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Mitochondria frozen with trehalose retain a number of biological functions and preserve outer membrane integrity

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In apoptosis, Bcl-2-family proteins regulate the barrier function of the mitochondrial outer membrane (MOM), controlling the release of proapoptotic proteins from the intermembrane space into the cytoplasm. This process can be studied *in vitro* with freshly isolated mouse liver mitochondria. Unfortunately, mitochondria frozen/thawed in standard sucrose–mannitol buffers become leaky and useless for apoptosis research. However, here we show that mitochondria frozen in buffer containing the sugar, trehalose, maintained MOM integrity and responsiveness to Bcl-2-family proteins, much like fresh mitochondria. Trehalose also preserved ultrastructure, as well as biological functions such as ATP synthesis, calcium-induced swelling, transmembrane potential, and the import and processing of protein precursors. However, bioenergetic function was somewhat reduced. Thus, trehalose-frozen mitochondria retained most of the biological features of mitochondria including MOM integrity. Although not ideal for studies involving bioenergetics, this method will facilitate research on apoptosis and other mitochondrial functions that rely on an intact MOM.
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Since the 1970s, there have been a number of attempts to freeze isolated mitochondria for storage, with various degrees of success. In most of these studies, the functionality of frozen–thawed mitochondria was assessed with regard to bioenergetic parameters. In a recent and relatively successful attempt, frozen mitochondria were reported to have roughly 50% of normal respiratory function, and the mitochondria were well coupled.[1] However, in all these studies, partial retention of respiratory functions was accomplished at the expense of disrupting mitochondrial outer membrane (MOM) integrity.

In recent years, it became apparent that mitochondria play another important role in the cell besides respiration: that of regulating apoptosis. Permeabilization of the MOM is a critical step in the mitochondrial apoptotic pathway,[2–5] leading to the release of soluble proteins such as cytochrome *c*, Smac and Omi from the mitochondrial intermembrane space (IMS). Once these proapoptotic proteins are released into cytosol, the Apaf-1 apoptosome and caspases are activated, resulting in death of the cell. MOM permeabilization is controlled primarily by the Bcl-2 family of proteins. In particular, the Bak and Bax proteins, when activated, form pores in the MOM, allowing IMS proteins to be released.

Much of what we know about the mitochondrial apoptotic pathway has been determined from experiments using isolated mouse liver mitochondria (which contain primarily Bak, not Bax). The outer membranes of these isolated organelles tend to deteriorate over time and rupture immediately upon freeze–thaw. Thus, the functional analysis of mouse liver mitochondria (and presumably mammalian mitochondria from other sources) currently requires freshly prepared organelles. To reduce the need for costly and time-consuming preparations, we sought to identify a storage medium that preserves mitochondrial function, particularly of the outer membrane. To that end, we analyzed the integrity of MOMs after rapid freeze–thaw in buffers containing various carbohydrates. Mitochondria frozen in almost all these solutions rapidly lost their MOM integrity and released cytochrome *c* when thawed and incubated at 37°C. However, we found that freezing mitochondria in buffer containing one particular sugar, trehalose, resulted in the preservation of MOM barrier function.

Trehalose is a naturally occurring disaccharide composed of two glucose molecules connected by an alpha, alpha-1,1 linkage. This sugar is implicated in the ability of plants and animals to withstand prolonged periods of desiccation, a process called xerobiosis. It has been shown that trehalose can protect proteins and cellular membranes from harmful effects of dehydration.[6] Womersley and colleagues[7] demonstrated that trehalose was more effective in preserving the structural and functional integrity of Ca^{2+}-transporting microsomes under dehydrating conditions than other

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Abbreviations: MOM, mitochondrial outer membrane; IMS, intermembrane space; TMRE, tetramethylrhodamine methyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; G3P, glycerol-3-phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; N/C-Bid, recombinant cleaved Bid; PTP, permeability transition pore; Su9-DHFR, fusion of the mitochondrial targeting signal of Su9 with dihydrofolate reductase
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carbohydrates, including lactose, maltose, cellobiose, sucrose, glucose, fructose, sorbitol, raffinose, myo-inositol, glycerol and alcohol sugars. In another study, trehalose was also found to be more efficient than glycerol and dimethylsulfoxide in the stabilization of sarcoplasmic reticulum during freeze–thaw.[8]

Here we provide a method for the long-term storage of frozen mouse liver mitochondria in trehalose-containing buffer and show that trehalose-frozen mitochondria retain a number of their important biological functions, most notably the preservation of MOM integrity.

Results

Cytochrome *c* is retained in trehalose-frozen mitochondria. To identify a suitable storage buffer that keeps mitochondria intact after freeze–thaw, we isolated mitochondria in buffers containing various concentrations of sugar and analyzed cytochrome *c* retention over time. We tested the following solutions: 0.6 mM sorbitol, 250–300 mM sucrose, 250–300 mM mannitol, 200 mM sucrose/75 mM trehalose, 200 mM mannitol/75 mM trehalose, 200 mM trehalose/75 mM sucrose, 200 mM trehalose/75 mM mannitol or 250–300 mM trehalose, all containing *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid (HEPES)–KEE mix (10 mM HEPES–KOH pH 7.7, 10 mM KCl, 0.1% bovine serum albumin (BSA), 1 mM ethylene diaminetetra acetic acid (EDTA) and 1 mM ethylene glycol-bis (*b*-aminoethyl ether) (EGTA)). We found that in almost all cases, if mitochondria were not frozen, they retained cytochrome *c* for 3–4 h at 22°C (data not shown). However, mitochondria frozen in almost all of these buffers lost a portion of their cytochrome *c* within the first 15 min after thawing at 22°C, suggesting that the freeze–thaw process damages the MOM (Figure 1a). Of the solutions tested, only 300 mM trehalose was effective for preserving cytochrome *c* content: mitochondria frozen and thawed in 300 mM trehalose buffer retained almost all their cytochrome *c* for at least 45 min (Figure 1a and b).

