Upregulation of the Mitochondrial Phosphate Carrier During Freezing in the Wood Frog *Rana sylvatica*: Potential Roles of Transporters in Freeze Tolerance

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Screening of a cDNA library prepared from liver of the freeze-tolerant wood frog (*Rana sylvatica*) identified a freeze-responsive clone containing a 1370-nt sequence with an open reading frame of 360 amino acids. Sequence analysis revealed 84–86% identity with the mammalian inorganic phosphate carrier (PiC) that spans the inner mitochondrial membrane. Northern blot analysis showed that *pic* transcript levels increased over a time course of freezing, reaching 60-fold upregulation after 24-h frozen. Transcript levels were also assessed under freezing-related stresses with results showing a strong increase in *pic* transcript levels in response to dehydration (elevated 9.0-fold in 40% dehydrated frogs) but not under anoxia. Western blotting revealed elevated PiC protein over a time course of freeze-thaw whereas other mitochondrial carriers (dicarboxylate carrier, oxoglutarate transporter) of the same family were not affected by freezing. This modulation of PiC protein levels may play a role in mitochondrial ionic and/or osmotic balance during freeze-induced cell volume reduction.

KEY WORDS: Mitochondrial inorganic phosphate carrier; freeze-tolerance; dehydration; anoxia; transporters.

INTRODUCTION

The wood frog *Rana sylvatica* is one of only a few vertebrate animals that can survive whole body freeze-thaw during its winter hibernation on the forest floor. This species has been widely used as a model for studying the physiological, biochemical, and molecular adaptations that endow vertebrate organs with freeze tolerance (for review see: Storey, 1999; Storey and Storey, 1996, 2001). Among the stresses that frogs endure during freezing are (a) potential physical damage from ice crystals forming in and around their organs, (b) extreme hyperglycemia due to the accumulation of glucose as a cryoprotectant (levels rise to 150–300 mM in blood and tissues compared with ~5 mM in controls), (c) long-term organ anoxia and ischemia due to the freezing of blood plasma, and (d) a major reduction in cell volume caused by the loss of intracellular water into extra-organ ice masses; indeed, as much as 65–70% of total body water may be converted to ice. Indeed, not only are wood frogs highly tolerant of anoxia and cell volume reduction during freezing but this species also shows excellent tolerance of these two stresses individually (Churchill and Storey, 1993; Holden and Storey, 1997) and we have found in several cases that freeze-induced metabolic responses appear to be derived from preexisting metabolic responses to anoxia or dehydration that are shared by both freeze tolerant and intolerant frog species.

Freeze tolerance involves a complex array of adaptations: changes in gene and protein expression, altered patterns of signal transduction, changes to the regulatory controls on selected enzymes, and the synthesis of high concentrations of glucose cryoprotectant (Storey, 1999; Storey and Storey, 1996, 2001). Recent studies by our lab have documented several examples of freeze-induced

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gene upregulation including genes encoding fibrinogen subunits (Cai and Storey, 1997a), ADP/ATP translocase (Cai *et al.*, 1997), and three genes encoding novel proteins (FR10, FR47, Li16) with different organ-, time-, and stress-specific distributions but as yet unknown functions (Cai and Storey, 1997b; McNally *et al.*, 2002, 2003).

The present study demonstrates that the nuclear-encoded gene (*pic*) for the inorganic phosphate carrier protein (*PiC*) is freeze upregulated in wood frog liver. PiC is an inner mitochondrial membrane protein that catalyzes the electroneutral movement of inorganic phosphate from the cytosol across the inner mitochondrial membrane into the matrix of mitochondria with H^+ ions (symport) or for OH^- (antiport) (Krämer, 1996; Stappen and Krämer, 1994). Both *pic* mRNA levels and PiC protein content increase substantially during freezing and also in response to dehydration suggesting that regulation of the transporter is responsive to changes in cellular water content and/or the consequent changes in intracellular or intramitochondrial ionic strength and osmolality.

MATERIALS AND METHODS

Animals

Male wood frogs between 5 and 7 g were collected in the spring of 2001 from the Ottawa, Ontario area. Animals were washed in a tetracycline bath and placed in plastic boxes with damp sphagnum moss at 5°C for at least 2 weeks before experimentation. Control frogs were sampled directly from this condition. Control and experimental frogs were both killed by pithing and then organs were rapidly excised, frozen in liquid nitrogen, and stored at −80°C until use. Protocols for animal care, experimentation, and euthanasia were approved by the Carleton University Animal Care Committee in accordance with the guidelines set down by the Canadian Council on Animal Care.

For freezing experiments frogs were placed in plastic boxes with damp paper toweling on the bottom and then put in an incubator set at −3°C. The damp toweling ensures that all animals are rapidly seeded when ice begins to form on the substrate and initial tests showed that, on average, prefreeze cooling prior to nucleation required ∼45 min (determined from initial trials with animals that had thermistors taped to their abdomens). All frogs were allotted an initial 45 min cooling after which freeze duration was timed for 2, 6, or 24 h followed by sampling. Other frogs were frozen for 24 h and then sampled after thawing at 5°C for 1, 2, 4, or 8 h.

For anoxia experiments, frogs were transferred to 700-mL bottles (5 frogs per bottle) held in crushed ice and containing 5 mL of distilled water, which had been bubbled with 2% CO_2 98% N_2 for 30 min. Bottles were tightly capped and sealed with a covering of parafilm and N_2 gassing was continued for another 30 min through syringe ports in the caps. Gas lines were then quickly removed and bottles were sealed and then placed into a 5°C incubator for 4 or 24 h. At the end of this time, bottles were transferred back to a crushed ice bath, gas lines were quickly reintroduced to maintain the anoxic atmosphere, and then animals were quickly sampled.

