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Phosphoglycerate Kinase 1 Expression Responds to Freezing, Anoxia, and Dehydration Stresses in the Freeze Tolerant Wood Frog, *Rana sylvatica*

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ABSTRACT Natural freezing survival by wood frogs (*Rana sylvatica*) involves multiple organ-specific changes in gene expression. Screening of a cDNA library made from brain of frozen frogs revealed freeze-responsive up-regulation of the glycolytic enzyme, phosphoglycerate kinase 1 (PGK1). Northern blots showed an approximately two-fold increase in *pgk1* transcripts in brain of frozen frogs whereas PGK1 protein levels rose by three- to five-fold within 4–8 hr of freezing. Freezing also elevated *pgk1* transcripts in liver but not in skin. Both transcript and protein levels also rose in response to two of the component stresses of freezing (anoxia and dehydration) with a particularly pronounced (11-fold) increase in PGK1 protein in brain in response to anoxia. Amino acid sequence analysis showed 92.5% identity between wood frog and *Xenopus laevis* PGK1 and 86–88% identity with the zebrafish, chicken, and human protein. Four unique amino acid substitutions in the wood frog protein could be important in maintaining the functional conformation of the wood frog protein at low body temperatures. Elevated amounts of PGK1, one of the ATP-generating reactions of glycolysis, in wood frog brain during freezing would enhance the glycolytic capacity of the organ and support the maintenance of cellular energetics under the ischemic conditions of the frozen state. *J. Exp. Zool. 311A:57–67, 2009.*

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Winter survival for a variety of animal species, including several kinds of terrestrial frogs, involves freeze tolerance, the ability to endure the freezing of 65–70% of total body water as extracellular ice and survive for weeks with no detectable vital signs (Storey and Storey, '96). Among these, the wood frog (*Rana sylvatica*) is the primary model used to study vertebrate freeze tolerance (Storey and Storey, 2004). Freezing places multiple stresses on animals and their cells including (a) the potential for physical damage to delicate tissues owing to ice crystal growth, (b) dehydration, osmotic and cell volume stress owing to the loss of intracellular water into extracellular ice, and (c) tissue ischemia caused by the freezing of blood plasma. Freeze tolerant animals exhibit multiple adaptations that deal with these stresses including the proliferation of ice nucleating proteins, cryoprotectant accumulation, metabolic rate depression, regulation of enzyme activity, and changes in gene/protein expression. In particular, our recent work has focused on freeze-stimulated changes in gene expression using cDNA library and DNA array screening technologies (Storey and Storey, '99, 2004; Storey, 2004). For example, screening of a cDNA library prepared from liver of frozen wood frogs identified freeze up-regulated

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genes including the α and γ subunits of fibrinogen, mitochondrial transporters (ADP/ATP translocase, inorganic phosphate carrier), and three novel freezing-responsive genes *fr10*, *li16,* and *fr47* (Cai and Storey, '97a,b; Cai et al., '97; McNally et al., 2002, 2003; DeCroos et al., 2004).

We recently turned our attention to the gene/protein expression responses that aid freezing survival of the brain. The brain is of particular interest because it is the most oxygen-sensitive organ in the vertebrate body and must be well protected from potential damage caused by low oxygen and/or ischemia during freezing. Furthermore, as the central regulator of behaviors and physiological functions, recovery of brain function after freezing is an absolute requirement for the survival of the whole animal. Screening of a cDNA library made from brain of freeze-exposed wood frogs revealed the differential up-regulation of several genes during freezing, as compared with unfrozen controls at 5°C. One of these was the gene for the acidic ribosomal phosphoprotein (P0) and that study, as well as another that used differential display PCR to show up-regulation of the ribosomal protein L7 during freezing, provided the first indication that protection and/or stabilization of the ribosomal translational machinery plays a role in freezing survival (Wu and Storey, 2005; Wu et al., 2008). The present study reports the freeze responsive up-regulation of the glycolytic enzyme, phosphoglycerate kinase 1 (PGK1), in wood frog brain and liver, suggesting that enhancement of the capacity for anaerobic ATP production by glycolysis is adaptive for survival under freeze-induced ischemia.

MATERIALS AND METHODS

Animal care and tissue sampling

Adult male wood frogs, *R. sylvatica*, were collected from breeding ponds near Ottawa (Ont., Canada) in April. Spring frogs newly emerged from hibernation retain freeze tolerance and are available to us in the large quantities that are needed for biochemical studies. The frogs were washed in a tetracycline bath and then placed in containers with damp sphagnum moss and held at 5°C for at least 1 week before use. The procedure for frog freezing is standard in our lab (Storey and Storey, '85); experimental dehydration was conducted as in Churchill and Storey ('93), and anoxic exposure was as in Holden and Storey ('97). All tissue samples were rapidly excised, frozen in liquid nitrogen, and then stored at −70°C. All animals were used according to the guidelines of the Canadian Council on Animal Care and all the experimental procedures had the earlier approval of the Carleton University Animal Care Committee. Tissues were sampled from control frogs that were cold acclimated at 5°C for 2 weeks, from frogs that were frozen for 4, 8, 12, or 24 hr at −2.5°C, and from frogs that were thawed at 5°C for 24 hr after a 24 hr freezing exposure. For dehydration experiments at 5°C, frogs were sampled after the loss of 20 or 40% of total body water or after full re-hydration at 5°C following 40% dehydration. For anoxia exposure, frogs were sampled after 1, 4, 12, or 24 hr under a nitrogen gas atmosphere at 5°C.