Similar results were obtained at the more physiologically relevant temperature of 37°C. When mitochondria that had been frozen in sucrose–mannitol buffer were incubated at 37°C, 95% of their cytochrome *c* was lost during the first 15 min. Incidentally, the same mitochondria lost only 20–30% of their content of cytochrome *c* when incubated at 22°C (Figure 1b, compare left middle and left bottom panels). The reason for this temperature dependence of cytochrome *c* efflux from mitochondria frozen/thawed in sucrose–mannitol buffer is unknown, but is hypothesized to involve temperature-dependent changes in the organization of cristae.[9]

In contrast, trehalose-frozen mitochondria retained almost all cytochrome *c* for at least 45 min either at 22 or 37°C (Figure 1b, right panels). Further analysis demonstrated that trehalose-frozen mitochondria retained cytochrome *c* for at least 2–3 h at 37°C (data not shown). Over 95% of total cytochrome *c* was retained in trehalose-frozen mitochondria during a 45-min incubation at 37°C (Figure 1c), compared to only 7% for mitochondria frozen in sucrose–mannitol buffer (data not shown). We conclude that trehalose-frozen mitochondria retain cytochrome *c* at 37°C much more effectively than mitochondria frozen in the standard buffer.

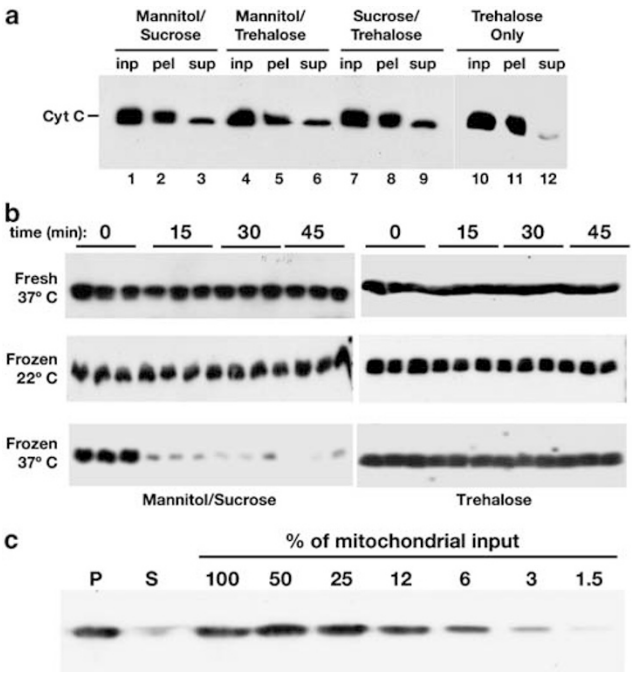

Figure 1 Retention of cytochrome *c* in trehalose-frozen mitochondria. (**a**) Mouse mitochondria were frozen in buffers containing each of the following sugar mixtures: 67 mM sucrose and 200 mM mannitol (lanes 1–3), 67 mM mannitol and 200 mM trehalose (lanes 4–6), 67 mM sucrose and 200 mM trehalose (lanes 7–9), or 300 mM trehalose (lanes 10–12) and quickly thawed in the same buffer containing 80 mM KCl. Aliquots of thawed mitochondria were either suspended in SDS sample buffer (lanes 1, 4, 7 and 10) or incubated at 22°C for 5 min and centrifuged for 10 min to separate mitochondrial pellets (lanes 2, 5, 8 and 10) from supernatants (lanes 3, 6, 9 and 12). Samples were resolved by SDS-15% PAGE and analyzed by Western blot using anti-cytochrome *c* antibody. (**b**) Fresh or frozen mitochondria in either the sucrose/mannitol (left) or trehalose (right) buffer were resuspended in the same buffer and incubated either at 22 or 37°C for 0–45 min. Mitochondria were isolated by centrifugation, triplicates of samples were subjected to SDS-PAGE, and the cytochrome *c* content of the samples analyzed by Western blotting. (**c**) Trehalose-frozen mitochondria were resuspended in trehalose buffer and incubated at 37°C for 45 min. The sample was fractionated by centrifugation and immunoblotted along with a series of dilutions of unfractionated samples, to estimate the percentage of released cytochrome *c*. The pellet fraction (P) contained more than 95% and the supernatant (S) less than 5% of the cytochrome *c* input. Although the rate of spontaneous release varied between mitochondrial preparations, it was usually below 5% of the total mitochondrial cytochrome *c* in the input

Trehalose-frozen mitochondria release cytochrome *c* in response to recombinant cleaved Bid. The retention of cytochrome *c* in trehalose-frozen mitochondria suggested that their OMs were intact. Alternatively, it was possible that the process of freezing mitochondria in the presence of trehalose altered the morphology of the mitochondria in some way, resulting in cytochrome *c* being trapped in compartments other than the IMS, such as the matrix. Therefore, we examined the ability of trehalose-frozen mitochondria to release cytochrome *c* in response to BH3-only proteins of proapoptotic Bcl-2 family members that activate Bax and Bak.

We compared the response of freshly isolated and trehalose-frozen mitochondria to BH3-only proteins first by incubating the organelles with 10 nM recombinant cleaved Bid (N/C-Bid) at 37°C. The results showed that both fresh and trehalose-frozen mitochondria released almost all of their cytochrome *c* within 15 min (Figure 2a). Thus, trehalose-frozen mitochondria released cytochrome *c* in response to cleaved Bid with kinetics similar to those of freshly isolated mitochondria.

N/C-Bid also induced the release of Omi, another IMS protein, from trehalose-frozen mitochondria. When trehalose-frozen mitochondria were treated with N/C-Bid and incubated at 37°C, Omi and cytochrome *c* began to appear in the supernatants at 12 min, and by 24 min both Omi and cytochrome *c* were almost entirely in the supernatants (Figure 2b, middle two panels). On the other hand, VDAC, a voltage-dependent channel protein that is integral to the MOM, was retained in the mitochondrial pellets throughout the experiment, as expected (Figure 2b, bottom two panels).

To test the specificity of the response to N/C-Bid in these experiments, we added a 10-fold excess of the antiapoptotic Bcl-2-family protein, Bcl-x_L, known to bind to and inhibit N/C-Bid. Bcl-x_L blocked the release of cytochrome *c* and Omi, suggesting that the regulation of MOM permeabilization by Bcl-2-family proteins[10] was normal in these frozen–thawed