Dehydration experiments were conducted as described by Churchill and Storey (1993). In brief, frogs were placed in closed desiccators at 5°C with a layer of silica gel desiccant on the bottom. Frogs were separated from the desiccant by a sponge pad. Frogs were allowed to dehydrate at an average rate of 1% of total body water lost per hour until 20% and 40% of body water was lost. Water loss was confirmed by weighing the frogs at intervals during the experiment. Some frogs were first dehydrated to 40% of total body water lost and were then placed in containers with 1 cm of distilled water on the bottom and allowed to fully rehydrate for 24 h at 5°C.

cDNA Library Screening

A cDNA library was constructed using the Lambda ZAP-CMV XR Library Construction kit (Stratagene) with mRNA isolated from liver of 24-h frozen frogs. The cDNA library was differentially screened using ^{32}P-labelled single-stranded total cDNA probes made from liver mRNA of control versus 24-h frozen frogs. Plaques that showed a much greater signal when exposed to the frozen versus control probes were collected and subjected to further rounds of purification and screening. Potential upregulated clones were rescued by in vivo excision and converted to pBluescript circular double-stranded plasmid according to the manufacturer instructions (Stratagene). One clone, termed *frozen liver 8* (*fl-8*), that was subsequently shown to contain the nucleotide sequence (*pic*) of the mitochondrial inorganic phosphate carrier protein (PiC), was chosen for further study.

RT-PCR

The reverse transcription-polymerase chain reaction (RT-PCR) was used to assess levels of *pic* mRNA transcripts in liver of control and stressed frogs. Tissue mRNA was purified using Oligotex Mini-Prep (QIAGEN)

and eluted two times with 40 μl of elution buffer provided with the kit (preheated to 70°C). mRNA was extracted using Oligotex beads (QIAGEN) and the purified mRNA was then reverse transcribed into cDNA using a reverse transcriptase enzyme (Superscript, GIBCO). The cDNA:mRNA hybrids were serially diluted and then RT-PCR was performed using frog-specific α-tubulin primers to normalize the amount of mRNA in controlled and stressed samples. The concentrations of control and stressed mRNA were adjusted using distilled-deionized water to ensure that there was the same concentration of starting material in serially-diluted control and stressed samples. The normalized samples were then amplified using primers designed specifically for frog *pic* (5′-GGCATCCTCAGTTGTGGTAT-3′; 3′-ACAAGACACGGTAGCAGAGG-5′). PCR was carried out under conditions of 94°C denaturation (1 min), 55°C annealing (1 min), and 72°C elongation (1 min), for 35 cycles using an MJ-Research PTC-100 thermocycler.

Northern Blot Analysis

Total RNA was extracted from tissues using Trizol (Gibco-BRL, Bethesda, MD) and methods previously described (Chomezynski, 1993). Briefly, ~50 mg of frozen tissue was placed in a 2-mL Eppendorf tube and homogenised in 500 μl of Trizol reagent. Chloroform was added (0.2 mL/mL of Trizol) and the samples were incubated at 21°C for 2–3 min. Samples were then centrifuged at $15,000 \times g$ for 15 min to separate the sample into an upper aqueous phase and a lower organic phase. After centrifugation, the upper aqueous phase was removed to fresh 1.5-mL tubes containing an equal volume of isopropanol to precipitate the RNA pellet. RNA was precipitated over 15 min at 21°C and then centrifuged at $12,000 \times g$ for 10 min. The supernatant was discarded and the pellets were washed with 70% ethanol and centrifuged again. The total RNA pellet was resuspended in 200 μl of RNAase free distilled water. Total RNA samples were then quantified using A_{260} and adjusted to ensure equal amounts of total RNA among samples.

Aliquots (16 μg) of total RNA were separated on a 1.5% denaturing formaldehyde gel for ~2 h at 90 V to ensure adequate separation. RNA was transferred onto nitrocellulose (Hybond, AMERSHAM) using a standard capillary transfer method. Blots were hybridized with radiolabeled *fl*-8 probe and visualized via exposure to phosphoimaging screens. The screens were scanned with a Personal Molecular Imager FX (BIO-RAD) and the images were analyzed using ImageQuant (Ver. 4.2) software to provide a densitometric analysis of band intensity.