Total RNA and mRNA isolation

Total RNA was isolated from frog tissues with Trizol reagent (GIBCO BRL, Grand Island, NY) and poly(A)$^+$ mRNA was purified by oligo(dT) cellulose affinity chromatography (New England BioLabs, Beverly, MA) following manufacturer's instructions. Both total RNA and mRNA were suspended in sterile diethylpyrocarbonate-treated water and stored in aliquots at −80°C. The purity and concentration of RNA were evaluated by spectrophotometry at 260 nm.

cDNA library construction and differential screening

mRNA was isolated from brains of frogs given three lengths of freezing exposure (8, 12, or 24 hr) at −2.5°C and then an equal amount of mRNA from each time point was pooled and used to construct a cDNA library using a Uni-ZAP cDNA synthesis kit (Stratagene, La Jolla, CA) following manufacturer's instructions; greater detail is available in Wu and Storey (2005). The cDNA library was differentially screened using ^{32}P-labeled cDNA probes derived from the poly(A)$^+$ mRNA isolated from tissues of control vs freezing-exposed frogs. Putatively up-regulated clones were further tested by Northern blot analysis with the DNA probe derived from the insert of the clone (see below).

Northern blotting analysis

Samples of total RNA (15 or 20 μg) or mRNA (1 μg) from tissues of control or stressed animals were loaded onto formaldehyde agarose gels following standard methods (Sambrook et al., '89). A 7 μg aliquot of RNA ladder (1 μg/μL, 0.24–9.5 kb, GIBCO BRL) was applied as a size marker in one lane. After electrophoresis, gels were rinsed with ddH_2O, stained with ethidium

bromide (0.5 μg/mL in ddH_2O), and photographed on a UV light transilluminator (Foto/UV 26, Fotodyne Inc., Hartland, WI) at 300 nm by a DS-34 Polaroid camera (Grand Island, NY). The 18S and 28S rRNA bands were used to confirm equal loading of RNA samples. Gels were then soaked in 10 × SSC solution (from 20 × SSC stock: 3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0), and the RNA was transferred onto a nylon membrane (pore size 0.2 μm, Schleicher & Schull, Keene, NH; or Hybond-N, Amersham, Piscataway, NJ) by capillary effection with the same buffer (10 × SSC) (Sambrook et al., '89). The bands of RNA ladder as well as ribosomal RNA in the total RNA sample were visualized by staining with methylene blue. Blots were treated in 5% acetic acid for 10 min, stained in 0.05% methylene blue (in 0.5 M sodium acetate, pH 5.2) for a few minutes, and then destained with ddH_2O until clear band resolution was obtained (Herrin and Schmidt, '88; Sambrook et al., '89). RNA was then fixed to the membrane in a UV cross-linking apparatus (CL-1000 UV cross-linker, UVP Inc., Upland, CA) at 120,000 μJ/cm^2 (254 nm) for 1 min. The membrane was hybridized with specific DNA probe derived from the cloned insert. The insert was isolated by restriction enzyme digestion of the plasmid DNA and restriction fragments were separated by agarose gel electrophoresis. Insert DNA was purified from the gel with a Geneclean III kit (Obiogene, Irvine, CA) (BIO 101).

Synthesis of ^{32}P-labeled DNA probe used the random priming method (Sambrook et al., '89). Northern blotting was conducted similar to the procedure for screening a cDNA library but with less radiolabeled probe ($1–2 \times 10^6$ cpm/per mL of hybridization solution) and higher stringency washing at 50–65°C. Each blot could be reused up to five times after stripping off old probe by heating the blot in a boiling solution of 0.1 × SSC and 0.2% sodium dodecyl sulfate (SDS) for 1–2 min. Autoradiography and X-ray film development were carried out as previously (Cai and Storey, '97a). Band intensity was quantified by densitometry using a Scan Jet3C scanner and Deskscan II v2.2 software (Hewlett-Packard, Palo Alto, CA) to acquire the images by using Imagequant v3.22 software (Innovative Optical Systems Research).

