10-1-2016

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Recommended Citation

Moore, D., & Hand, S. (2016). Cryopreservation of lipid bilayers by LEA proteins from Artemia franciscana and trehalose. *Cryobiology, 73* (2), 240-247. https://doi.org/10.1016/j.cryobiol.2016.07.003

Cryopreservation of Lipid Bilayers by LEA Proteins from *Artemia franciscana* and Trehalose

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Running Head: Protection of lipid bilayers against freezing damage

Key Words: late embryogenesis abundant protein, trehalose, liposome, *Artemia franciscana*, freezing, intrinsically disordered protein, mitochondria.

Abbreviation: Cho, Cholesterol; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; mitochondrial permeability transition pore (MPTP); large unilamellar vesicles (LUVs); 5(6)-carboxyfluorescein (CF).

Abstract

The capacity of Late Embryogenesis Abundant (LEA) proteins and trehalose to protect liposomes against freezing-induced damage was examined by measuring the leakage of 5(6)-carboxyfluorescein (CF). Liposomes were prepared to simulate the lipid compositions of the inner leaflet of the plasma membrane, outer mitochondrial membrane (OMM), and inner mitochondrial membrane (IMM). Two recombinant LEA proteins belonging to Group 3 (AfrLEA2 and AfrLEA3m) were expressed and purified from embryos of *Artemia franciscana*. Only OMM-like liposomes were significantly protected by AfrLEA2 and AfrLEA3m against freeze-thaw damage; at the highest protein:lipid mass ratio tested, leakage of CF was 56.3% of control with AfrLEA3m and 29.3% with AfrLEA2. By comparison, trehalose provided protection to all compositional types. The greatest stabilization during freezing occurred when trehalose was present on both sides of the bilayer. When mitochondria isolated from rat liver were freeze-thawed in trehalose solution, the OMM remained intact based on the absence of increased oxygen consumption when cytochrome *c* was added during oxidative phosphorylation (OXPHOS). Respiratory control ratios (OXPHOS/LEAK) were depressed by only 30% after freeze-thawing in trehalose compared to non-frozen controls, which indicated some retention of OXPHOS capacity by the IMM. Trehalose then was loaded into the matrix (0.24 μmol/mg mitochondrial protein) by transient opening of the permeability transition pore, a procedure optimized for retention of OMM integrity. Surprisingly, respiratory control ratios were not improved after freeze-thawing with external plus matrix trehalose, when compared to external trehalose alone. This result could perhaps be explained by insufficient accumulation of matrix trehalose.

Introduction

Anhydrobiotic organisms like embryos of brine shrimp, *Artemia franciscana*, are able to survive the virtual absence of water [15,20,24]. Many organisms that tolerate extreme water stress utilize common protective mechanisms like depressed cellular metabolism and accumulation of specialized proteins and solutes of low molecular weight [14,16,17,20,33,64]. Prominent among the protective molecules found to accumulate in embryos of brine shrimp are late embryogenesis abundant (LEA) proteins [10,34,41,54,57,58] and the sugar trehalose [12,13,27,31,43]. LEA proteins are a family of hydrophilic and often intrinsically disordered proteins expressed in desiccation-tolerant plants and animals [35,55]. Trehalose is a non-reducing disaccharide that accumulates in desiccation tolerant organisms in response to water deficit or is constitutively maintained in developmental stages commonly encountering water stress [13,17,39,49]. In the present study, we evaluate the ability of two LEA proteins from *A. franciscana* to protect liposomes of different lipid compositions against freeze-induced damage, and we compare their effectiveness to the well-established capacity of trehalose for liposome stabilization. Finally, we expand upon existing information regarding the ability of trehalose to preserve the function of isolated mitochondrial during freezing and thawing.

Several LEA proteins form amphipathic alpha-helices capable of interacting with lipid bilayers [30,42,44,45,51,52,53]. However, stabilization of lipid bilayers during water stress has only been experimentally demonstrated for three LEA proteins from plants and two from animals. COR15a and COR15b (LEA23 and LEA24) from *Arabidopsis thaliana* protect chloroplast membranes from freeze-induced damage both *in vitro* and *in vivo* [4,47,50]. PsLEAm from the pea plant (*Pissum savitum*) improves the stability of either dried or frozen liposomes [52]. AfrLEA2 and AfrLEA3m from embryos of *A. franciscana* protect liposomes

87 from desiccation-induced damage [42]. These latter two LEA proteins were used in this
88 current freezing study with liposomes that mimic the lipid content the inner leaflet of the
89 plasma membrane (ILPM), outer mitochondrial membrane (OMM) and inner mitochondrial
90 membrane (IMM).

91 Previous studies have demonstrated the ability of trehalose to protect liposomes and
92 membranes from freezing and desiccation-induced damage [11,17,21,23,38,48,62,63]. Like
93 many sugars, trehalose forms a glass at low water contents and/or temperatures [18].
94 Trehalose is sometimes superior to other sugars at protecting macromolecules due to its
95 relatively high glass transition temperature [22,23]. Sugar glasses provide structural support
96 and immobilize cellular components, which are important for preventing deleterious
97 interactions. In addition to sugar glass formation, there is evidence that trehalose has the
98 ability to bind to dry membranes in the place of water [25,59]. This "water replacement"
99 favors the retention of the liquid crystalline phase of lipid bilayers. For maximal protection of
100 a membrane trehalose must be present on both sides of a lipid bilayer [23,25]. There is some
101 evidence that trehalose protection during drying is dependent on the lipid composition of the
102 target membrane [36,37].

103 For cells to survive water stress, it is critical that cellular components such as the
104 mitochondria be preserved [5,32]. To optimally preserve mitochondria, it would appear
105 important that trehalose contact both surfaces of the relevant bilayers [26,38]. The outer
106 membrane of mitochondria is permeable to solutes with a molecular weight less than 5 kDa
107 due to non-specific protein channels like porin [40,56]. While chemically unmodified
108 trehalose is apparently not permeable across the IMM of mammalian cells [cf. 1], it is possible
109 to load disaccharides into the matrix of isolated mammalian mitochondria by transient opening

of the mitochondrial permeability transition pore (MPTP) [2,38]. The MPTP is a mega-pore of the inner membrane that is comprised of dimers of the ATP synthase [7,28]. Based on (1) results below that show protection by trehalose is greatest when present on both sides of IMM-like liposomes during freeze/thawing and (2) previous experiments on drying of isolated mitochondria [38], we viewed it appropriate to test whether loading trehalose into the matrix of mitochondria isolated from rat liver prior to freezing improves retention of respiratory function after thawing.