Western Blot Analysis

Levels of selected mitochondrial carrier proteins were quantified using Western blotting. Frozen tissue samples were homogenized (1:9, w/v) in a solution of 25-mM MOPS, 50-mM β-glycerophosphate, 2-mM EDTA, 2-mM EGTA, 1-mM Na-orthovanadate, and 1-mM DTT with a few crystals of phenylmethylsulfonyl fluoride added immediately prior to homogenization. After centrifugation for 10 min at $10,000 \times G$, the supernatants were removed and mixed (1:1 v/v) with sample buffer containing 100-mM Tris-HCI (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, and 10% (v/v) 2-mercaptoethanol. Protein concentration was measured using the Bradford assay (Bradford, 1976) with the Bio-Rad prepared reagent and 20-μg of total protein was loaded into each sample well. Samples were loaded onto sodium-dodecyl-sulfate (SDS) polyacrylamide gels and electrophoresis was carried out for 2 h at 150 V. Proteins were then electroblotted onto polyvinylidene difluoride (PVDF) (Immobilon-P Transfer Membrane, Millipore, Bedford, MA) by wet transfer using a transfer buffer solution containing 25-mM Tris (pH 8.5), 192-mM glycine, and 10% (v/v) methanol at 4°C for 1.5 h at 0.3 mA. The membranes were washed in TBST (10-mM Tris, pH 7.5, 150-mM NaCl, 0.05% v/v Tween-20) and then blocked for 1 h at RT in a 1.0% w/v solution of nonfat dry milk (NFDM). Membranes were then probed with a primary antibody diluted (1:3000 v/v) in blocking solution (TBST, 1.0% NFDM) overnight at 4°C followed by washing three times in TBST for 5 min at 21°C. The membranes were then incubated in secondary antibody (conjugated with horseradish peroxidase) diluted 1:2000 v/v in blocking solution for 1 h at 21°C. Bands were detected using enhanced chemiluminescence (ECL) (DuPont NEN, Boston, MA); the membrane was exposed to X-ray film and the resulting image was scanned on a HP ScanJet 3c using HP Deskscan II (v.2.9) software and quantified using ImageQuant (v.4.2, Innovative Optical Systems Research, Molecular Dynamics). After immunodetection was complete, blots were stained with Coommassie blue to reconfirm equal loading of samples and proper transfer by analysis of multiple bands.

All primary antibodies were polyclonal antibodies raised in rabbits as described previously for PiC (Capobianco *et al.*, 1991), the oxoglutarate transporter (OGC) (Fiermonte *et al.*, 1993) and the dicarboxylate carrier (DIC) (Fiermonte *et al.*, 1999) and, in all cases, cross-reaction with frog samples produced a single band of the expected molecular weight (~35 kDa for each). For anti-PiC, the source antigen was bovine, which shared 86.6% identity with the frog sequence. For anti-OGC and

anti-DIC the source antigens were bovine and rat, respectively. Due to high sequence conservation among different species, we are confident that antibodies had a high cross-reactivity with the frog mitochondrial transporters. Secondary antirabbit antibodies conjugated with horseradish peroxidase and prestained protein markers (broad range) were from New England Biolabs (Mississauga, Ontario). Statistical significance of the data was tested using analysis of variance (Model I) followed by the Dunnett's test (two-tailed).

DNA and Protein Sequence Analysis

DNA sequencing was conducted by Canadian Molecular Research Services (Orleans, Ontario). Alignments of sequence data and prediction of the open reading frame (ORF) used DNAMAN (v.4.11). Primers for RT-PCR were designed using Primer Designer for Windows (v.3.0). Protein features were predicted based on searches of the Conserved Domain Database (NCBI-BLAST). Predictions of transmembrane regions were done using HMMTOP (Tusnády and Simon, 1998).

RESULTS

Differential screening of a cDNA library made from liver of frozen wood frogs using ^{32}P-labeled probes made from mRNA of control (5°C-acclimated) versus frozen (24 h at −3°C) frogs revealed several clones that were putatively upregulated in response to freezing. One clone, termed frozen liver 8 (*fl-8*), was chosen for further analysis. Nucleotide sequencing revealed a 1370-bp sequence that contained a potential full open reading frame running from nucleotide 44 to 1126 as well as a polyadenylation signal (nucleotides 196–201) and a poly A^{+} tail; the sequence was submitted to Genbank with the accession number AF536222. The sequence encoded a protein of 360 amino acids. BLAST search analysis revealed a high degree of sequence identity with the mammalian mitochondrial inorganic phosphate carrier (PiC), a nuclear-encoded gene (Kramer, 1996). Figure 1 shows a comparison of the amino acid sequence of the wood frog protein with PiC from three mammalian species. Sequence differences between the frog and mammalian proteins were primarily limited to a small segment of the protein from amino acids 21–52. Overall, wood frog PiC showed 84.3, 86.2, and 86.6% sequence identity with the sequences of bovine (Runswick *et al.*, 1987), human (Dolce *et al.*, 1991), and rat (Ferreira *et al.*, 1989) PiC, respectively.

Analysis of frog PiC revealed the presence of three repeats of about 100 amino acids each that are character-

istic of the inner mitochondrial membrane protein family. These span amino acid residues 59–152, 163–246, and 262–338 predicted using HMMTOP (Tusnády and Simon, 1998). Within each of the repeats are two hydrophobic regions (shown by Roman numerals in Fig. 1) that are separated by a hydrophilic segment and result in six transmembrane regions.
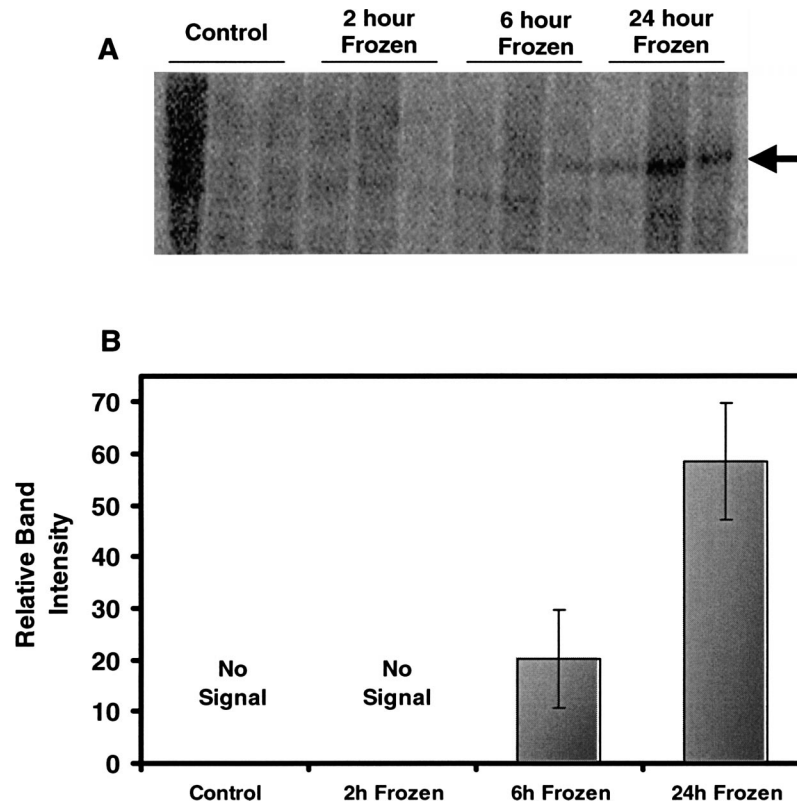
Northern Blotting