DNA sequencing and analysis

DNA sequencing was performed by the enzymatic chain-termination method (Sanger et al., '77) using the kit from United States Biochemical (Cleveland, OH) and [α-^{35}S]dATP (1,000 Ci/mmol, Amersham). Initial analysis for isolated clones was done from both ends. Subsequently, complete DNA sequences were obtained and/or confirmed by sending samples for automated sequencing by Canadian Molecular Research Services Inc., Ottawa. Nucleotide sequences and deduced polypeptide sequences were analyzed by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). DNASTAR (DNASTAR Inc., Madison, WI) and DNAMAN programs (Lynnon BioSoft, Vaudreuil, Que., Canada) were applied for sequence comparison and alignment.

5′ rapid amplification of cDNA ends (RACE)

5′ RACE was performed using the RLM-RACE kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. Wood frog liver poly(A) mRNA (~250 ng) was used for first strand cDNA synthesis together with random decamers and M-MLV reverse transcriptase following the manufacturer's protocol. The first PCR was performed with the kit's 5′ RACE outer primer and a reverse primer PGK1-R1 (5′ CGGAAAGCTTCTACCTTAGCAGCATC 3′) derived from a cDNA clone of PGK1 whereas the nested PCR was carried out with the 5′ RACE inner primer and the second reverse primer PGK1-R2 (5′ CCTCTTCTACATGGAATCGCAGG 3′) with Advantage-Taq mix kit (Clontech, Mountain View, CA). The 5′ RACE products were isolated with the PCR purification kit (Roche Applied Science, Laval, Que., Canada) according to the manufacturer's instructions and were then cloned into the pGEM T easy vector (Promega, Madison, WI). Plasmid DNA was isolated with High Pure Plasmid Isolation kit (Roche Applied Science, Laval, Que., Canada) and sequenced by the DNA Sequencing and Synthesis Facility, Ottawa Health Research Institute (Ottawa, Ont., Canada).

Western blotting analysis

Frozen tissue samples were weighed and homogenized 1:10 w/v in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1 mM EGTA, and 1 mM EDTA with protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 0.1 mM phenylmethylsulfonyl fluoride) added immediately earlier to homogeniz-

ing. After centrifugation at 14,000 rpm for 15 min at 4°C in a Biofuge 15 (Baxter Canlab, Mississauga, Ont., CA), supernatants were removed and centrifuged again at the same speed for 15 min. Final supernatants were removed and stored at −70°C. Soluble protein concentration was determined by the Bio-Rad protein assay (Bradford, '76). SDS-polyacrylamide gel electrophoresis used the discontinuous system of Laemmli ('70). Protein extracts were diluted 1:1 (v/v) with 2 × sample loading buffer, boiled for 3–5 min, loaded onto 10–12% minigels with 20 μg of protein per lane, and separated by electrophoresis at 180 V for 1 hr using the Bio-Rad mini-gel apparatus. Proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P Transfer Membrane, Millipore Corp., Billerica, MA) by wet transfer with prechilled blotting solution containing 25 mM Tris (pH 8.5), 192 mM glycine, 0.01% SDS, and 20% v/v methanol at 4°C for 1.5–2 hr at 50 V. Western blotting was performed with an anti-PGK1 rabbit polyclonal antibody (a synthetic peptide derived from the C-terminus of PGK1 was used as the immunogen) kindly provided by Dr. John A. Bryant (University of Exeter, Exeter, UK). The protein blot was rinsed with TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and then incubated in blocking buffer (TBST with 1–2% bovine serum albumin or 5% nonfat dry milk) for 1 hr. The blot was then added to blocking buffer containing diluted primary antibody and incubated at room temperature for 1 hr or at 4°C for overnight. Following 3–5 washes in TBST, the blot was incubated with secondary antibody (goat antirabbit immunoglobulin G, horseradish peroxidase conjugated, from Santa Cruz Biotechnology) at 1:5000 v:v. Protein was visualized by enhanced chemiluminescence (NEN Life Science Products, Inc. Boston, MA).

RESULTS

cDNA library screening and Northern blot confirmation of freeze-responsive clones

A cDNA library constructed from brain of frozen wood frogs was screened using ^{32}P-labeled cDNA probes synthesized from the mRNA of control frogs (acclimated at 5°C) vs frozen frogs, as described previously (Wu and Storey, 2005). After primary, secondary, and tertiary screening, five unique clones (named Br3, Br4, Br15, Br30, Br34) were retrieved that were putatively up-regulated during freezing and these were confirmed by Northern blotting. Clone Br15 was identified as the acidic ribosomal phosphoprotein, P0, and characterized by Wu and Storey (2005). Analysis of clone Br30 is presented here.