Materials and Methods

Biochemicals

Lipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids were naturally derived in order to mimic the fatty acid distribution found in eukaryotic cells. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were derived from bovine liver (Avanti Polar Lipids; product numbers 840055, 840026, 840042). Because these phospholipids were naturally derived, they contain a mixture of fatty acids. However, the predominate phospholipid species in each sample is reported by Avanti to be 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, and 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoinositol. Phosphatidylserine (PS; predominantly 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine) was purified from bovine brain tissue (Avanti Polar Lipids; product number 840032) and cardiolipin (CL; predominantly 1',3'-bis[1,2-dilinoleoyl-sn-glycero-3-phospho]-sn-glycerol) from bovine heart (Avanti Polar Lipids; product number 840012). Cholesterol was isolated from ovine wool (Avanti Polar Lipids; product number 700000P). 5(6)-Carboxyfluorescein (CF) (product number 21877) was obtained from Sigma-Aldrich (St. Louis, MO). Trehalose (product number T-104-4) was purchased from Pfanstiehl (Waukegan, IL). Bovine serum albumin (product number A6003, essentially fatty acid free) was purchased from Sigma-Aldrich.

Liposome Preparation

Lipids were combined with mass ratios simulating the inner leaflet of the plasma membrane (ILPM), outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) inner mitochondrial membrane of mammalian cells as previously reported [42]: ILPM

(50 % PC, 30 % PE, 10 % PS, 10 % Cho), IMM (50 % PC, 30 % PE, 20 % CL), OMM (55 % PC, 30 % PE, 15 % PI). For comparison to a non-physiological bilayer, liposomes composed entirely of phosphadidylcholine were also prepared. Large unilaminar vesicles (LUVs) extruded and rinsed precisely as described previously [42]. Liposomes were mixed with 250 mM trehalose (19:1 sugar:lipid mass ratio) and/or LEA proteins to yield various protein:lipid mass ratios between 0.1 and 0.4. Recombinant AfrLEA3m and AfrLEA2 were purified as described by Boswell et al. [10]. Liposomes were mixed with equal amounts of BSA in order to compare protection by LEA proteins to that of a protein not associated with membrane stabilization. For some experiments (i.e., Fig. 2B), 250 mM trehalose was included during the preparation of LUVs, so that the sugar also was present inside of the liposomes. The final concentration of liposomes was approximately 5 mg total lipid/ml.

Liposome Freezing and Measurements of CF Leakage

For CF leakage assays, 2 µl droplets of liposome mixtures were placed in the wells of opaque 96-well plates (OptiPlate 96-F, PerkinElmer, Waltham, MA). Plates were covered in plastic wrap and frozen at -80°C for at least 1 week. Fluorescence of CF is strongly quenched at the high concentration of 100 mM contained inside of the liposomes, but increases when CF is released into the medium [60]. CF fluorescence was measured as described previously [42]. Due to inherent differences in control leakage across compositional types, experimental treatments were normalized to the controls (no protectant) when comparing stabilization of liposomes with various lipid compositions.

Isolation of Mitochondria

Mitochondrial isolation from rat liver was performed as outlined by Gnaiger et al. [29]. Briefly, male Spraque-Dawley rats were euthanized by CO_2 asphyxiation in accordance with

protocol 12-061 approved by the LSU Institutional Animal Care and Use and Committee. The liver (approx. 10-15 g) was excised and immediately placed in ice-cold homogenization medium A (HMA), which was composed of 250 mM trehalose, 10 mM Tris, 1 mM EGTA (pH 7.4). The large lobes of the liver were finely diced and any connective tissue observed was removed. The minced liver was rinsed several times in HMA and transferred to a 40 ml Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The liver was suspended in 30 ml of homogenization medium B (HMB), which was HMA fortified with 0.2% BSA. The homogenate was centrifuged at 700 x *g* for 10 min at 4°C. The supernatant was centrifuged at 9,000 x *g* for 10 min at 4°C. The pellet was resuspended in 30 ml of fresh HMB and centrifuged once more at 9,000 x *g* for 10 min at 4°C. The resulting mitochondrial pellet was resuspended in HMA at 40-60 mg protein/ml as determined by Coomassie Plus (Bradford) protein assay (Thermo Fisher Scientific Inc., Rockford, IL).

Transient Opening of the MPTP

Loading the matrix of isolated mitochondria with trehalose via transient opening of the mitochondrial permeability transition pore (MPTP) was performed as described by Al-Nassar and Crompton [2]. Mitochondria suspended in HMA were diluted to 0.5-1 mg/ml in HMA that contained no EGTA, which resulted in a buffer composed of 250 mM trehalose, 10 mM Tris, and 25 μM EGTA (pH 7.4). MPTP formation was induced by the addition of glutamate (10 mM), malate (2 mM), KH_2PO_4 (1 mM), and Ca^{2+} [6,8,65]. For freezing studies, MPTP opening was induced by the addition of 35 μM Ca^{2+}. After 1 min the pore was closed by the addition of 2 mM EGTA, 1 mM $MgCl_2$, 1 mM NAD^+, and 2 mg/ml BSA [9,65]. Mitochondria were incubated at room temperature for ~8 min after the addition of EGTA to allow complete closure of the pore [2,65]. Control (i.e. non-permeabilized) mitochondria were incubated

under identical conditions with the exception that calcium was not added to the medium. For comparison of trehalose-incubated mitochondria to ones that were not exposed to trehalose, mitochondria were isolated and frozen in a buffer composed of 150 mM KCl, 10 mM Tris, 2 mM EGTA, and 2 mg/ml BSA (pH 7.4).

Quantification of Trehalose by HPLC

After opening and reclosing of the MPTP, mitochondria were sedimented by centrifugation (10,000 x *g* for 10 min at 4°C). To remove extra-mitochondrial trehalose, mitochondrial pellets were resuspended in buffer composed of 250 mM sucrose, 10 mM Tris, and 1 mM EGTA (pH 7.4) and centrifuged once more at 10,000 x *g* for 10 min at 4°C, after which the supernatant was discarded and the mitochondrial pellet resuspended in water. The suspension was frozen at -80°C and thawed to ensure complete release of trehalose from the matrix. The sample was heated at 95°C for 20 min then centrifuged at 20,000 x *g* for 30 min at 4°C to remove precipitated protein. HPLC analysis was performed as described by Patil et al. [43]. Values were expressed as μmol trehalose per mg mitochondrial protein.