Both Northern blot and RT-PCR analysis using the *fl-8* clone as a probe showed that levels of the *pic* transcript increased in wood frog liver during freezing. Northern blot analysis of changes in *pic* transcript levels over a time course of freezing is shown in Fig. 2. Transcripts were virtually undetectable in liver of control frogs and frogs sampled 2 h after freezing began. However, a marked induction of the gene was seen after 6 h of freezing and transcript levels continued to rise with longer freezing.

Because of the difficulty of detecting *pic* transcripts in liver extracts of control frogs, we turned to RT-PCR to further assess the changes in *pic* transcript levels during freezing and thawing. RT-PCR was carried out using mRNA isolated from liver of control, 24-h frozen, and 8-h thawed frogs and primers specific to the *fl-8* clone (Fig. 3). RT-PCR is more sensitive than Northern blotting, allowing detection of minute amounts of transcript. Control, frozen, and thawed samples were serially diluted and PCR was performed on each dilution. For each sample condition, the intensity of the band should decrease as the dilution is increased (higher dilutions have less starting material). The control sample (Fig. 3(A)) shows a product up to the 10^{-2} dilution whereas the 24-h frozen sample showed product even at high dilutions (10^{-4}) (Fig. 3(B)). This indicates much higher levels of *pic* transcripts in extracts from liver of frozen frogs. The thawed sample (Fig. 3(C)) showed product at the 10^{-3} dilution indicating reduced levels compared with frozen liver but still greater levels than controls.

Figure 4 shows changes in *pic* transcript levels in liver of wood frogs exposed to anoxia or dehydration stresses. Each of these stresses mimics an element of freezing—the anoxia/ischemia induced by plasma freezing versus the strong cell volume reduction caused by water exiting into extracellular ice crystals. The data show that *pic* transcript levels did not respond to anoxia but that transcripts were strongly upregulated by the loss of 20 or 40% of total body water (Fig. 4). Mean transcript levels were 3.1-fold higher than control values in liver of 20% dehydrated frogs (although this was not a significant increase) and rose to 9.0-fold higher than controls in 40% dehydrated animals ($P < 0.05$).

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Frog     MYSTVAHLARANPFNAPHFQVGQECATLRKKKNTSEIQPVR            40
Rat      -f-s--------------l-lvhdvsgp-sppgp.....p             35
Human    -f-s-----------t--l-lvhdglgdlrssspgptgqp             40
Bovine   -f-s-----------t--l-lvhdglgdlrssspgptgqp             40

                                            I
Frog     RLAAAATAAEGD..YSCEYGSTKFYAFCGFGGILSCGITH             78
Rat      -rsrhla--aveg.----f--m-y--l-----v----l--             74
Human    -rprnla--aveeq---d---gr-fil--l---i---t--             80
Bovine   -rprnla--avee.----f--a-y--l-----v----l--             79

Frog     TAVVPLDLVKCRMQVDPQKYKSIFSGFSVTLKEDGVRGLA            118
Rat      ---------------------g--n---i-----------            114
Human    --l------------------g--n--------------            120
Bovine   ---------------------g--n--------------            119

                 II
Frog     KGWAPTFIGYSMQGLCKFGFYEVFKVLYSNLLGEENTYLW            158
Rat      ------l-----------------a----i---------            154
Human    -------l----------------------m---------            160
Bovine   -------l----------------------m---------            159

                     III
Frog     RTSLYLAASASAEFFADIALAPMEAAKVRIQTQPGYANTL            198
Rat      ---------------------------------------            194
Human    ---------------------------------------            200
Bovine   ---------------------------------------            199

                          IV
Frog     RQAAPKMYAEEGIWAFYKGVAPLWMRQIPYTMMKFACFER            238
Rat      -e-v----k---ln--------v----------------            234
Human    -d------k---lk-------------------------            240
Bovine   -d------k---lk-------------------------            239

                              V
Frog     TVEALYKHVVPKPRSECSKSEQLVVTFVAGYIAGVFCAIV            278
Rat      -------f---------t-a-------------------            274
Human    -------f----------p-------------------            280
Bovine   -------f----------p-------------------            279

Frog     SHPADSVVSVLNKEKGSTATQVLKRLGPKGVWKGLTAXII            318
Rat      ------------------s---q---fr------f-r--             314
Human    ----------------s-sl------f-------f-r--             320
Bovine   ----------------s-sl------f-------f-r--             319

             VI
Frog     MIGTLTALQWFIYDSVKVYFRLPRPPPPEMPESLKKKLGLTE           360
Rat      ------------------------------------------          356
Human    ------------------------------------------q         362
Bovine   ------------------------------------------q         361
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Fig. 1. Predicted amino acid sequence of the ORF of wood frog PiC (Genbank accession number AF536222) aligned with the sequences of rat liver (AAA41634), human heart (CAA42641), and bovine heart (CAA28951) PiC. Dashes replace amino acids that are identical with those in the frog sequence and periods indicate amino acid residues that are not present in some sequences. Lowercase amino acids underneath the frog sequence indicate amino acid changes in other species. Segments I–VI are the potential transmembrane portions of the frog PiC. The three repeat segments characteristic of mitochondrial membrane carriers are shaded.