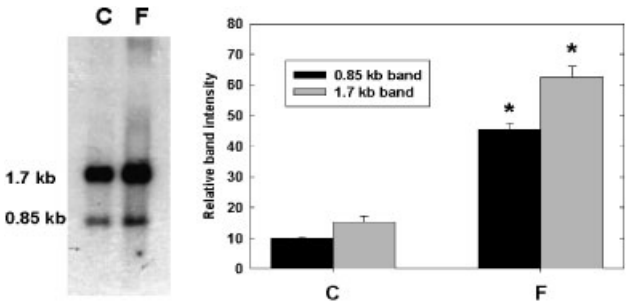

Fig. 1. Northern blots of brain mRNA probed with clone Br30, comparing mRNA sampled from control (5°C acclimated) and 24 hr frozen (−2.5°C) frogs. Each lane was loaded with 1 μg of mRNA and equal loading was confirmed by staining the blot with methylene blue. Relative RNA band intensity was normalized to the 0.85 kb RNA transcript band of the control group. Data are mean ± SEM, $n = 3$ independent determinations. * Significantly different from the corresponding control value using the student's t-test, $P < 0.05$.

Two transcript bands were detected by Northern blotting of mRNA isolated from brain of control (5°C acclimated) and −2.5°C frozen frogs using clone Br30 cDNA as a probe (Fig. 1). The band at ~1.7 kb was the dominant one with a lower intensity band at ~0.85 kb. In an analysis of mammalian *pgk1* mRNA levels, two mRNA bands were also observed (Semenza et al. '94) with the ~1.7 kb band being consistent with the known size of the full *pgk1* transcript. Intensities of both bands increased significantly in brain samples from frozen frogs by 38% for the 1.7 kb band and 50% for the 0.85 kb band.

Cloning, characterization, and identification of clone Br30

Nucleotide sequencing showed that clone Br30 contained a 1608 bp cDNA insert and a BLAST search showed a single long open reading frame (nt 1-1185) that encoded phosphoglycerate kinase isozyme 1 (*pgk1*). The cDNA had a 423 nucleotide 3′ untranslated region that contained a polyadenylation signal (AATAAA) and ended with a poly(A) tail. Hence, the cDNA covered the complete 3′ end of the gene but the 5′ end appeared to be missing a portion of coding sequence as well as the 5′ untranslated region. The technique of 5′RACE was then used to extend the N-terminal part of the sequence. The 5′RACE product was 434 bp; it contained 319 bp that overlapped with

```
Wood frog     MSLSNKLTLDKVDMKDKRVVMRVDFNVPMKNNQITNNQRIKAAVPSIQYCLDNGAKSVVL     60
Clawed frog   ------------v-g-------------------------------------------     60
Zebrafish     -------h-----v-g---i------------v--------------------c--a-a-     60
Chicken       ------------v-g--------------dhk------------t-kh---h-------     60
Human         -----------l-v-g-------------------------------kf-----------     60

Wood frog     MSHLGRPDGVPMPDKYSLAPVAVELKALLKRDVVFLKDCVGPEVEASCAAPATGTVFLLE     120
Clawed frog   ---------------------e---s-m--ei-------------a-sd------l---     120
Zebrafish     ------------------e---a---n--gk--q--------d--k---d-pa-s-i---     120
Chicken       ---------------f-fs----------g-e-s-----------ka--n--n-s-i---     120
Human         -------------------e-------s--gk--l-----------ka--n--a-s-i---     120

Wood frog     NLRFHVEEEGKGKDAAGNKIKADAAKVEAFRASLSKLGDVYINDAFGTAHRAHSSMVGVK     180
Clawed frog   -------------------------n-ds-------------------------------     180
Zebrafish     ------a--------s---t--sq-eid-------------v-----------------n     180
Chicken       ---------------s-----------------------v-----------------h     180
Human         ---------------s---v--ep--i--------------v-----------------n     180

Wood frog     LPQRAAGFLMKKELEYFAKALENPERPFLAILGGAKVKDKIQLINNMLDQVNEMIIGGGM     240
Clawed frog   --------------d-------------------------s----------------     240
Zebrafish     ---k----------d---m---k-q-----------------------k----------     240
Chicken       ---k----------d-------s--------------q------s----k----------     240
Human         ---k-g--------n-------s--------------a-----------k----------     240

Wood frog     AFTFLKVLNNMEIGTSLYDDEGAKIVKDLMAKAEKNLVKITLPVDFTTADKFDENATTGQ     300
Clawed frog   -----------s-------e---n------------g------------n------s---     300
Zebrafish     --------k--------f-e--st------------g---------i-------k----t     300
Chicken       -----------q--n--f-e--s-------------g---------i-------h-q--e     300
Human         -----------------f-e----------s-----g---------v---------k---     300

Wood frog     ATVAGGIPAGWMGLDCGPESMKLFVEAVGRAKQIVWNGPVGVFEWNEFAKGTKAVMDKVV     360
Clawed frog   -s-st---d-----------v------------------------dn-------------     360
Zebrafish     ----e---------------s--ya---a----------------dn--h---nm-----     360
Chicken       ----s---------------v-k---v------------------dk-s-----l-----     360
Human         ----s---------------s-kya---t----------------ea--r----l--e--     360

Wood frog     EVTAKGTITIIGGGDTATCCAKWDTEDKVSHVSTGGGASLELLEGKVLPGVDALSSV       417
Clawed frog   ---g--c-------------------------------------------------       417
Zebrafish     -a-kn-----------------------------------------------------n-     417
Chicken       ---g--c----------------n---------------------------------       417
Human         ka-sr-c----------------n-----------------------------ni       417
```