Freezing of Mitochondria

Mitochondria were frozen as described by Yamaguchi et al. [63]. Mitochondria were suspended at 40 mg mitochondrial protein/ml in HMB. Plastic microcentrifuge tubes (0.6 ml) were placed on a bed of dry ice pellets, and a 5 μl droplet of mitochondrial suspension was pipetted into each tube. The tubes were transferred in to liquid nitrogen and then placed in a -80°C freezer overnight. Droplets were thawed quickly by warming between fingers.

Measurements of Mitochondrial Respiration

Oxygen consumption of mitochondria was measured at 37°C with an Oxygraph 2K (OROBOROS Instruments, Innsbruck, Austria), and the data were analyzed with DatLab

software (OROBOROS Instruments). The rate of oxygen consumption was calculated as the time derivative of oxygen concentration and normalized per mg mitochondrial protein (nmol O_2 s^{-1} mg mitochondrial $protein^{-1}$). Complex II-dependent respiration was measured in the presence of succinate (10 mM) and rotenone (0.5 μM; inhibitor of Complex I). Proton leak respiration (LEAK state, or state 4) was measured in the presence of substrate without ADP. The addition of ADP (5 mM) stimulated maximum oxidative phosphorylation (OXPHOS state, or state 3). The respiratory control ratio (RCR) was calculated by dividing OXPHOS by LEAK respiration. Cytochrome *c* (cyt *c;* 4 mM) was added to the respiration medium during the OXPHOS state in order to assess the integrity of the outer membrane; an increase in oxygen consumption was indicative of compromised membrane integrity. Finally, carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) was titrated step-wise to estimate the maximum respiratory capacity of the electron transport system in the uncoupled state (ETS state).

Statistics

Two-way ANOVA paired with a Tukey post-hoc test were used to compare protection of each type of liposome by AfrLEA3m, AfrLEA2, or BSA across the range of protein:lipid mass ratios tested (Prism 6; GraphPad Software, La Jolla, CA). A Bonferroni multiple comparisons test of sample means was used to identify statistical differences between trehalose and protein combinations compared to each component alone (Prism 6). 1-way ANOVA paired with a Tukey multiple comparisons test was used to compare protection of each type of liposome by trehalose alone. Paired and unpaired t-tests were used to compare trehalose content and mitochondrial respiration rates and RCRs before and after freezing or permeabilization (Prism 6). Significance level was set at $p \leq 0.05$.

Results

Stability of Liposomes Frozen with LEA Proteins

Protection of liposomes by LEA proteins varied depending on the lipid composition of the liposomes (Fig. 1). The only statistically significant (2-way ANOVA, Bonferroni multiple comparisons test, $p>0.05$, n=3-6) improvement in stability during freezing and thawing occurred with liposomes that simulated the composition of the outer mitochondrial membrane (OMM). When quantified at the highest protein:lipid mass ratio tested, leakage of CF from OMM-like liposomes was 56.3±2.8% (mean ± SD, n=6) of control leakage in the presence of AfrLEA3m and 29.3±2.5% (mean ± SD, n=6) in the presence of AfrLEA2 (Fig. 1A and B; 2-way ANOVA, Bonferroni multiple comparisons test, $p>0.05$, n=3-6). After freeze-thawing in the presence of AfrLEA3m, there were no statistical differences compared to controls in CF loss from liposomes that mimicked the inner leaflet of the plasma membrane (ILPM), the inner mitochondrial membrane (IMM), or in those composed of 100% phosphatidylcholine (Fig. 1A, PC; 2-way ANOVA, Bonferroni multiple comparisons test, $p>0.05$, n=3-6). Likewise, AfrLEA2 did not significantly diminish CF leakage after freeze-thawing from IMM, ILPM, or PC liposomes (Fig. 1B; 2-way ANOVA, Bonferroni multiple comparisons test, $p>0.05$, n=3-6). The control protein, BSA, did not depress CF leakage from any liposomes tested except at the highest protein:lipid mass ratio tested with OMM-like liposomes, and this depression was very modest (Fig. 1C; 2-way ANOVA, Bonferroni multiple comparisons test, $p>0.05$, n=3-6). It is noteworthy that the ILPM-like liposomes generally displayed greater variability in leakage during freezing experiments compared to other compositional types for reasons that are unclear.

Stability of Liposomes Frozen with Trehalose

Relative to controls (no sugar added), 250 mM external trehalose significantly depressed CF leakage from liposomes for all compositional types tested (Fig. 2A; 2-way ANOVA, Bonferroni multiple comparisons test, $p<0.05$). Trehalose protected non-biological liposomes composed of 100% PC and liposomes with lipid compositions similar to the OMM and ILPM with equal efficacy. However, CF loss from IMM-like liposomes in the presence of trehalose was significantly greater than for the other liposomes (1-way ANOVA, Tukey multiple comparisons test, $p<0.05$, n=3-6). Therefore, lipid composition did affect the degree to which trehalose improved liposome stability. The relatively low protection afforded to IMM-like liposomes is consistent with loss of inner membrane function by isolated mitochondria frozen with external trehalose [63]. This result may indicate that the requirement for trehalose to be present on both sides of a membrane to provide maximal protection could be particularly important for preservation of the IMM during freeze/thawing. In order to evaluate maximal protection by trehalose, IMM-like liposomes were prepared with 250 mM trehalose inside and outside. Loss of CF from IMM-like liposomes significantly decreased (1-way ANOVA, Tukey multiple comparisons test, $p<0.05$, n=3) from 22.9±1.3% (mean ± SD, n=6) in the presence of external trehalose alone to 7.1±0.2% (mean ± SD, n=3) when liposomes were provisioned with both internal and external trehalose (Fig. 2B). Low leakage was observed in liposomes even without protectant when frozen at -80 C as performed in this study (Fig. 2B), an issue to which we will return in the Discussion section.

Stabilization of liposomes by LEA proteins in combination with trehalose was also examined. No significant increase in protection beyond that seen with trehalose alone was detected for any of the liposomes when LEA proteins were added in conjunction with trehalose (data not shown).