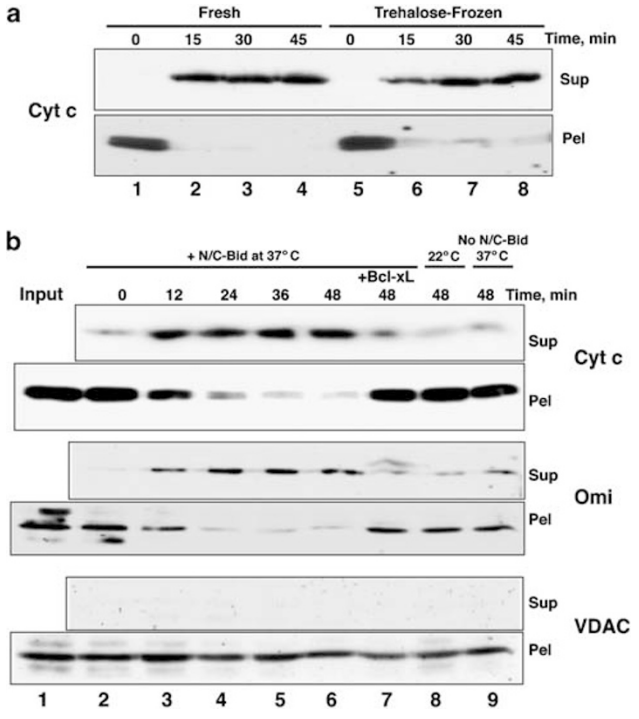

Figure 2 Trehalose-frozen mitochondria retained MOM integrity. (**a**) Fresh and trehalose-frozen mitochondria were resuspended in the assay buffer and incubated with 10 nM of N/C-Bid at 37°C for 0, 15, 30 or 45 min. Mitochondrial pellets were precipitated by centrifugation and pellets and the cytochrome *c* content of the samples was analyzed by Western blotting. (**b**) Trehalose-frozen mitochondria were resuspended in the assay buffer supplemented with either 10 nM N/C-Bid (lanes 2–6 and 8), 100 nM Bcl-x_L (lane 7), or left untreated (lane 9). Samples were incubated at 37°C (lanes 2–7 and 9) or at 22°C (lane 8). At the indicated time points, samples were centrifuged to separate pellets from supernatants and their contents analyzed by Western blot

mitochondria. We also observed that N/C-Bid-induced release of cytochrome *c* and Omi only occurred at physiological temperature (37°C). When mitochondria were incubated with the same concentration of N/C-Bid at 22°C for as long as 45 min, neither cytochrome *c* nor Omi was released (lane 8). Similar results were obtained using peptides corresponding to the BH3 domains of Bid and Bim[11]): these peptides (20 μM) triggered the release of cytochrome *c* and Omi from trehalose-frozen mitochondria with kinetics comparable to those seen with freshly isolated mitochondria (data not shown).

In summary, trehalose-frozen mitochondria responded to BH3-only proteins by releasing cytochrome *c* and Omi from their IMS. The evidence strongly suggests that MOMs of trehalose-frozen mitochondria remained intact after freeze–thaw and also remained responsive to BH3-only proteins. We also observed that N/C-Bid-induced oligomerization of Bak in trehalose-frozen mitochondria occurred in the same manner as with fresh mitochondria (not shown), a further indication that the MOMs of trehalose-frozen mitochondria were intact and functional.

ATP synthesis in trehalose-frozen mitochondria. To ensure further that trehalose-frozen mitochondria are indeed biologically similar to freshly prepared mitochondria, we assayed other biological properties. ATP synthesis is a critical function of mitochondria. We first compared the ATP content of 2-week-old trehalose-frozen mitochondria with that of freshly isolated mitochondria, using bioluminescence assays (Figure 3, 'input'). Frozen–thawed mitochondria contained less than half the amount of ATP ($\sim$19 ng of ATP/μg protein) present in fresh mitochondria ($\sim$40 ng of ATP/μg protein). This difference suggests that a slow hydrolysis of ATP occurred during storage.

To stimulate ATP synthesis, mitochondria were suspended in buffer containing 67 μM ADP and incubated at 37°C. At the outset, the ATP concentration of fresh mitochondrial suspensions (10 μg total protein) was about 95 μM, and within 30 min, it increased by 20% to 118 μM (Figure 3, top). No further increase was seen with longer incubation (data not shown). The addition of glycerol-3-phosphate (G3P) did not stimulate ATP synthesis more than ADP alone and indeed produced a slightly smaller increase, to 115 μM. With suspensions of frozen mitochondria, in contrast, 67 μM ADP stimulated a 110% increase in ATP concentration in 30 min, producing a final ATP concentration of 95 μM, comparable to that of unstimulated fresh mitochondria (Figure 3, bottom). In the presence of both ADP and G3P, the ATP concentration of frozen mitochondria increased by 155%, reaching nearly 115 μM. Again, this concentration is comparable to those of ADP or ADP + G3P-stimulated fresh mitochondria.

Antimycin A is a potent inhibitor of electron transfer through complex III. When antimycin A was added to either ADP-stimulated or ADP + G3P-stimulated frozen mitochondria (Figure 3), no increases in ATP levels were seen upon incubation; rather, the ATP concentrations declined to less than 20 μM in both cases. When fresh mitochondria were treated with antimycin A along with ADP or ADP + G3P, there were precipitous declines in ATP concentration to nearly 15 μM. The larger decreases seen in fresh mitochondria in the presence of antimycin A may indicate faster rates of ATP

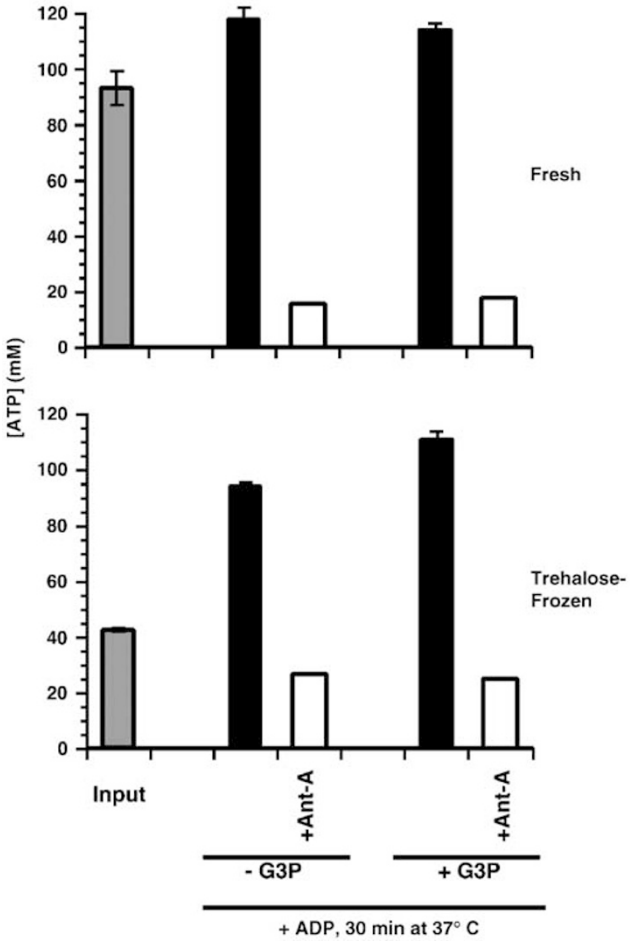

Figure 3 Trehalose-frozen mitochondria can synthesize ATP. Fresh and trehalose-frozen mitochondria were incubated in ATP synthesis assay buffer and incubated at 37°C for 30 min. To stimulate ATP synthesis, 67 μM of ADP was added. Where indicated, 5 mM G3P, 0.2 μM antimycin A (Ant-A) or both were added to samples. All samples were analyzed in triplicates and ATP concentrations were plotted. The experiment shown was representative of three independent experiments