Fig. 2. Comparison of the deduced amino acid sequence of wood frog (*Rana sylvatica*) PGK1 with the sequences from African clawed frog (*Xenopus laevis*), zebrafish (*Danio rerio*), chicken (*Gallus gallus*), and human (*Homo sapiens*); GenBank accession numbers are AF175978, BC043781, NM_213387, P51903, and P00558, respectively. Dashes replace amino acid residues that are identical with wood frog PGK1. Amino acid substitutions that are unique to the wood frog sequence are in bold underline.

clone Br30 whereas the extended portion of 115 bp contained 66 bp encoding 22 amino acid residues at the N-terminal as well as 49 bp from the 5′UTR. The full length deduced amino acid sequence of wood frog PGK1 contained 417 amino acids as in other species. Comparison of the wood frog sequence with African clawed frog (GenBank accession: BC043781), zebrafish (NM_213387), chicken (P51903) (Rauen et al., '94), and human (P00558) (Michelson et al., '83) PGK1 sequences showed that the frog sequence shared 92.5, 86, 88, and 87% identity with the other sequences, respectively (Fig. 2). Wood frog PGK1 contained four instances where a unique substitution occurred that was fully conserved in the other four species: two amino acids in the N-terminal region (residues 14 and 16) and two more centrally placed (residues 260 and 277). The wood frog *pgk1*sequence was submitted to GenBank (accession number **AF175978)**. Based on this sequence, the size of the protein was 44,751 daltons and the isoelectric point was 6.8.

Northern blot analysis of pgk1 transcript levels in response to freezing in wood frog organs

A major RNA band (1.7 kb) cross reacting with the Br30 probe was detected on total RNA blots of wood frog brain, liver, and skin (Fig. 3). Transcript levels rose significantly by 80–100% in wood frog brain over 12–24 hr freezing at −2.5°C and by 50% in liver of 24 hr frozen frogs, as compared with controls at 5°C. Transcript levels remained elevated during thawed recovery in brain and increased further in liver (to 2.5-fold higher than controls). In skin the *pgk1* probe hybridized with two distinct bands (1.7 and 3.4 kb) but no significant changes in band intensity were seen during freezing or thawing.

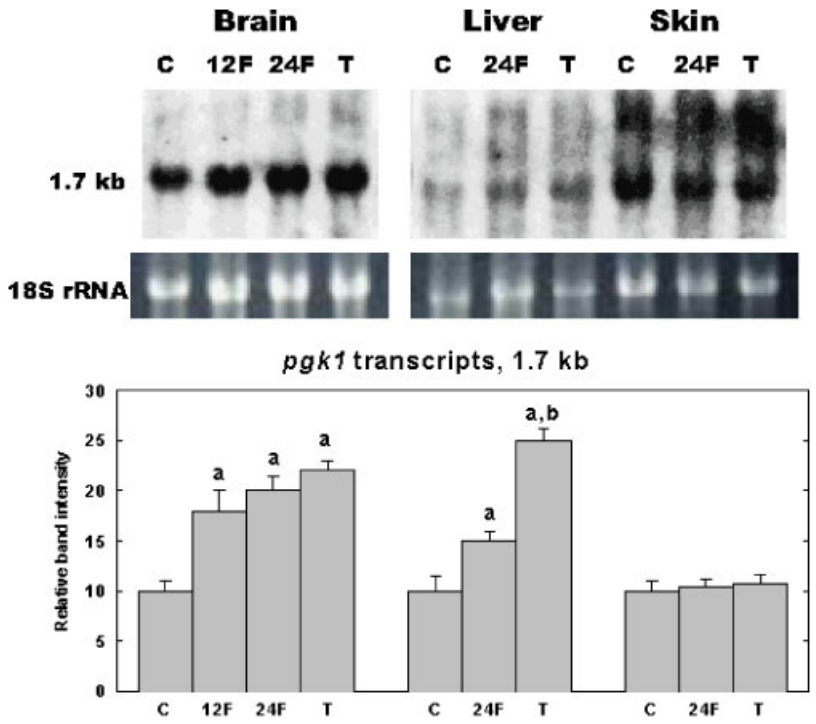

Fig. 3. Expression of *pgk1* gene transcripts during freezing and thawing in brain, liver, and skin of wood frogs. The gene probe was derived from the insert of frog brain Br30 and bound to a 1.7 kb band on the blot. Experimental groups are C, control cold-acclimated frogs at 5°C for 2 weeks; 12F or 24F, frogs frozen at −2.5°C for 12 or 24 hr; T, 24 hr thawed recovery at 5°C after 24 hr freezing at −2.5°C. For liver and skin, only 24 hr frozen was evaluated. Ethidium bromide staining of 18S rRNA bands was used to assess equal loading of lanes. Band intensities of *pgk1* transcripts in each lane were normalized against their corresponding rRNA band intensities and then mean relative intensities for each group were plotted in the histogram. Values for experimental treatments were normalized against the controls for each tissue that were set to 10. Data are means ± SEM, $n = 3$ independent trials. [a] Significantly different from the corresponding control value after analysis of variance (ANOVA) followed by the Student Newman Keuls test, $P < 0.005$; [b] significantly different from the 24 hr frozen value, $P < 0.005$.