Optimization of Transient Opening of the MPTP

Because the presence of trehalose on both sides of the lipid bilayer improved the integrity of IMM model liposomes during freezing, we wished to compare stabilization of isolated mitochondria frozen with and without trehalose in the matrix. Transient opening of the MPTP has been used to load sugars into the matrix of mitochondria isolated from rat liver [2,38]. However, the opening and closing of the pore required optimization to preserve the respiratory function of mitochondria. Mitochondria that were permeabilized by the addition of 35 μM Ca^{2+} for 1 min and then resealed accumulated significant trehalose and also retained respiratory function and outer membrane integrity (Fig. 3). By inducing transient opening of the MPTP in this manner, 0.24 ± 0.03 μmol trehalose/mg mitochondrial protein (mean ± SD, n = 6) was incorporated into the matrix (Fig. 3A). The increase in OXPHOS respiration after the addition of exogenous cytochrome *c* was less than 10% and not significantly different from that measured for non-permeabilized mitochondria (Fig. 3B; Student's paired t-test, $p>0.05$, n=6), which indicated that the outer membrane remained substantially intact after transient permeabilization with 35 μM Ca^{2+}. The respiratory control ratio (RCR) was unchanged as well after permeabilization with 35 μM Ca^{2+} (Fig. 3C; Student's paired t-test, $p>0.05$, n=6). In contrast, when 100 μM Ca^{2} for 1 min was used to induce pore formation, RCR values significantly decreased (Fig. 3C; Student's paired t-test, $p<0.05$, n=6), which was a result of diminished OXPHOS with no change in LEAK (data not shown). Stimulation of OXPHOS by cyt *c* was increased (Fig. 3B; Student's paired t-test, $p<0.05$, n=6). The amount of trehalose loaded into the matrix of mitochondria after permeabilizing with of 100 μM Ca^{2+} (0.31±0.03 μmol trehalose/mg mitochondrial protein) was significantly greater than achieved with 35 μM Ca^{2+} (Fig. 3A; Student's paired t-test, $p<0.05$, n=3-5). Nevertheless, to test whether matrix

trehalose could improve mitochondrial function after freeze-thawing, 35 μM Ca^{2+} was chosen for MPTP opening due the lack of impact on OMM integrity and RCR.

Cryoprotection of Isolated Mitochondria by Trehalose

Respiratory function of mitochondria before and after freezing was measured to assess protection of mitochondria by trehalose (Fig. 4). Prior to freezing, all RCR values were > 4 when succinate was used as a substrate. In the absence of trehalose (replaced by 150 mM KCl), the respiratory control ratio was diminished to 0.98±0.36 (mean ± SD, n = 4) after freezing and thawing (Fig. 4A, right panel). This RCR value reflects the lack of respiratory stimulation above LEAK upon ADP addition; there was both an increase in LEAK and a decrease in OXPHOS after freezing in KCl medium compared to non-frozen controls (Fig. 4A, left panel; unpaired t-tests, Holm-Sidak post-hoc, $p<0.05$, n=4). The addition of cyt *c* resulted in an increase in respiration above OXPHOS after freeze-thawing, which indicated compromised integrity of the outer membrane when frozen with KCl (Fig. 4A, left panel; unpaired t-tests, Holm-Sidak post-hoc, $p<0.05$, n=4). In contrast, mitochondria frozen with 300 mM external trehalose showed improved RCR values (3.8 ± 0.4, mean ± SD, n=4-5) compared to those frozen in KCl (Fig. 4A and B, right panels; Student's unpaired t-test**,** $p\leq 0.05$, n = 4). Additionally, the external trehalose eliminated any cyt *c* effect (compared to OXPHOS) post-freezing (Fig. 4B, left panel; unpaired t-tests, Holm-Sidak post-hoc, $p>0.05$, n=4-5); thus, the outer membrane was protected. Providing trehalose to the matrix compartment along with external trehalose did not improve freeze tolerance compared to mitochondria frozen with external trehalose alone (Fig. 4C). Indeed, the RCR value post freezing (2.6 ± 0.2, mean ± SD, n = 4) was actually less than that measured with external trehalose alone (Student's unpaired t-test**,** $p\leq 0.05$, n = 4-5). It may be that the amount of

trehalose loaded into the matrix with 35 µM Ca^{2+} was insufficient to improve the RCR or to compensate for any negative effect that opening/closing the MPTP caused in the RCR that only became evident after freezing (see Discussion).

Discussion

In this study we have shown that cryopreservation of liposomes by AfrLEA2 and AfrLEA3m was dependent on the lipid composition of the bilayer. Only liposomes mimicking the outer mitochondrial membrane were significantly stabilized by LEA proteins from *A. franciscana* during freezing and thawing. External trehalose significantly decreased damage to all liposomes tested. However, when trehalose was present on both sides of the lipid bilayer during freezing, as tested with IMM-like liposomes, the stabilization was much greater. Based on this result, we investigated whether isolated mitochondria provisioned with trehalose in the matrix would retain better respiratory function than mitochondria frozen with trehalose present only outside of the matrix. Isolated mitochondria maintained outer membrane integrity and exhibited improved respiratory function when frozen in a medium that contained 300 mM trehalose, compared to mitochondria in a KCl solution without trehalose. However, loading trehalose into the matrix (at least to the modest levels obtained by transient opening of the MPTP) did not improve the RCR after freeze-thawing compared to external trehalose alone.

LEA Proteins and the Stability of Frozen Liposomes

Freezing damage to liposomes with a lipid composition mimicking the OMM was substantially reduced by AfrLEA2 and AfrLEA3m. However, these LEA proteins did not have any effect on freeze-induced damage to IMM-like, ILPM-like, and PC liposomes, which indicated that protection is dependent on the lipid composition of the bilayer. This lack of stabilization of PC liposomes was unexpected because Tolleter et al. [52] report a ~15-25% decrease in CF leakage from PC liposomes frozen with PsLEAm at similar protein:lipid mass ratios. Additionally, COR15a and COR15b offset freeze-induced damage to chloroplast-like liposomes [50]. Liposomes were frozen at -18°C in the study by Tolleler et al. [52] and at -

353 20°C by Thalhammer et al. [50]. In our study liposomes were frozen at -80°C, which may
354 have been less damaging. Indeed, our leakage values for control liposomes (without
355 protectants) across all compositional types were similar to the smallest amount of leakage
356 reported by Tolleter et al. [52] for liposomes protected with PsLEAm. Thus, in order to detect
357 broader protection of liposomes by AfrLEA2 or AfrLEA3m, perhaps freezing conditions that
358 promote greater damage are needed. It may also be that AfrLEA2 and AfrLEA3m are simply
359 less effective stabilizers of liposomes during freezing than other LEA proteins, or that freezing
360 at -80°C alters the nature of the final secondary structure gained by LEA proteins. Slow versus
361 fast drying modifies the final percentages of α-helix versus β-sheet for plant LEA proteins for
362 example [61].