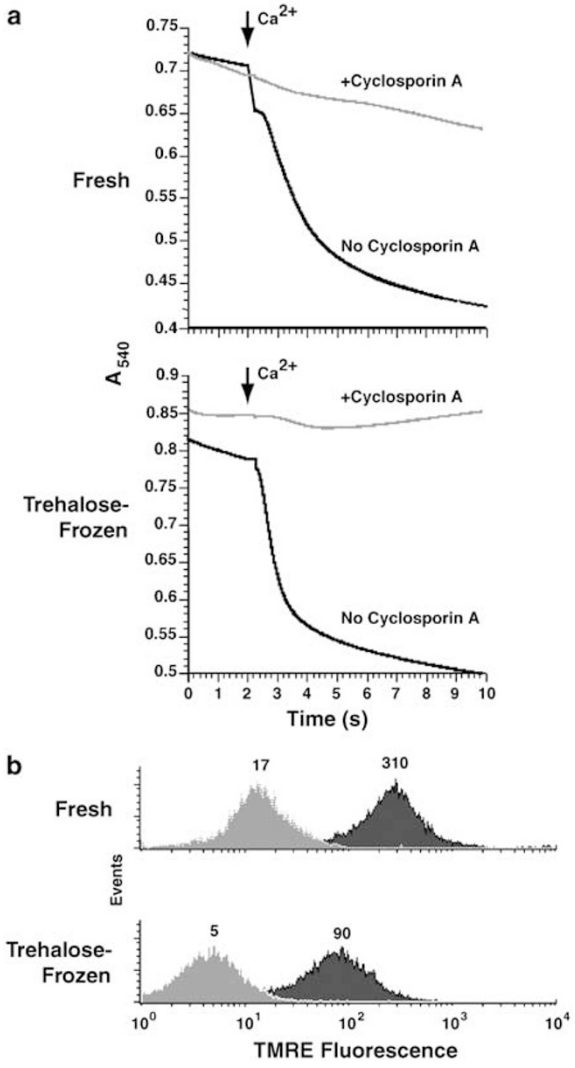

Figure 4 Trehalose-frozen mitochondria display normal PT. (**a**) Fresh and frozen mitochondria were resuspended in isotonic buffer (see Materials and Methods) with or without Cyclosporin A and light scattering was measured 5 times every second at 540 nM for 10 min. For each fresh or frozen mitochondria sample, samples containing 25 μM Cyclosporin A were analyzed simultaneously. At 2 min, 100 μM calcium was added to both samples and measurement resumed for another 8 min. In both frozen and fresh mitochondria, with the addition of calcium, absorbance at 540 nm changes dramatically in the absence of cyclosporin A. (**b**) Mitochondria were suspended in PBS and 100 nM TMRE was added to the suspensions which were mixed well and immediately analyzed by flow cytometry (black fill). CCCP (1 μM) was added to the samples to dissipate transmembrane potential (gray fill)

hydrolysis in freshly prepared mitochondria. These data suggest firstly that the increases in ATP concentration we observed with the addition of ADP and ADP + G3P were mitochondrially generated and secondly that there is constitutive ATP hydrolysis in the mitochondria. Taken together, our data clearly indicate that trehalose-frozen mitochondria are capable of ATP synthesis.

Trehalose-frozen mitochondria swell in response to increased calcium concentration. Cytosolic calcium concentrations are tightly regulated by the endoplasmic reticulum and mitochondria.[12–14] Calcium is imported into, and exported out of, the mitochondrial matrix through ion channels located in the inner membrane. Calcium sequestered in mitochondria plays important physiological roles including: (1) controlling the rate of oxidative phosphorylation, (2) modifying the shape of cytosolic Ca^{2+} pulses or transients and (3) under conditions of Ca^{2+} overload, inducing the permeability transition (PT), which causes mitochondrial matrix swelling, rupture of the MOM and the release of cytochrome *c*, followed by cell death.

Thus, we determined whether trehalose-frozen mitochondria can respond to increasing concentrations of calcium by activating the permeability transition pore (PTP). Calcium-induced mitochondrial swelling was assayed by measuring the decrease in light scattering at 540 nm. The addition of exogenous calcium (100 μM, or $\sim$200 nmol/mg of mitochondrial protein) induced a comparable degree of swelling in fresh and trehalose-frozen mitochondria (Figure 4a). Pretreatment with 25 μM cyclosporin A, an inhibitor of PTP, blocked the

swelling in both cases. This suggests that in both cases, swelling was caused by calcium-induced PTP opening, as expected.

Trehalose-frozen mitochondria retain transmembrane potential. Mitochondrial electron transport generates an electrochemical proton gradient of approximately 180–200 mV across the inner membrane that drives ATP synthesis. As trehalose-frozen mitochondria are capable of ATP synthesis, we expected the transmembrane potential, $\Delta\Psi_m$, to be present in frozen mitochondria. We used flow cytometry to compare the retention of the potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRE) in fresh vs trehalose-frozen mitochondria. Both types of mitochondria retained TMRE (Figure 4b; graphs filled in black), and the amount of retained TMRE was greatly reduced by the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Figure 4b; graphs filled in gray), indicating that both mitochondrial preparations possessed a measurable inner membrane potential. In this experiment, we did observe a lower overall TMRE fluorescence in the frozen mitochondria. This may merely reflect variation in staining perhaps due to different total numbers of mitochondria in the two preparations, or it may reflect altered respiratory function in the trehalose-frozen mitochondria (see below). Addition of a higher concentration of CCCP (10 μM) did not cause further loss of TMRE staining (data not shown) suggesting that CCCP had dissipated $\Delta\Psi_m$ completely in each case.