Analysis of organ-specific pgk1 gene expression in response to anoxia and dehydration

Transcript levels of *pgk1* also increased significantly in frog brain under anoxia exposure, being elevated by about 2.5-fold after just 1 hr under a nitrogen gas atmosphere (Fig. 4). Transcript levels in brain were similarly increased by ~2.4-fold when frogs were dehydrated to either 20 or 40% of total body water lost.

In liver, short anoxia exposures enhanced *pgk1* expression (levels were ~2.5-fold higher than controls after 4 hr anoxia exposure) but with longer anoxia exposures *pgk1* levels were reduced (Fig. 5). Transcript levels also increased in liver by 2.8-fold in frogs that had lost 20% of total body water but levels fell again when frogs experienced more extensive dehydration to 40% of total body water lost.

Frog PGK1 protein production in response to stresses

Western blotting showed that a protein band of ~45 kDa in frog brain extracts was recognized by the anti-PGK1 antibody (Fig. 6). The intensity of the protein band increased under freezing, anoxia, or dehydration stresses and was particularly pronounced under anoxia. PGK1 protein content increased by 3.3-fold after 4 hr freezing and reached 4.9-fold higher than controls in 8 hr frozen frogs as compared with 5°C controls. Protein levels declined with longer freeze-exposure (12–24 hr) but after 24 hr thawed, PGK1 protein was again elevated with levels that were 4.2-fold higher than control values. Anoxia exposure (4 or 24 hr) strongly increased brain PGK1 content with levels rising by 8.9–10.6 fold. Brain PGK1 protein was rose by 6.3-fold in 40% dehydrated frogs but levels were reduced again after rehydration.

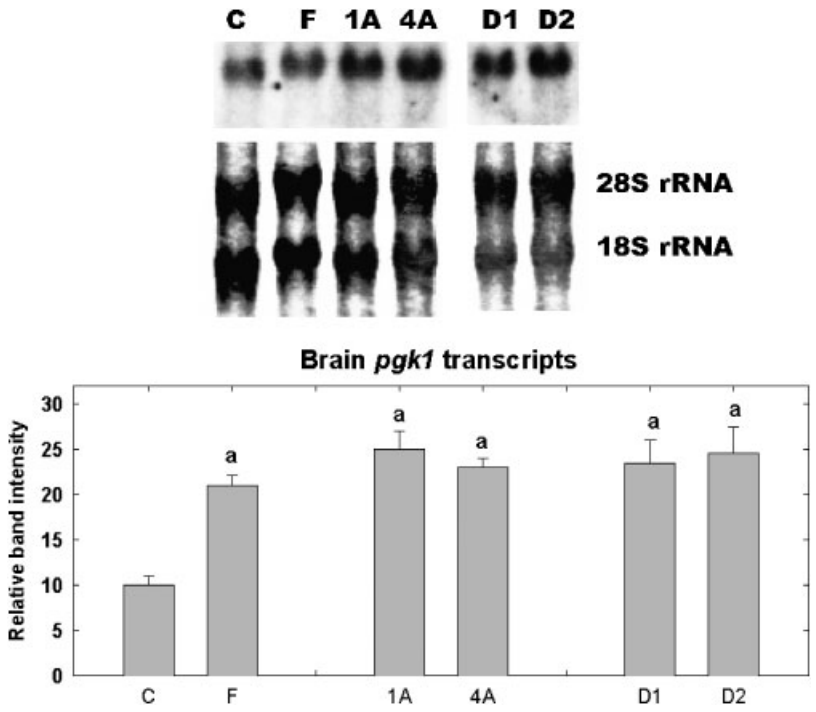

Fig. 4. Differential expression of the *pgk1* gene in wood frog brain in response to freezing, anoxia and dehydration. Total RNA was isolated from tissues and 15 μg was loaded per lane. Frog brain clone Br30 was used as the probe. Controls (C) were cold acclimated at 5°C for 2 weeks. Freeze-exposed (F) frogs were given 24 hr at −2.5°C. Anoxia (A) exposures were 1 or 4 hr under a nitrogen gas atmosphere at 5°C and dehydration at 5°C was to 20% (D1) or 40% (D2) of total body water lost. Methylene blue staining shows 18S and 28S ribosomal RNA band intensities. Band intensities of *pgk1* transcripts in each lane were normalized against the corresponding 18 S rRNA band intensity and then mean relative intensities for each group were plotted. Histograms show means ± SEM for $n = 3$ independent trials; experimental values are expressed relative to the RNA band intensity of controls. [a] Significantly different from the control value after analysis of variance (ANOVA) followed by the Student Newman Keuls test, $P < 0.005$.