Fig. 2. (A) Northern blot analysis of *pic* transcript levels (probed with clone *fl-8*) in wood frog liver over a time course of freezing. Arrow points to a band of ~1400 bp which corresponds to the *pic* transcript. (B) Densitometric analysis of northern blot showing mean intensity ± SEM for $n = 3$ separate isolations of mRNA from frog liver under each experimental condition.

Western Blotting

The effects of freeze/thaw on the content of mito-chondrial phosphate carrier (PiC) protein in wood frog liver were assessed via Western blotting using a polyclonal antibody raised against bovine PiC. Figure 5 shows that the PiC antibody crossreacted with a single band at the expected molecular weight of ~35 kDa. PiC protein content was not affected by short-term freezing but levels had increased by 3.3-fold after 24 h frozen ($P < 0.05$). PiC protein content remained elevated (2.8-fold higher than controls; $P < 0.05$) after 1 h of thawing but then gradu-ally decreased until levels similar to control values were re-established after 8-h thawed.

Immunoblotting was also used to assess PiC protein levels in other organs of wood frogs during freeze/thaw, comparing levels in control, 24-h frozen, and 8-h thawed frogs (Fig. 6). However, unlike the situation in liver, freeze/thaw had no significant effect on PiC levels in brain, gut, heart, skin, or kidney of *R. sylvatica*.

PiC is one of a family of transmembrane carriers that are responsible for moving metabolites into or out

of the mitochondria. Hence, we might expect that freeze-stimulated increases in PiC protein content would be ac-companied by similar changes in the levels of other mito-chondrial carriers. To test this idea, two other carriers that belong to the same family were also assessed via western blotting. Mammalian polyclonal antibodies to the OGC and DIC carriers showed good cross-reaction with the frog liver proteins, in each case showing one strong band that cross-reacted with the antibody at the expected molecular weight of each protein. However, as Fig. 7 shows, there were no significant changes in the amounts of either pro-tein in wood frog liver over the freeze-thaw time course.

DISCUSSION

PiC belongs to a class of structurally similar inner mitochondrial membrane carrier proteins. These are char-acterized by the presence of three repeats of ~100 amino acids that are found in all members of the family. Each repeat contains two transmembrane α-helical domains for a total of six membrane-spanning domains per protein

10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}
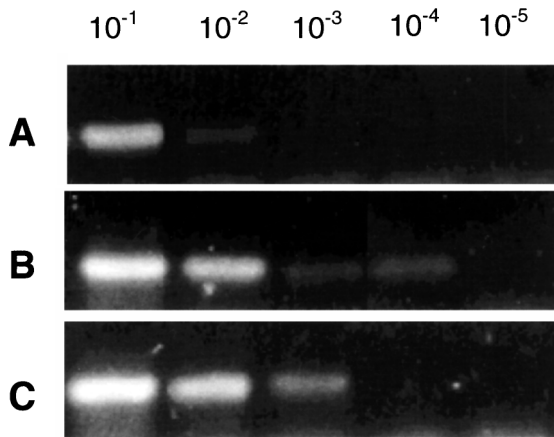
Fig. 3. Relative RT-PCR of *pic* amplified from wood from liver. Dilutions of mRNA isolated from liver of (A) control, 5°C acclimated frogs, (B) 24-h frozen frogs, and (C) 8-h thawed frogs were amplified using PCR conditions described in the Materials and Methods. Samples of mRNA were normalized using primers for α-tubulin. PCR products were run on a 1.0% TAE agarose gel and stained with ethidium bromide. Product is visible in the 24-h frozen sample even at high dilutions (10^{-4}).

(Kuan and Saier, 1993, Palmieri in press). Other similar mitochondrial transporters such as the dicarboxylate carrier (DIC) and the oxoglutarate transporter (OGC) also belong to the same family. DIC is a carrier protein that exchanges dicarboxylates like malate or succinate for inorganic phosphate (Kaplan and Pedersen, 1985). OGC catalyzes an exchange of oxoglutarate for malate (or certain other dicarboxylates) (Palmieri *et al.*, 1993). All catalyze exchange reactions that are electroneutral (Palmieri *et al.*, 1996).

Wood frog PiC showed these same structural features as well as a high overall sequence identity (84–86%) with the mammalian proteins and a comparable length (360 amino acids compared with 356–362 for the mammalian protein) (Fig. 1). However, the sequence near the *N* terminus showed much higher variability in the frog protein compared with the mammals although the mammalian sequences are also variable in this region. This segment of the protein forms the extramembranous region that extends into the mitochondrial intermembrane space (Kramer, 1996). This variability near the *N* terminus has been reported for all mitochondrial transporters whereas the transmembrane regions are much more highly conserved (Kuan and Saier, 1993; Palmieri *et al.*, 1993). Mammalian PiC does not have any known phosphorylation or glycosylation sites and the frog protein is similar in this regard. Mammalian PiC exists as two splice variants, isoforms A (PiC-A) and B (PiC-B) (Dolce *et al.*, 1994; Fiermonte *et al.*, 1998). Bovine PiC-A was found in muscle mitochondria (heart, skeletal muscle, diaphragm)

whereas PiC-B had a wider distribution (Fiermonte *et al.*, 1998). PiC-B showed a higher V_{max} and lower K_m compared to PiC-A (Fiermonte *et al.*, 1998). The present study used a polyclonal antibody that cross-reacts with both PiC isoforms to assess the organ distribution of frog PiC; the protein was found in all six organs examined but the present results cannot comment on isoform distribution.