Trehalose-frozen mitochondria are capable of protein import and processing. Most mitochondrial proteins are encoded on nuclear genes and synthesized as precursor proteins in the cytoplasm. These precursors are imported into the organelle via a multistep pathway that includes binding to surface receptors on the MOM, unfolding of the precursor, translocation across both mitochondrial membranes, processing to the mature form, refolding of the imported protein and assembly into multisubunit enzyme complexes.

To investigate whether trehalose-frozen mitochondria are capable of protein import, we introduced an ^{35}S-labeled fusion of the mitochondrial targeting signal of Su9 with dihydrofolate reductase (Su9-DHFR).[15] As a negative control, we used the nuclear export receptor Crm1. When trehalose-frozen mitochondria were incubated at 37°C with an energy regenerating system in AT buffer, Su9-DHFR was observed in the mitochondrial pellets within 30–60 min (Figure 5, lanes 4, 6 and 8). The mature form of Su9-DHFR, indicated by mSu9 in Figure 5, appears in the pellet fractions at 30–90 min, indicating that Su9-DHFR maturation took place in the mitochondria. As expected, very little Crm1 was associated with mitochondrial pellets as it remains in the cytosol (lanes 2–9). When we added a metalloprotease inhibitor, phenanthroline, Su9-DHFR was still associated with the mitochondria. However, the absence of the mature form of Su9-DHFR in the mitochondrial pellet indicated that although the protein was imported into mitochondria, its processing was blocked by phenanthroline (lanes 16 and 17), suggesting that processing of Su9-DHFR is dependent on metalloprotease activities.

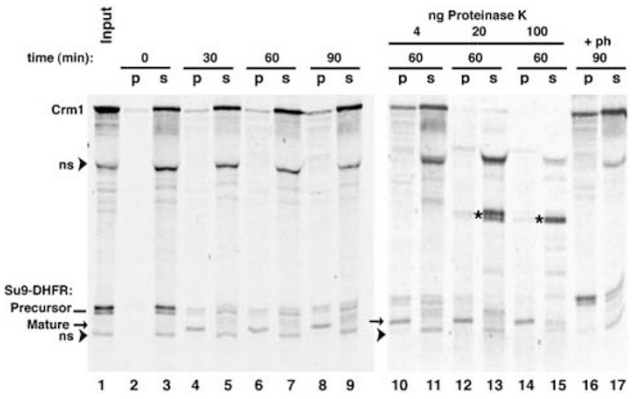

Figure 5 Trehalose-frozen mitochondria can import a model precursor protein and display mitochondrial peptidase activity. Frozen mitochondria were resuspended in AT buffer containing 80 mM KCl and supplemented with an energy regenerating system and ^{35}S-labeled Su9DHFR and Crm1 proteins made in rabbit reticulocyte lysates. At the time points indicated at the top, mitochondria were pelleted and supernatants (s) and pellets (p) were analyzed by SDS-PAGE and phosphorimaging. Following the import reaction, the samples analyzed in lanes 10–15 were treated with 4–100 ng/ml of proteinase K for 30 min. Samples analyzed in lanes 16 and 17 contained 200 μM phenanthroline to inhibit metalloproteinase activities in the mitochondria. Positions of Su9-DHFR precursor and mature processed forms (arrows) are indicated. Arrowheads indicate nonspecific proteins labeled in rabbit reticulocyte lysates (ns). The asterisks (*) in lanes 13 and 14 indicate degradation products of Crm1

We used proteinase K digestion as an assay for protein import. Limited concentrations of proteinase K can degrade proteins outside mitochondria, whereas proteins inside the MOM or imported into the matrix are largely resistant to degradation. We added three different concentrations of proteinase K to mitochondria that had been incubated with Su9-DHFR for 60 min. After a further 30 min incubation, we observed the disappearance of Su9-DHFR from the supernatant. Not surprisingly, the reduction of protein in the supernatant was greater with higher amounts of proteinase K added. Crm1 degradation in the supernatant was also observed with addition of proteinase K (asterisks in lanes 10–15 denote apparent degradation products). We also note that at higher concentrations of proteinase K, only the mature form of Su9-DHFR remained in the mitochondrial pellet (lane 14). Thus our data indicate that the mature form of Su9-DHFR had translocated into mitochondria and was inaccessible to proteinase K. We also tested the mitochondrial import of natural substrates such as Mgm1/OPA1, Dpk1, AIF1 and others. Trehalose-frozen mitochondria efficiently imported and processed each of these (data not shown).

Trehalose preserves ultrastructure of mitochondria. To examine the degree of structural preservation that accompanied the freezing processes, we used transmission electron microscopy. We observed significant differences between isolated mitochondria frozen in trehalose or mannitol/sucrose revealed (Figure 6). Isolated mitochondria often display a range of conformational states from 'orthodox' to 'condensed' with the majority being in the condensed state (for definitions of orthodox and condensed, see