DISCUSSION

Phosphoglycerate kinase is one of the two ATP-generating reactions of glycolysis and, as such, has a key role to play in energy metabolism under conditions when mitochondrial ATP output is limiting (e.g. anoxia, ischemia). Not surprisingly, then, elevated amounts of PGK1 (EC 2.7.2.3) have been reported as a response to hypoxia stress in mammalian cells (Firth et al., '94) and *pgk1* gene expression is known to be regulated by the hypoxia-inducible transcription factor 1 (HIF-1) (Semenza et al., '94; Li et al., '96; Okino et al., '98). The up-regulation of *pgk1* transcripts and PGK1 protein levels in selected frog organs during freezing is probably a response to the ischemia that develops when plasma freezes. Freezing of blood plasma halts oxygen delivery to tissues and results in a shift to glycolytic ATP output as evidenced by the accumulation of lactate and alanine as glycolytic end products in organs of frozen frogs (Storey, '87). Peripheral ischemia develops quickly (within 1–4 hr) as skin and muscles freeze but slow circulation to core organs persists for 6 hr or more (Costanzo et al., '95) until cardiac arrest occurs. Hence, PGK1 protein accumulation in brain after 4 and 8 hr freezing probably represents a compensatory response by this oxygen-sensitive organ to hypoxia/ischemia and aims to elevate the amounts of selected glycolytic enzymes by stimulating transcription and translation. Steady-state mRNA levels of several glycolytic enzymes including selected isozymes of PGK, hexokinase, aldolase, enolase, glyceraldehydes-3-P dehydrogenase, pyruvate kinase, and lactate dehydrogenase are known to be elevated under low oxygen conditions in hypoxia-sensitive species (Semenza et al., '94; Wang et al., '95; Haddad, 2002). With longer freezing, however, such a compensatory response that relies on ATP-expensive protein synthesis is impractical because oxygen limitation is severe and ATP is in short supply. A strategy of metabolic rate depression is then initiated, which

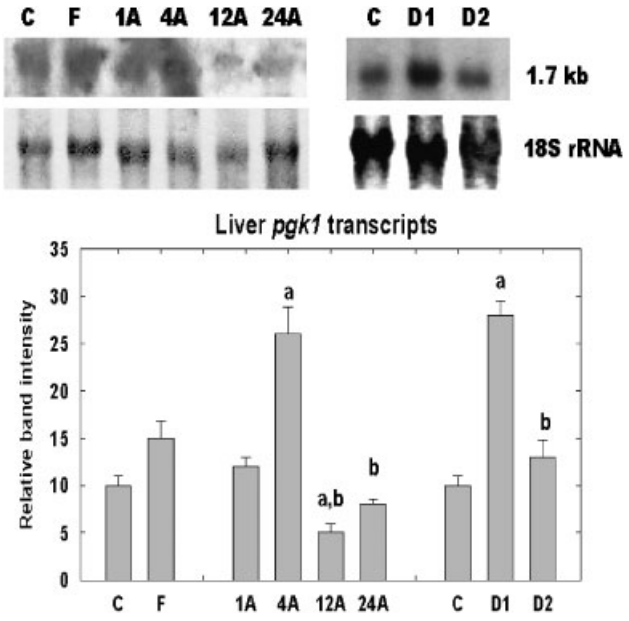

Fig. 5. Differential expression of *pgk1* in wood frog liver under freezing, anoxia, and dehydration stresses. Each lane was loaded with 15 μg of total RNA. The 18S ribosomal RNA bands were stained with methylene blue. Groups are control (C) (cold acclimated at 5°C for 2 weeks); frozen (F) (24 hr at −2.5°C); anoxic (A) (1, 4, 12, or 24 hr under a nitrogen gas atmosphere); and dehydrated to 20% (D1) or 40% (D2) of total body water lost. Band intensities of *pgk1* transcripts in each lane were normalized against the corresponding 18 S rRNA band intensity and then mean relative intensities for each group were plotted. Histograms show means ± SEM for $n = 3$ independent trials; experimental values are expressed relative to the RNA band intensity of controls. [a] Significantly different from the corresponding control value after analysis of variance (ANOVA) followed by the Student Newman Keuls test, $P < 0.005$; [b] significantly different from the corresponding 4 hr anoxic or 20% dehydrated value, $P < 0.005$.

strongly inhibits all ATP-expensive cellular processes and lowers net ATP expenditures to a level that can be met during long-term freezing by the ATP output of glycolysis alone (Storey and Storey, 2004).