The data demonstrate that pic is a freeze-responsive gene in the wood frog. Transcript levels were strongly upregulated after 6 or 24 h of freezing (Fig. 2) with RT-PCR analysis suggesting that the increase in *pic* transcript levels may be as much as 60-fold after 24-h frozen (Fig. 3). PiC protein also rose during freezing with a maximum increase of 3.3-fold higher than control values seen after 24-h frozen. The marked discrepancy between the fold changes in mRNA transcripts and protein levels is interesting. In some cases we have observed strong increases in the transcript levels of freeze-responsive genes during the freezing exposure but a delay in reaching peak protein levels until the recovery period after thawing (McNally *et al.*, 2002, 2003). The freezing of blood plasma imposes an ischemic state on organs that gradually halts ATP-expensive processes such as protein synthesis. Hence, a high-fold induction of transcripts may be necessary to achieve significant protein synthesis before protein synthesis is compromised by ATP restriction. A high-fold induction during freezing also makes sense as a means of achieving a rapid increase in the synthesis of the protein product as soon as thawing begins, for example, as is needed for proteins involved in the recovery of cell function after the stress of freezing (McNally *et al.*, 2002, 2003). In the present study, however, we did not find enhanced PiC protein synthesis during thawing; indeed, PiC levels in liver were beginning to decline even after 1 h of thawing. The high transcript levels, but lower (yet significant) protein levels suggest that possibly not all transcripts are translated into functional protein products. During freezing with a global decrease in protein synthesis any increase in the synthesis of selected proteins would be physiologically relevant.

The role of PiC upregulation during freezing appears to be liver-specific since PiC protein levels did not change during freezing in any of the other five organs tested (Fig. 6). Some other freeze-responsive proteins are also liver-specific including the novel protein FR47 and fibrinogen (very minor amounts of fibrinogen transcripts were found in two other organs) (Cai and Storey, 1997; McNally *et al.*, 2003). In the case of fibrinogen this is because vertebrate liver is typically the sole organ responsible for the synthesis and secretion of this plasma protein. However, the situation with PiC is different because this carrier is found in the mitochondria of all cell types. Furthermore, although PiC was strongly upregulated during
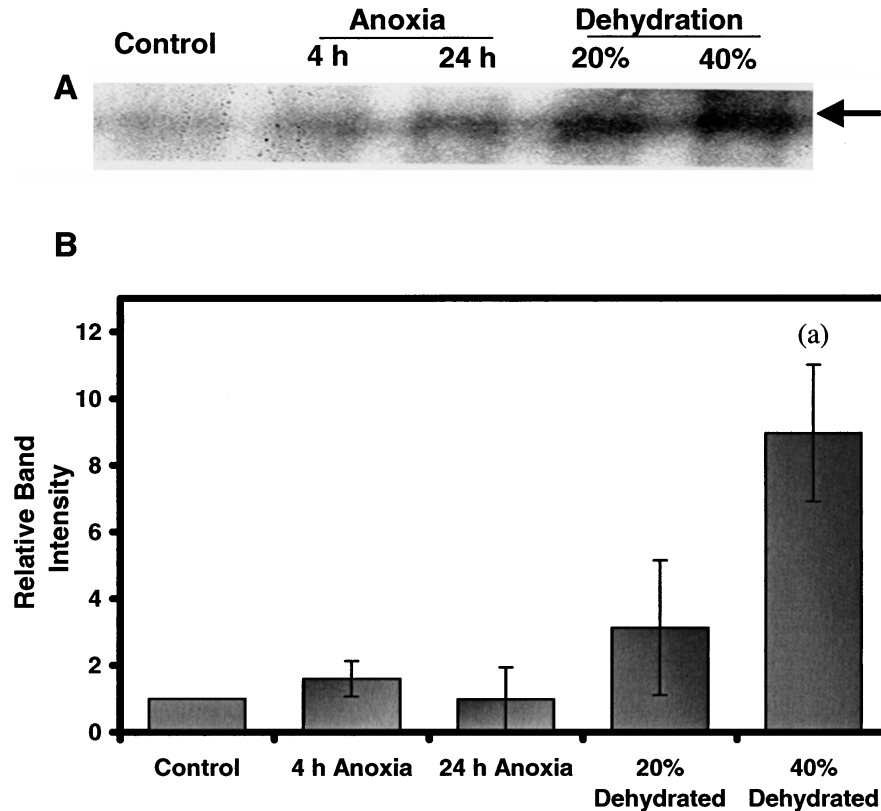
Fig. 4. (A) Northern blot analysis of total RNA isolated from liver of anoxia- or dehydration-exposed frogs probed with *fl-8*. Arrow points to a band of ∼1400 bp in size which corresponds to *pic*. (B) Densitometric analysis of northern blots showing mean intensity ±SEM for $n = 3$ blots using separate isolations of liver mRNA from different animals. [a]Significantly different from controls, $P < 0.05$.

freezing in liver, two other mitochondrial inner membrane carriers of the same family, OGC and DIC, showed no significant changes in protein content over the course of freeze-thaw (Fig. 7).