363 *Protection of Liposomes by Trehalose*

364 In the present study, liposomes frozen with 250 mM trehalose retained significantly
365 more CF than liposomes frozen without trehalose, regardless of the lipid composition of the
366 liposomes. However, IMM-like liposomes were not protected as well compared to all others.
367 Therefore, lipid composition can affect the ability of trehalose to stabilize lipid bilayers. It
368 must be emphasized that most experiments herein were performed with trehalose only on the
369 outside of liposomes. Many studies with trehalose have demonstrated that virtually no damage
370 occurs to liposomes during freezing if at least 0.2 M trehalose is present on both sides of the
371 lipid bilayer [e.g.,(3,19,46]. However, liposomes with lipid compositions similar to the IMM of
372 mammalian mitochondria were not previously investigated. Consequently, IMM-like
373 liposomes were frozen with internal and external trehalose (Fig. 2B), and CF leakage was
374 significantly reduced, which extends the established paradigm to this compositional type as
375 well.

Trehalose Loading Via Transient Opening of the MPTP

The impact of transient pore opening on the respiratory function of mitochondria has not been the primary focus of studies involving the MPTP. Often respiratory measurements before and after pore opening in isolated mitochondria are not reported. Al-Nasser and Crompton [2] found that mitochondria isolated from rat liver retained RCR values after loading sucrose via transient opening of the MPTP comparable to those of non-permeabilized mitochondria. However, respiration was measured in the presence of exogenous cyt *c*, and therefore any damage to the outer membrane would not have been detected. Liu et al. [38] demonstrated that mitochondria loaded with trehalose in the matrix via transient MPTP formation retained a greater capacity to generate membrane potential after drying compared to mitochondria dried without trehalose or with trehalose present only outside of the matrix. However, RCR values were diminished from >5 before drying to less than 3 after rehydration. Respiratory measurements were not taken after transient MPTP opening prior to drying, so it is possible the OMM was compromised to some degree before desiccation. Nevertheless, matrix trehalose does appear able to stabilize the inner mitochondrial membrane during drying based on the results of Liu et al. [38]. Our results indicate that OXPHOS can be compromised by cyt *c* loss if high Ca^{++} concentrations (100 µM) are used for pore opening.

Stability of Isolated Mitochondria Frozen with Trehalose

Mitochondria isolated from rat liver retain significant respiratory function when frozen in 300 mM trehalose, similar to that reported for mouse mitochondria by Yamaguchi et al. [63]. The fact that mammalian mitochondria exhibit some degree of coupling after freeze-thawing is noteworthy. Mitochondria isolated from mouse liver also retain outer membrane

integrity when frozen in 300 mM trehalose medium [63], as shown here for rat liver mitochondria by the cyt *c* test (Fig. 4B).

Surprisingly, loading trehalose into the matrix of mitochondria did not improve the respiratory performance of the organelle after freeze-thawing. We speculate that the lower RCR after freezing was largely due to compromised OXPHOS, although the apparent decrease in the mean value did not reach statistical significance (unpaired t-tests, Holm-Sidak post-hoc, $p>0.05$, n=4-5). In any case, the RCR result is possibly due to an insufficient amount of trehalose loaded into the matrix. Transient opening of the MPTP was optimized to preserve outer membrane integrity and respiratory function. As Figure 3 shows, there is a trade-off between retention of these characteristics and the quantity of trehalose loaded into the matrix. Stabilization of mammalian cells and mitochondria during freezing is dependent on the concentration of trehalose [11,63]. Yamaguchi et al [63] reported that mitochondria isolated from mouse liver retained outer membrane integrity when frozen in 300 mM trehalose but not in 250 mM trehalose. Thus small differences in effective trehalose concentration in the matrix could explain why stabilization is observed in some cases and not others, coupled with differences in the type and severity of water stress imposed.

Acknowledgements. This work was supported by National Science Foundation grants IOS-0920254 and IOS-1457061/IOS-1456809. We would like to thank Prof. Ramachandra Devireddy, Department of Mechanical and Industrial Engineering at LSU, for his advice with preparation of liposomes.

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Figure Legends

Figure 1. Leakage of carboxyfluorescein (CF) from liposomes frozen and thawed in the presence of LEA proteins and BSA. Lipid compositions simulated those of the inner mitochondrial membrane, the outer mitochondrial membrane, the inner leaflet of the plasma membrane, or a non-biological composition of 100 % phosphatidylcholine. Liposomes were frozen at the indicated protein:lipid mass ratios for AfrLEA3m **(A)**, AfrLEA2 **(B)**, or BSA **(C)**. Data are expressed as means ± SD (n = 3-6 samples). Where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly less CF leakage than that of liposomes frozen without protectants (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n = 3-6).

Figure 2. (A) Cryoprotection by external trehalose of liposomes with different lipid compositions. Liposome stability was assessed by leakage of carboxyfluorescein (CF) Lipid compositions simulated those of the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM), the inner leaflet of the plasma membrane (ILPM), or a non-biological composition of 100 % phosphatidylcholine (PC). Data are expressed as means ± SD (n = 3-6 samples). Asterisks indicate significant decrease of CF loss compared to controls (1-way ANOVA, Tukey multiple comparisons test, $p<0.05$). **(B)** IMM-like liposomes were prepared with and without 250 mM trehalose present on the inside of the liposomes and frozen in a 250 mM trehalose solution. Control liposomes were frozen without any trehalose. Stability of liposomes was determined by CF leakage. Data are expressed as means ± SD (n = 3-6 samples). An asterisk indicates significant decrease of CF loss compared to controls. Double asterisks indicate significant decrease of CF leakage compared to liposomes frozen

613 with external trehalose only (1-way ANOVA, Tukey multiple comparisons test, $p<0.05$, n = 3-
614 6).