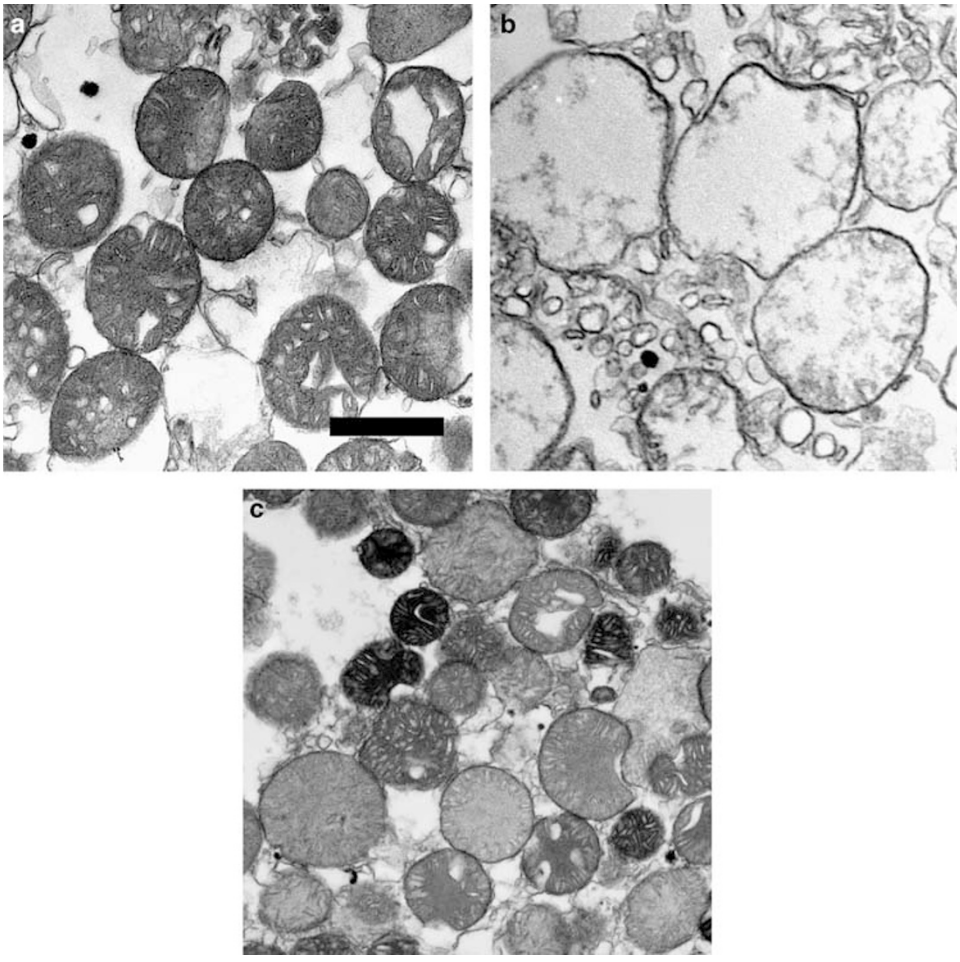

Figure 6 Electron microscope images of isolated mitochondria prepared freshly or frozen in mannitol/sucrose or trehalose. The mitochondria in the fresh and trehalose samples (**a** and **c**) were nearly all in the condensed state with outer and inner membranes close together. The mannitol/sucrose mitochondria (**b**) show large-amplitude swelling and degraded cristae; however, outer membranes did not display obvious breaks. The bar represents 1 μM

Hackenbrock[16]; examples in Kuwana *et al*.,[10] and von Ahsen *et al*.[17]) In condensed forms, the matrix is more electron dense, indicative of more tightly packed material. Concomitantly, the intracristal space is more expanded and in contrast to the matrix is quite light. In this condensed state, the IMS, the region bounded by the mitochondrial outer and inner boundary membranes, is often (but not always) expanded, that is, the inner boundary membrane is pulled away from the outer membrane. The freshly prepared and trehalose-frozen mitochondria shown in Figure 6(a and c) are nearly all in the condensed state, with the freshly prepared mitochondria appearing slightly more condensed (generally darker matrix and more expanded intracristal spaces). In both samples, the outer membrane is seen to be in close apposition to the inner boundary membrane. In contrast, the mitochondria frozen in mannitol/sucrose exhibited significant structural damage. Most of the mitochondria were highly swollen (example of this type of swelling in von Ahsen *et al*.[17]) and contained degraded cristae (Figure 6b). Even with this large-amplitude swelling, often no breaks in outer membranes could be seen. In summary, no significant differences in ultrastructure were apparent between the freshly prepared and trehalose samples, whereas the mannitol/sucrose mitochondria were extensively damaged.

Bioenergetic function is partially preserved in trehalose-frozen mitochondria. To assess quantitatively the functional integrity of the inner membrane, we measured respiration of mitochondria in different metabolic states (Figure 7). Trehalose-frozen mitochondria showed significantly decreased rates of both phosphorylating (state 3) and maximally uncoupled respiration (Figure 7a) that resulted in decreased respiratory control and acceptor control ratios (Figure 7b). However, these mitochondria displayed essentially intact activity of cytochrome *c* oxidase (Figure 7a). Taken together these data indicate that freezing in trehalose partially preserves bioenergetic function, consistent with the ability of these mitochondria to maintain a membrane potential (Figure 4b) and, therefore, to perform membrane potential-dependent functions: ATP synthesis (Figure 3) and protein import (Figure 5), as well as Ca^{2+} accumulation with consequent opening of the PTP (Figure 4a).