Some insights into the reason for PiC upregulation in wood frog liver can be drawn from an analysis of the responses by *pic* transcript levels to anoxia and dehydration stresses. Transcripts of *pic* did not change when frogs were given experimental anoxia exposure but levels increased by 9-fold in frogs that had lost 40% of their total body water (Fig. 4). Freezing imposes multiple stresses on cells, two of the most important being (a) anoxia/ischemia due to the interruption of oxygen delivery when plasma freezes, and (b) cell and organ dehydration due to the withdrawal of a high percentage of body water into extra-organ ice masses masses (Storey and Storey, 1996). When first exploring the regulation of cryoprotectant synthesis in freeze-tolerant frogs, we found that the hyperglycemic response to freezing was reproduced when frogs were dehydrated (but not under anoxia stress) and

that freeze-intolerant frogs also showed a significant hyperglycemic response to dehydration masses (Storey and Storey, 1996). From this, we proposed that the cryoprotectant response to freezing evolved out of a preexisting volume-regulatory hyperglycemic response by frogs to dehydration. Since then we have found that all freeze-responsive genes can also be categorized as responding to either anoxia (ADP/ATP translocase, FR47, Li16) (Cai *et al.*, 1997; McNally *et al.*, 2002, 2003), or dehydration (fibrinogen, FR10, PiC) (Cai and Storey, 1997a,b) and this helps us to understand both the cellular signal that triggers their expression and their potential role in freezing survival. The very strong response of *pic* transcripts to dehydration stress, but not to anoxia, suggests that an increase in PiC protein content in the liver mitochondrial membrane may be involved in adjusting ionic, osmotic, or volume regulatory parameters between the mitochondrial and cytoplasmic compartments during cellular dehydration. Indeed, during freezing at −2.5°C that converted 65% of total body water into ice, liver lost ∼58%

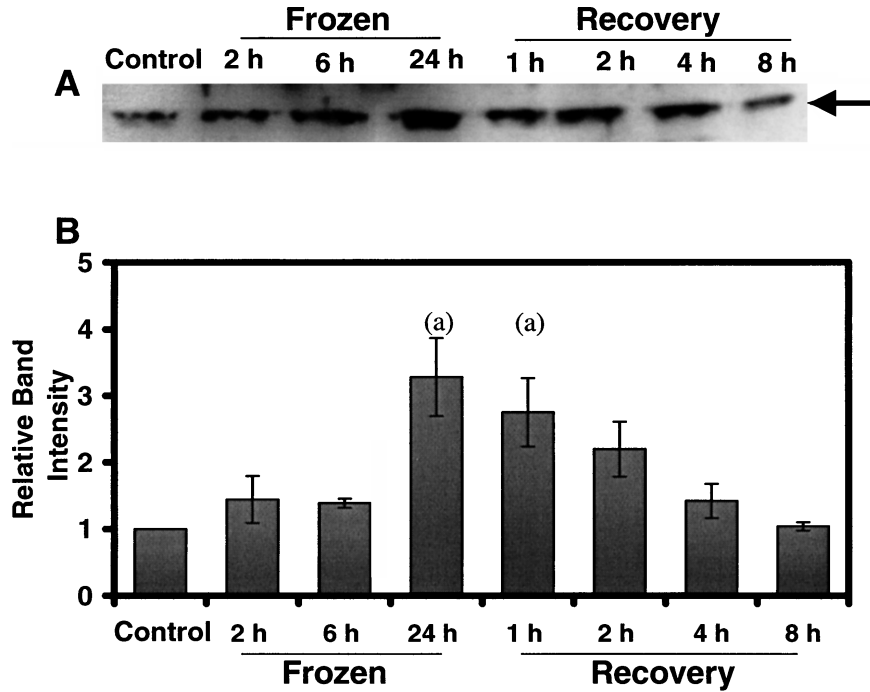**Fig. 5.** (A) Western blot showing changes in PiC protein content in *R. sylvatica* liver over a time course of up to 24-h freezing at $-3°C$ and followed by 8-h thawing recovery at $5°C$. Proteins were probed using polyclonal antibodies directed against the bovine form of PiC. (B) Histogram shows densitometric analysis of western blots, means $\pm$ SEM for $n = 3$ blots using protein extracts from different animals. [a]Significantly different from the control, $P < 0.05$.
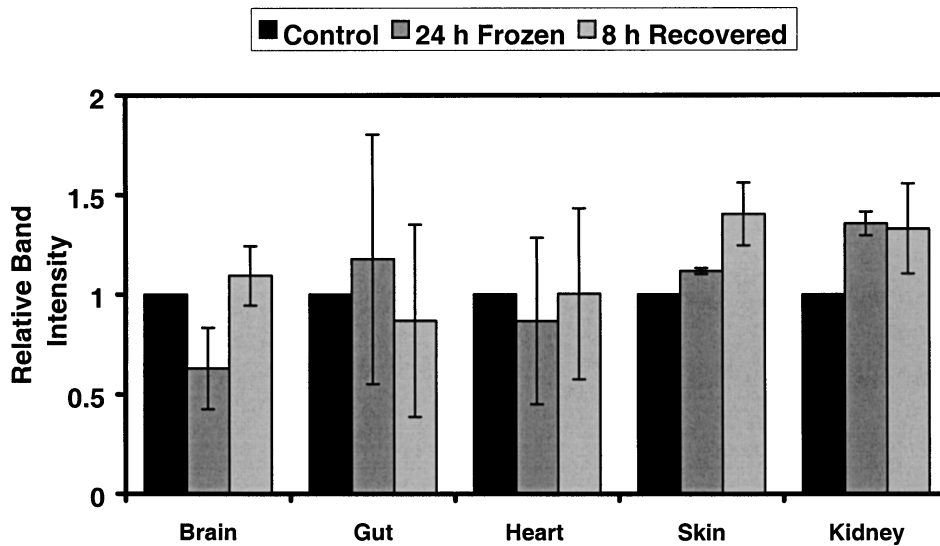

