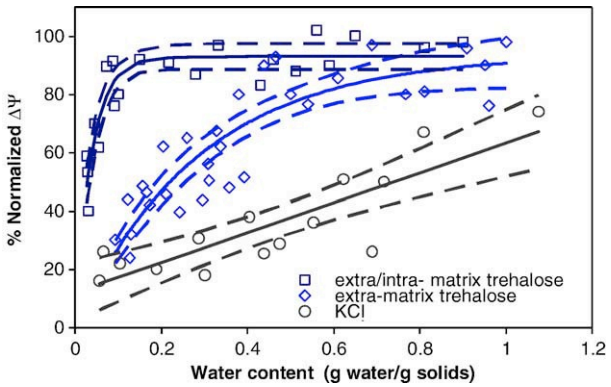

Fig. 4. Normalized membrane potential of rehydrated mitochondria as a function of the final water content reached during desiccation. Isolated rat mitochondria were dried to different water contents in SB-KCl (0.125 M KCl without trehalose), SB-trehalose (with 0.25 M extra-mitochondria trehalose only), and SB-trehalose after permeabilization (i.e., with both extra-mitochondrial and matrix trehalose present). After rehydration, DW was measured by the JC-1 fluorescence intensity ratio (590/538 nm), which was normalized to that of non-desiccated (control) mitochondria. Dash lines show 95% confidence interval for the data.

mitochondria as a function of the final water content. In all experimental cases, after re-hydration, DW decreased with decreasing water content. However, at any given water content, the trehalose-loaded mitochondria had the highest DW; mitochondria suspended in KCl solution without any trehalose had the lowest DW. For example, when mitochondria were dried to about 0.1 g water/g solids, the trehalose-loaded mitochondria retained 86% of the original DW while the non-permeabilized mitochondria dried in trehalose or KCl solutions had 38% and 21% of DW, respectively. Thus, the maximum protective effect of trehalose on integrity of the inner membrane during desiccation is seen when the sugar is present on both sides of the membrane.

These findings are in agreement with other studies for liposomes [29], bacteria [30], and mammalian cells [10], which show that desiccation protection by sugars such as trehalose is greatest when they are present on both surfaces the lipid bilayers or cell membranes. According to the "water replacement hypothesis" [3,4], sugars like trehalose depress the phase transition temperature (T_m) of membrane lipids by hydrogen bonding to phospholipids, thereby preventing phase transitions during rehydration. Hydrogen bonding between proteins and sugars can also inhibit protein unfolding and aggregation during drying. Because trehalose needs to be in direct contact with membrane proteins and lipids, it is reasonable to assume that trehalose should also be present in the mitochondrial matrix to confer maximum protection. Our data suggest that species that are naturally dehydration-tolerant must also have mechanisms in place to provision the mitochondrial matrix with trehalose. Preliminary data (G. Elliot, M. Menze, J. Reynolds, M. Toner, and S. Hand, unpublished) indicate that trehalose is present in the mitochondrial matrix of desiccation-tolerant embryos of the brine shrimp Artemia franciscana, and that greatly reduced levels are found in larval stages that are not resistant to drying. This observation is a further indication that

trehalose in the mitochondrial matrix may be necessary for desiccation tolerance.

Not surprisingly, the functional status of rehydrated mitochondria, as assessed by the respiratory control ratio, was significantly compromised. RCR values were between 2 and 3 post-desiccation, versus greater than 5 in control mitochondria (data not shown). This situation is likely due to loss and/or damage of key components necessary for re-establishing fully-coupled oxidative phosphorylation. Because trehalose-loaded mitochondria after desiccation (limited to water contents above 0.1 g water/g solids) display DW values nearly equivalent to control mitochondria, one would predict that components of the electron transport chain are functional and that proton leak across the inner membrane is not excessive. Indeed, state 4 respiration for trehalose-loaded mitochondria after desiccation was similar to controls (data not shown), which explains the capacity to re-establish DW; it was state 3 respiration that appeared to be compromised. Perhaps it is the adenine nucleotide transporter and/or the F_oF_1 ATP synthase that are most sensitive to the drying process. Studies addressing the mechanism(s) for this damage are currently underway. Nevertheless, the ability of trehalose to protect the capacity to re-establish DW is quite noteworthy.

3.4. Implication for desiccation of mammalian cells

This present study shows that introduction of trehalose into the matrix of isolated mitochondria improves the desiccation tolerance of this organelle in vitro. It is interesting to note that opening of MPTP and swelling of mitochondria in some cases can occur reversibly in situ without causing cytochrome c release and cell death [31,32]. Therefore, it may also be possible to introduce cytoplasmic trehalose into mitochondria in vivo by opening the MPTP without compromising cell viability. We think these findings may have important implication for desiccation storage of mammalian cells. Mitochondria play a pivotal role in many cellular functions including energy production, intracellular homeostasis of inorganic ions, cell motility, cell proliferation, and apoptosis. Damage to mitochondria would inevitably cause the disruption of cell function and eventually lead to cell death [33,34]. This scenario could be an explanation for the observation that cytoplasmic trehalose fails to completely protect mammalian cells during drying. Mitochondria could still be damaged due to the impermeability of the inner mitochondrial membrane to trehalose, even though the plasma membrane and cytoplasmic proteins are well protected. Protection of membrane-bound organelles such as mitochondria by introducing trehalose and/or other protective molecules into them may be crucial to the final successful desiccation of mammalian cells.

Acknowledgements

We thank Dr. Gloria D. Elliott and Ms. Jennifer Cusick for their expert technical assistance with HPLC. This work was supported by grants from the Defense Advanced Research Projects Agency/Naval Research Laboratories (N00173-01-1 G011), and National Institutes of Health (GM71345).

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