615 **Figure 3.** Matrix trehalose content **(A)**, stimulation of OXPHOS by exogenous cytochrome *c*
616 **(B)**, and respiratory control ratios (RCR) **(C)** after transient opening of the MPTP for
617 mitochondria isolated from rat liver. Addition of 35 or 100 μM $Ca^{2+}$was used to induce MPTP
618 opening for 1 min prior to closing the pore by chelation of Ca^{2+} with 2 mM EGTA. Control
619 mitochondria were incubated under identical conditions as permeabilized mitochondria except
620 that calcium was omitted. As a test for integrity of the OMM, OXPHOS was measured before
621 and after addition of 4 mM cyt *c* and expressed as percent increase after the addition. An
622 asterisk indicates significant difference from control values, and double asterisks indicate
623 significant difference from values obtained with 35 μM Ca^{2+} (Student's unpaired t-test, $p<0.05$,
624 n = 3-6).

625 **Figure 4.** Respiratory measurements of mitochondria isolated from rat liver before and after
626 freezing. Mitochondria were frozen in media that contained 150 mM KCl **(A)** or 300 mM
627 trehalose **(B** and **C)**. Trehalose was loaded into the matrix of mitochondria in **(C)** by transient
628 (1 min) opening of the permeability transition pore as promoted with 35 μM Ca^{2+} (see legend
629 to Fig. 3). Cytochrome *c* (+ cyt *c*) was added during the OXPHOS state and represents a test
630 for integrity of the OMM. Data represent the mean ± SD of 4-5 samples. Asterisks indicate a
631 significant change from non-frozen values and the double dagger symbol (‡) indicates a
632 significant increase in OXPHOS respiration after the addition of cytochrome *c* (Multiple
633 unpaired t-test, Holm-Sidak post hoc, $p\leq 0.05$, n=4-5).
634

Figure 1

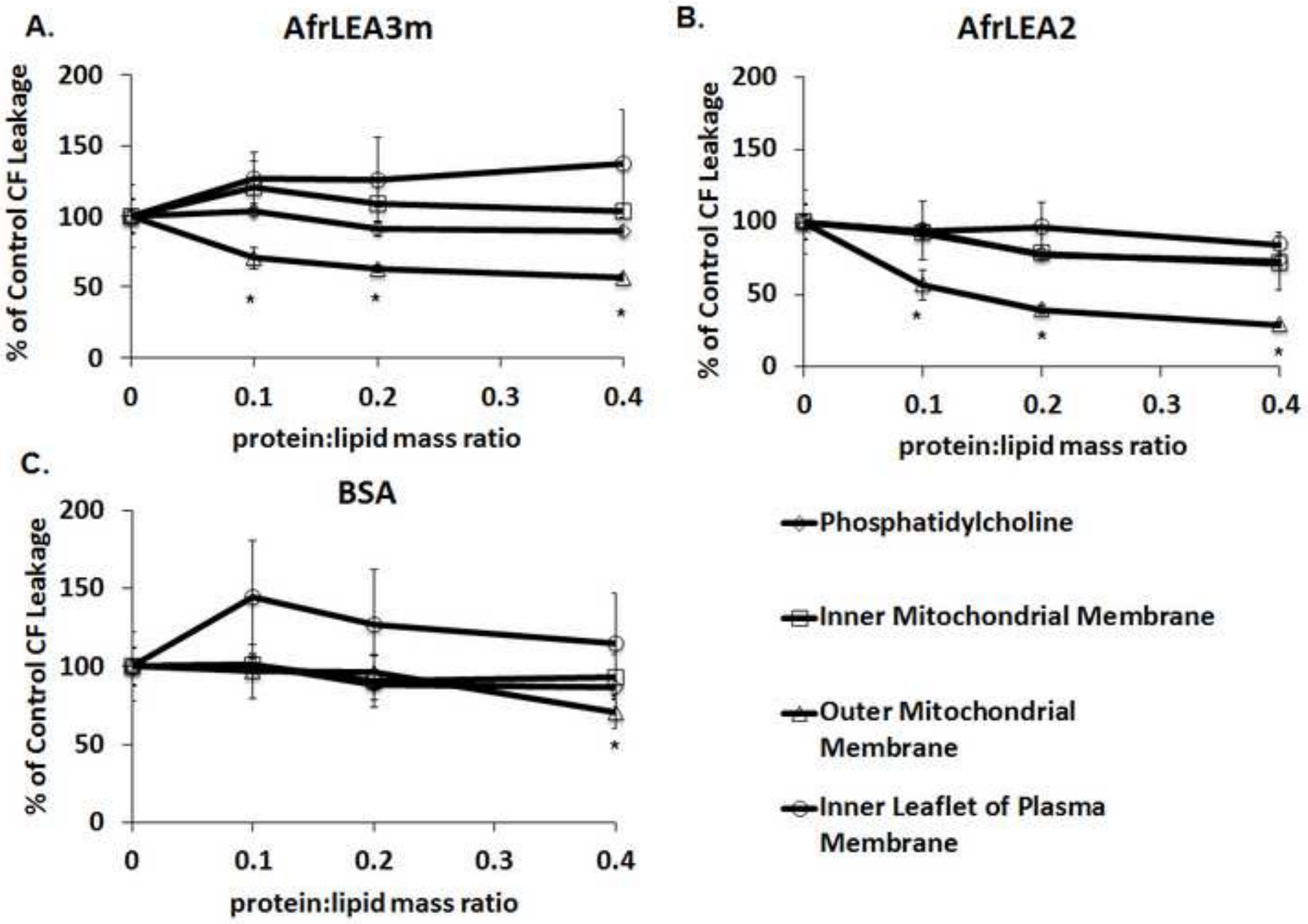

A.
AfrLEA3m
% of Control CF Leakage
200
150
100
50
0
0
0.1
0.2
0.3
0.4
protein:lipid mass ratio
B.
AfrLEA2
% of Control CF Leakage
200
150
100
50
0
0
0.1
0.2
0.3
0.4
protein:lipid mass ratio
C.
BSA
% of Control CF Leakage
200
150
100
50
0
0
0.1
0.2
0.3
0.4
protein:lipid mass ratio
Phosphatidylcholine
Inner Mitochondrial Membrane
Outer Mitochondrial Membrane
Inner Leaflet of Plasma Membrane