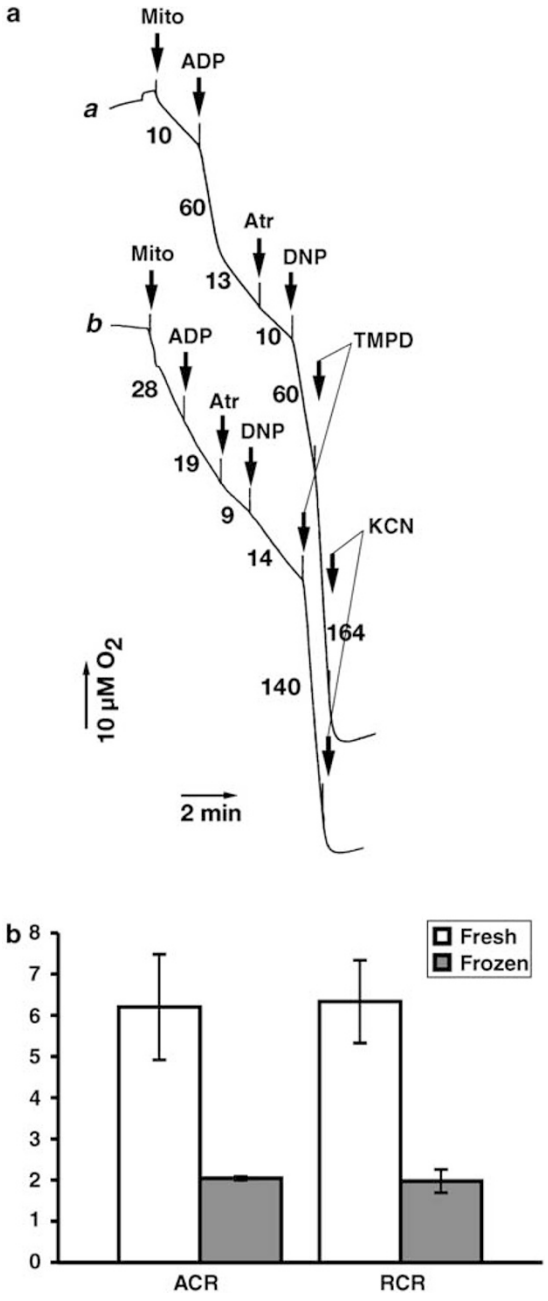

Figure 7 Bioenergetic function is compromised but not completely disrupted in trehalose-frozen mitochondria. (**a**) Respiration of freshly prepared and trehalose-frozen mitochondria. 1 mg/ml of freshly prepared (trace a) or trehalose-frozen (trace b) mouse liver mitochondria were incubated as described in Material and Methods. ADP (200μM; ADP) was used to induce metabolic state 3 (phosphorylating respiration). Next, 100 μM atractyloside (Atr), an inhibitor of the adenine nucleotide translocator, was added to induce resting (state 4) respiration, and then 40 μM DNP was added to measure the maximal rates of electron transport chain activity (state 3u or uncoupled respiration). Additions of 2 mM *N,N, N',N'*-tetramethyl-1,4-phenylenediamine (TMPD) and 0.5 mM potassium cyanide (KCN) were used to assess cytochrome oxidase activity and nonmitochondrial oxygen consumption, respectively. The data are a paired experiment representative of three separate preparations. (**b**) Respiratory control in freshly prepared and trehalose-frozen mitochondria. Acceptor control ratios (ACR) and respiratory control ratios (RCR) were calculated as the ratios of rates of respiration in metabolic state 3 or state 3u, respectively, to the rates of respiration in metabolic state 4. The data are mean $\pm$S.E.M., $n=3$

Discussion

We have shown that mouse liver mitochondria can be frozen in trehalose buffer in such a way that much of their biological function and ultrastructure is preserved. In particular, this procedure maintains the integrity of the MOM and the regulation of its permeability by Bcl-2-family proteins. In contrast, outer membranes of mitochondria frozen in standard sucrose–mannitol buffer deteriorate quickly when thawed and then incubated at 37°C. In one experiment, nearly 97% of cytochrome *c* was lost from mitochondria during the first 15 min of incubation at 37°C. Interestingly, the same mitochondria incubated at 22°C retained over 80% of cytochrome *c* for 45 min. One possible reason for this temperature dependence of cytochrome *c* release may be that mitochondria frozen in sucrose–mannitol buffer already have compromised MOMs and are simply more unstable during the 37°C incubation, perhaps as a result of increased membrane fluidity. However, a more intriguing possibility is that a temperature-dependent change in the structure of mitochondrial cristae occurs, similar to the changes observed by Korsmeyer and colleagues.[9]

Temperature also caused differences in the mitochondrial response to N/C-Bid; at 37°C, 10 nM N/C-Bid induced the complete release of cytochrome *c* and Omi from the IMS in 24 min, whereas at 22°C, mitochondria released neither cytochrome *c* nor Omi over 45 min (Figure 2). Furthermore, Bak oligomerization was observed only at 37°C and not at 22°C (data not shown). One possible explanation might be that the fluidity of the MOM is lower at 22°C, thus hindering Bak-mediated lipidic pore formation. Alternatively, some unknown enzymatic activity might be required.

Interestingly, mitochondria from *Xenopus laevis* eggs are inherently resistant to freeze–thaw even in mannitol–sucrose buffers, retaining most of their cytochrome *c* content and their ability to be permeabilized by proapoptotic Bcl-2-family proteins.[15,18] It is possible that *Xenopus* eggs, which must be fertilized outside the mother's body, contain protectants like trehalose to help them resist adverse conditions in the wild, such as freezing or desiccation.

It also seems likely that temperature played a role in lowering the ATP concentration of trehalose-frozen mitochondria during storage (Figure 3). As mitochondrial ATP synthesis is a temperature-dependent reaction requiring the activities of proton pumps, whereas ATP hydrolysis can take place even at low temperatures, freezing could potentially tip the balance towards net loss of ATP. Despite their lowered initial ATP concentrations, however, when trehalose-frozen mitochondria were stimulated with ADP or ADP + G3P, they reached final ATP concentrations comparable to those seen with fresh mitochondria within 30 min (Figure 3). Thus, our data indicate that trehalose-frozen mitochondria are capable of ATP synthesis and can maintain normal ATP levels when warmed to 37°C.

Closer examination of mitochondrial respiration revealed that the inner membrane function of trehalose-frozen mitochondria is somewhat compromised (Figure 7) as reflected by their low respiratory control ratios. Freezing the mitochondria appeared primarily to compromise rates of phosphorylating (ADP-stimulated) and uncoupler-stimulated

(DNP-stimulated) respiration rather than dramatically increasing the extent of mitochondrial uncoupling (measured in the presence of atractyloside). The reason for this is unclear; however, one possibility is that freezing disrupts the interaction of the proteins that form one or more of the electron transport chain complexes, yet has a minimal effect on the integrity of the lipid membrane. As well, frozen mitochondria may release some of their matrix solutes, such as ADP and NAD/NADH. A high extramitochondrial concentration of ADP could explain, for example, the finding that the mitochondria were in state 3 from the start (i.e., they displayed a high initial rate of oxygen consumption that was not increased by exogenous ADP.) Nevertheless, trehalose-frozen mitochondria can maintain sufficient transmembrane potential to synthesize ATP (Figure 3), to import and process mitochondrial precursor proteins (Figure 5) and to swell in response to elevated external Ca^{2+} (Figure 4), indicating a substantial degree of functional preservation. Furthermore, electron microscopy showed that the ultrastructure of trehalose-frozen mitochondria was largely intact (Figure 6).

While this manuscript was in preparation, a publication appeared that described the loading of liver mitochondrial matrix with trehalose via the intentional reversible opening of the PTP using 150 μM calcium, a manipulation that was reported to facilitate dessication of mitochondria.[19] The claim was made that trehalose-loaded and desiccated mitochondria retain a high degree of inner membrane integrity. However, no direct comparison with freshly isolated mitochondria was made, nor was the integrity of the MOM examined. Indeed, the activation of permeability transition, at least under some conditions, is known to rupture the MOM.[15] Thus, it is possible that mitochondria that are loaded with trehalose via induction of PT may better preserve inner membrane function, but perhaps at the expense of compromising outer membrane integrity and possibly the content of other solutes in the matrix that could, in principle, leak out through the PTP.

Our method of freezing isolated mitochondria in the presence of trehalose preserves a number of their important biological activities, in particular the regulation of MOM permeability by Bcl-2-family proteins. The protocol developed here will make the use of isolated mitochondria more convenient for research on apoptosis and other mitochondrial functions.