Fig. 6. Western blot analysis of PiC protein levels in five tissues of *R. sylvatica* after 24-h freezing or 8-h thawing, compared with controls. Bars show densitometric analysis of western blots, means $\pm$ SEM, $n = 3$ blots using protein extracts from different animals. Bars are: control (solid), 24-h frozen (open), and 8-h thawed (shaded).
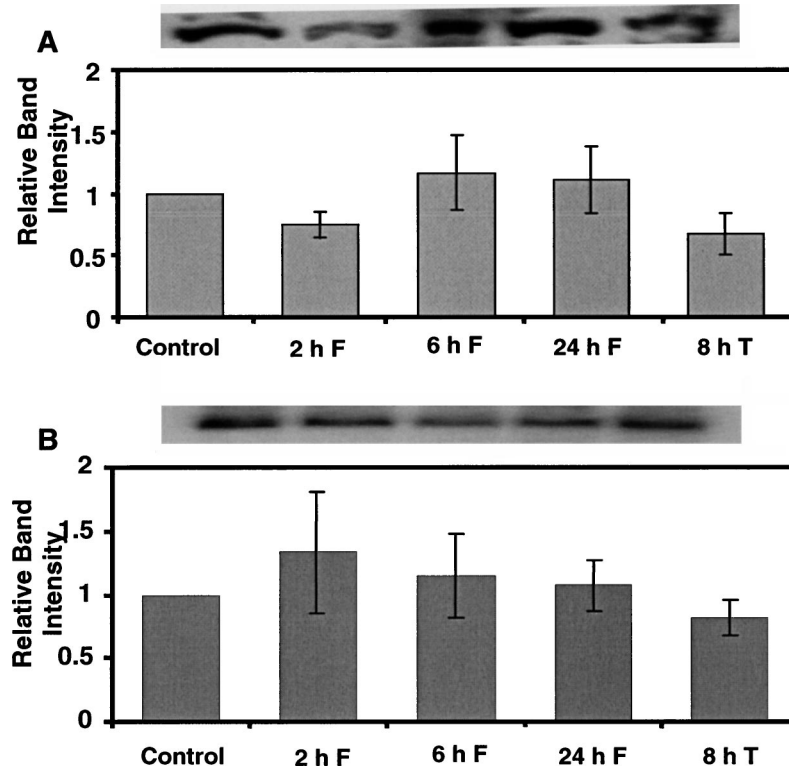
Fig. 7. Western blot analysis showing levels of (A) the dicarboxylate carrier protein and (B) the oxoglutarate carrier in *R. sylvatica* liver over a time course of freezing (F) and thawing (T). Bars show densitometric analysis of band intensities, means $\pm$ SEM, $n = 3$ samples of protein extracts from different animals.

of its water (Lee *et al.*, 1992) and accompanying this sustained a large increase in ionic strength and osmolality of liver intracellular fluids, including an increase in glucose levels of at least 200 mM (Storey and Storey, 1996).

Although the OGC and DIC carriers were unaffected by freeze-thaw in wood frog liver, a fourth carrier of the same family, the ADP/ATP translocase (AAT) (Palmieri, 1994) is freeze-responsive (Cai *et al.*, 1997). *Aat* transcripts levels peaked after 8-h freezing and AAT protein content was maximal after 24 h. Interestingly, however, *Aat* transcript levels changed during freezing in six of eight organs tested and, in liver, *Aat* transcripts responded to anoxia exposure but not to dehydration (Cai *et al.*, 1997). Hence, expression of *Aat* and *pic* genes, although both freeze-responsive, are clearly differentially regulated. This may not be surprising because although both AAT and PiC are involved in the energy metabolism in the cell they respond to different controls in vivo. PiC catalyzes Pi and H^+ uptake into the mitochondria using the chemical gradient of the proton motive force generated by the respiratory chain whereas and the AAT catalyzes an electrophoretic exchange of cytosolic ADP^{3-} for matrix ATP^{4-} using the electrical component of the proton motive force (Klingenberg, 1989). In fact, PiC is regulated by the pH gradient and AAT by the membrane potential.

Maintenance of a chemiosmotic gradient across the inner mitochondrial membrane is essential for cellular health (Huizing *et al.*, 1996, 1998). Disruption of the gradient has been linked to apoptosis (Green and Reed, 1998) and various mitochondrial disorders (Huizing *et al.*, 1996, 1998)]. Since *R. sylvatica* can survive freezing, they must be able to maintain their mitochondria in an intact and viable state despite the stresses imposed by the freezing or thawing processes. It has been suggested (Huizing *et al.*, 1996) that defects in PiC and AAT result in osmotic imbalances that ultimately affect energy metabolism. Hence, the upregulation of both AAT and PiC during freezing in wood frog liver suggests that both have roles in readjusting mitochondrial metabolism or ionic/osmotic balance during freezing although their roles are different and are undoubtedly responsive to different intracellular signal transduction systems. Whether the upregulation of PiC and AAT seen in freezing is beneficial or protective to mitochondrial survival remains to be determined.

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