Figure 2

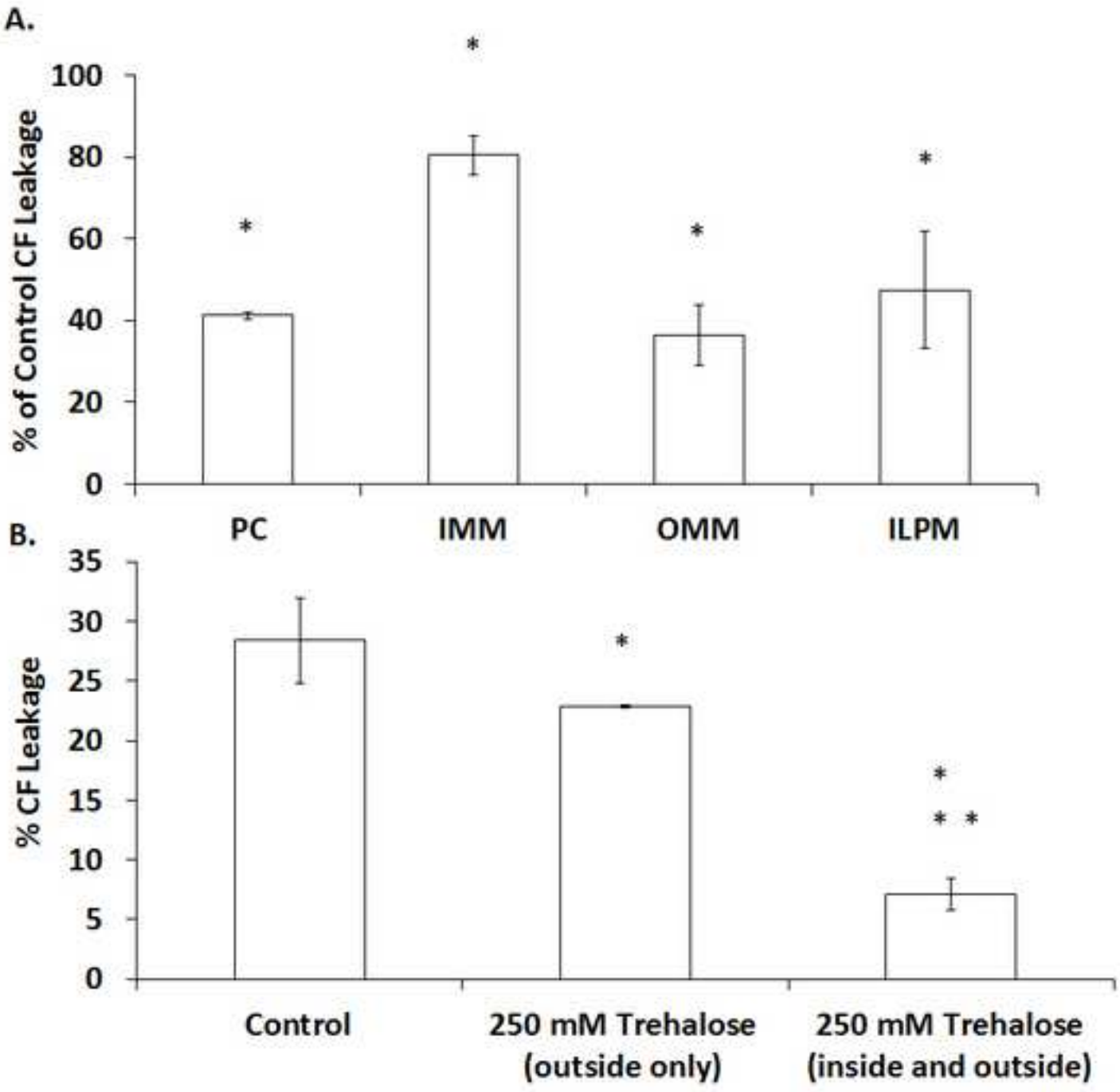

A.
% of Control CF Leakage
100
80
60
40
20
0
PC
IMM
OMM
ILPM
B.
% CF Leakage
35
30
25
20
15
10
5
0
Control
250 mM Trehalose (outside only)
250 mM Trehalose (inside and outside)