Materials and Methods

Isolation and storage of mouse liver mitochondria. Mouse liver mitochondria were isolated by Dounce homogenization in AT buffer (300 mM trehalose, 10 mM HEPES–KOH pH 7.7, 10 mM KCl, 1 mM EGTA, 1 mM EDTA and 0.1% BSA). The homogenate was centrifuged at 600 $\times$ *g* for 10 min. The supernatant was removed and centrifuged at 3500 $\times$ *g* for 15 min, and the resulting pellet was resuspended in 10 ml of isolation buffer and centrifuged at 1500 $\times$ *g* for 5 min. The mitochondrial supernatant was centrifuged at 5500 $\times$ *g* for 10 min, and the last two steps were repeated. The mitochondrial pellet was resuspended in a small volume of trehalose buffer at 50 mg of protein/ml. For freezing, 5-μl samples of mitochondrial suspension were pipetted into 0.6-ml Eppendorf tubes placed on the bed of dry ice. It was important to avoid touching the tube wall with the pipet tips, as this could cause mitochondria inside the tip to freeze immediately. The tubes were immediately thrown into liquid nitrogen and then stored at -80°C. For defrosting, tubes were held between the fingers for rapid thawing.

Cytochrome *c* release assay. Frozen or freshly isolated mitochondrial pellets were washed once in AT buffer containing 80 mM KCl and resuspended in the same buffer supplemented with an energy regenerating system (5 mM succinate, 2 mM ATP, 10 μM phosphocreatine and 10 μg/ml creatine kinase). Samples were incubated with the indicated amounts of recombinant proteins or peptides at 37°C for 30 min unless otherwise specified. For the cytochrome *c* release assay, we typically used 30–60 μg of mitochondria in 30 μl of trehalose buffer. Mitochondrial pellets were separated from supernatants by centrifugation at 10 000 $\times$ *g* for 10 min at 4°C. Pellets and supernatants were suspended in SDS sample buffer and separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then immunoblotted onto PVDF membrane using anti-cytochrome *c* antibody (BD Bioscience). In some experiments, the membranes were reprobed with antibodies to Htra2/Omi (R&D Systems) or VDAC (BD Bioscience).

ATP bioluminescence assay. Mitochondrial ATP concentrations were measured as described.[20] Fresh or frozen mitochondria pellets were resuspended in 75 μl of ATP synthesis buffer (20 mM Tris-HCl pH 7.4, 15 mM KH_2PO_4, 0.6 M sorbitol, 10 mM $MgSO_4$ and 2.5 mg/ml BSA). To induce ATP synthesis, 67 μM ADP (with or without G3P) was added. Where indicated, mitochondrial ATP synthesis was inhibited by the addition of antimycin A (0.2 μM). After incubation at 37°C for 30 min, 1.75 μl of 60% perchloric acid was added, and samples were left on ice for 10 min followed by centrifugation at 15 000 $\times$ *g* for 10 min. A volume of 60 μl was collected and neutralized with 11.5 μl of 1 M KOH. The reaction was incubated on ice for 5 min and centrifuged as before. Finally, the ATP concentration for 10 μl of the resulting supernatant was determined using the ATP Bioluminescence Assay Kit CLS II (Roche) according to the manufacturer's instructions.

Calcium-induced mitochondrial swelling. Mitochondrial pellets were suspended (at 500 μg/ml as protein) in assay buffer containing 150 mM KCl, 5 mM KH_2PO_4 and 5 mM Tris-HCl (pH 7.4). A pair of samples, one containing 25 μm cyclosporin A and the other without, were placed in cuvettes side by side in a Cary 100 Bio UV-Visible Spectrophotometer and light scattering measurements at 540 nm were taken from both samples simultaneously. Measurements were taken five times every second. Two minutes after the initiation of the assay, 100 μM of $CaCl_2$ ($\sim$200 nmol/mg of mitochondria) was added to both samples and the assay was continued for another 8 min.

Flow cytometric analysis of isolated liver mitochondria. Mitochondrial transmembrane potential was measured as described.[21] Fresh or frozen mitochondrial pellets were suspended in phosphate-buffered saline (PBS). TMRE (100 nM) was added to the suspension, which was mixed well and was immediately analyzed by flow cytometry, with care taken to gate out noise from particulate contaminants. Addition of 1–10 μM CCCP resulted in an immediate downward shift in TMRE staining.

Mitochondrial protein import assay. Frozen mitochondrial pellets were treated as in the cytochrome *c* release assay. Twenty-microliter samples containing 0.2 μl of the indicated ^{35}S methionine-labeled proteins, prepared in rabbit reticulocytes, were incubated at 37°C for the indicated time. Pellets were precipitated as before and supernatants and pellets were subjected to 12% SDS-PAGE. Gels were dried and exposed overnight with Kodak MR film.

Electron microscopy. Electron microscopy was performed essentially as described.[10,17,22] Briefly, fresh or frozen resuspended mitochondrial pellets (1.5 mg/ml protein) isolated by differential centrifugation (thus containing contaminating ER and Golgi membranes) were incubated in 1% formaldehyde on ice for 10 min, and centrifuged at 6000 $\times$ *g* for 5 min. Pellets were fixed with 2% paraformaldehyde–2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4 for 1 h on ice. Afterwards, they were rinsed in ice-cold 0.15 M sodium cacodylate buffer containing 3 mM calcium chloride for membrane preservation. Postfixation was accomplished with ice-cold 1% osmium tetroxide in 0.15 M sodium cacodylate containing 0.8% potassium ferrocyanide and 3 mM calcium chloride. Following repeated rinses in ice-cold, double-distilled water, the mitochondria were stained and internal membranes were stabilized in ice-cold 2% uranyl acetate. Dehydration was initiated with a graded ethanol series on ice – 20, 50, 70 and 90%. Dehydration was completed at room temperature with 100% ethanol. Infiltration was performed with well-mixed 50% ethanol/50% Durcupan ACM resin. Infiltration continued with multiple exchanges of 100% Durcupan ACM. The resin was hardened in a 60°C oven. Sections from the samples were cut with a thickness of $\sim$80 nm using a Leica ultramicrotome. After poststaining in uranyl acetate and lead salts, these sections

were examined using a JEOL 1200FX electron microscope. Images were recorded on film at 6000 × magnification. The negatives were scanned at 4000 dpi using a Nikon CoolScan giving a pixel size of 0.96 nm. Contrast adjustment was performed with Adobe Photoshop.

Measurement of respiration. Respiration of mitochondria was measured using a Clark-type oxygen electrode (Diamond General) connected to an oxygen monitor (Yellow Springs Instruments) in incubation medium containing 250 mM sucrose, 2 mM inorganic phosphate, 1 mM EGTA, 2 mM $MgCl_2$ and 10 mM HEPES–KOH, pH 7.4 at 22°C. Glutamate (5 mM) and malate (5 mM) were used as respiratory substrates.

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