Figure 3

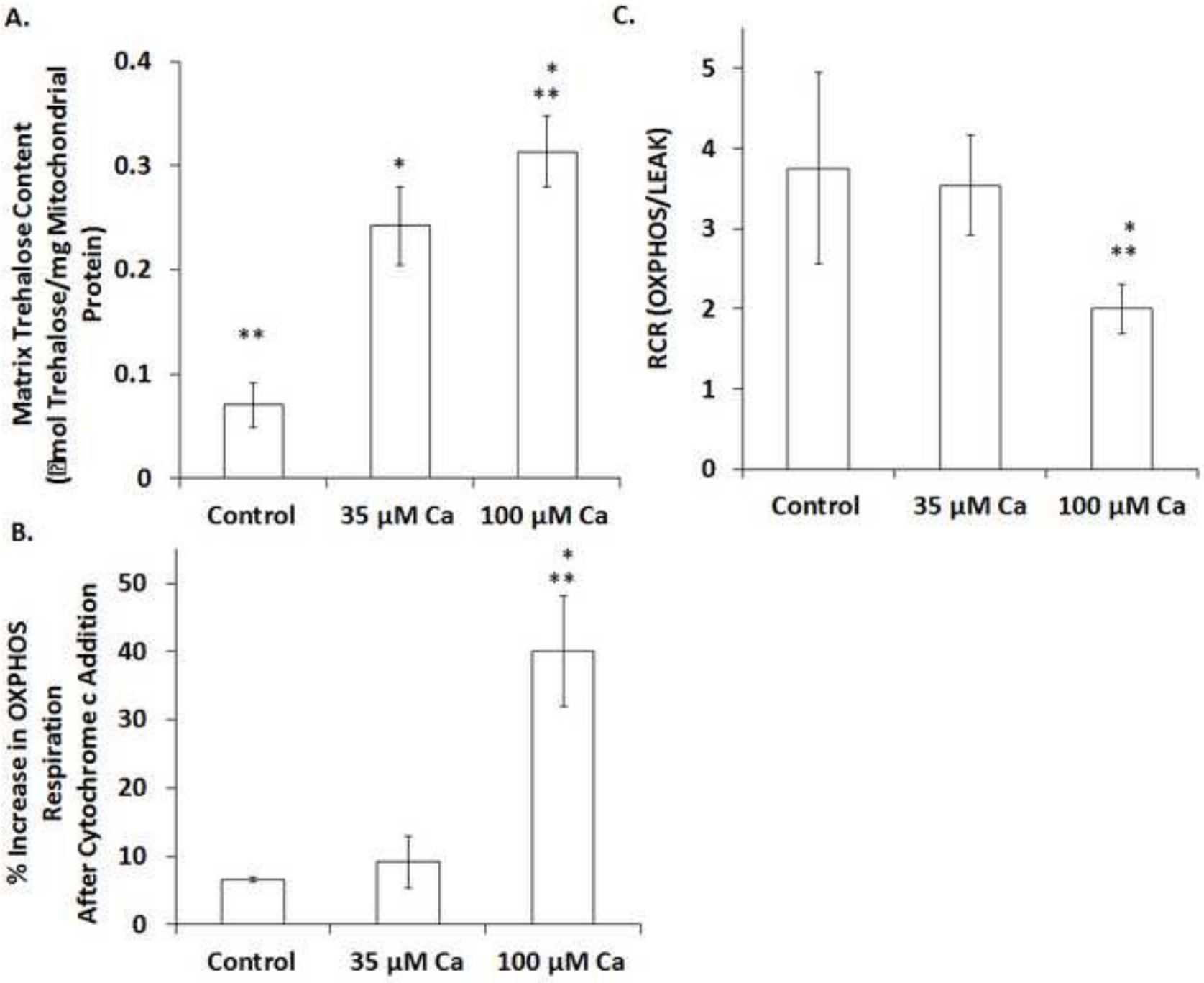
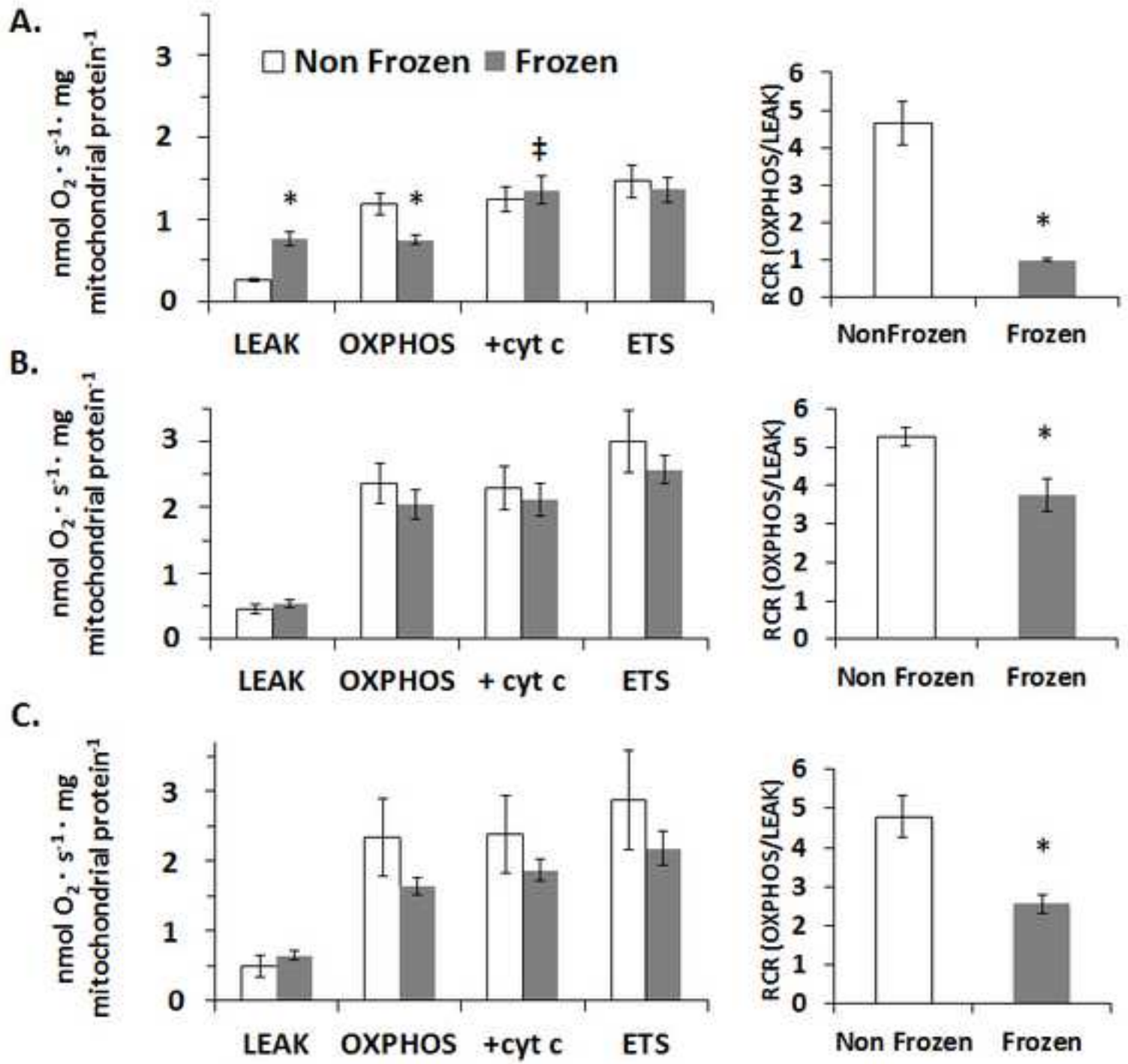

A.
Matrix Trehalose Content (nmol Trehalose/mg Mitochondrial Protein)
0.4
0.3
0.2
0.1
0
**
*
*
**
Control
35 µM Ca
100 µM Ca
C.
RCR (OXPHOS/LEAK)
5
4
3
2
1
0
*
**
Control
35 µM Ca
100 µM Ca
B.
% Increase in OXPHOS Respiration After Cytochrome c Addition
50
40
30
20
10
0
*
**
Control
35 µM Ca
100 µM Ca

Figure 4


A.
B.
C.
Non Frozen
Frozen
nmol $O_2 \cdot s^{-1} \cdot$ mg mitochondrial protein^{-1}
LEAK
OXPHOS
+cyt c
+ cyt c
ETS
RCR (OXPHOS/LEAK)
NonFrozen
Non Frozen
Frozen
*
‡