An up-regulation of PGK-1 (and perhaps other HIF-1 regulated glycolytic enzymes) during freezing might also seem to be at odds with the need to produce and maintain high levels of glucose as a cryoprotectant in wood frog organs, glucose being an excellent substrate for anaerobic glycolysis. It must be remembered, however, that up-regulation of glycolytic enzymes is a conserved response to low oxygen across vertebrate phylogeny and it is a response that wood frogs need during normal unfrozen life. Cells would be unlikely to distinguish, at least initially, between oxygen limitation owing to freezing vs any one of several other causes of low oxygen availability. Hence, the early response, seen clearly in brain after 4 or 8 hr frozen is an increase in PGK1 protein (Fig. 6). However, PGK1 levels during freezing never reach the comparable values seen after the same lengths of anoxia exposure and, indeed, PGK1 protein in brain fell again to near control values by 12 or 24 hr frozen (Fig. 6). Although any consumption of cryoprotectant glucose by brain of frozen frogs would be gated primarily by hexokinase, a reversal of the earlier elevation of PGK1 protein in brain when freezing is prolonged could contribute to minimizing glycolytic rate to support long-term metabolic suppression in the frozen state. After 24 hr thawing, however, *pgk1* transcripts in liver and PGK1 protein in brain were substantially increased as compared with values in 24 hr frozen frogs (Figs. 3 and 6) and this, we believe, represents a recovery response. As organs thaw and rehydrate, oxygen-sensing mechanisms would perceive low oxygen signals that would persist until proper circulation is re-established. Resumption of breathing and the return of a bright red coloration to blood can take as long as 12 hr when frogs are thawed at 5°C (J. Storey, personal observations). Hence, it is likely that HIF-1 mediated responses that aim to enhance glycolytic capacity are engaged again when animals thaw.

The present study shows that *pgk1* transcripts increased significantly in wood frog brain and liver under freezing, anoxia, or dehydration stresses. PGK1 protein levels in brain rose in parallel and, in particular, were strongly enhanced by >10-fold under anoxia. This provides strong evidence that, as in mammals, increased levels of PGK1 enhance the glycolytic capacity of wood frog organs under situations that impose hypoxia or ischemia. Studies of the expression of several freeze-responsive genes in wood frogs have shown that these genes are also typically responsive to one of two component stresses of freezing: anoxia (representing freeze-induced ischemia) or dehydration (representing freeze-induced cell volume reduction) (Storey, 2004). For example, up-regulation of fibrinogen and of the mitochondrial phosphate carrier in liver responded to freezing and dehydration but not to anoxia (Cai and Storey, '97a; DeCroos et al., 2004) whereas levels of the mitochondrial ADP–ATP translocase in liver and the ribosomal phosphoprotein P0 in brain responded only to freezing and anoxia (Cai et al., '97; Wu and Storey, 2005). The present study shows that *pgk1* transcripts and PGK1 protein levels in brain were significantly elevated under both anoxia and dehydration stresses although the

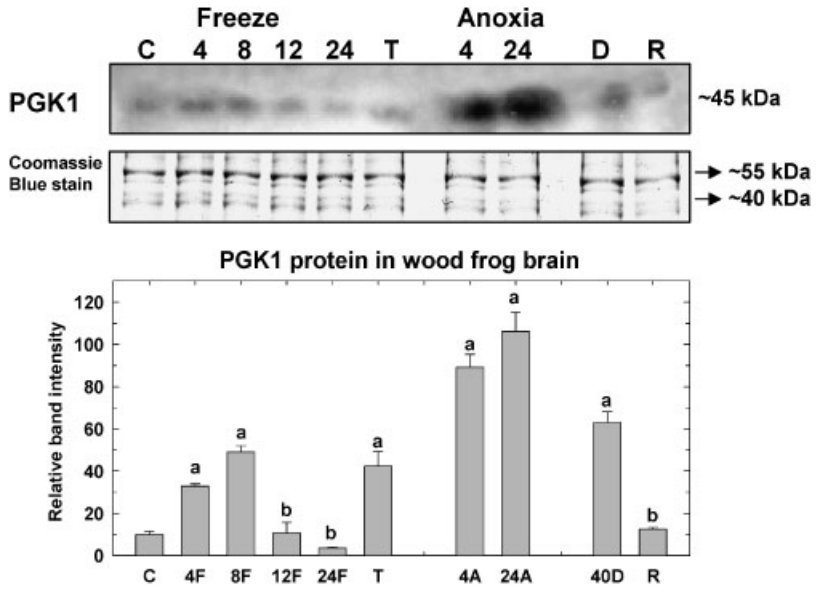

Fig. 6. Western blotting to detect PGK1 protein levels in wood frog brain. Equal amounts of protein (20 μg) were loaded in each lane. Lanes are C, control, cold-acclimated at 5°C for 2 weeks; Freeze-exposed at −2.5°C for 4, 8, 12, or 24 hr; T, thawed at 5°C for 24 hr after 24 hr frozen at −2.5°C; A, anoxia-exposed for 4 or 24 hr at 5°C; D, dehydrated to 40% of total body water lost; R, fully re-hydrated after 40% dehydration. After immunoblotting, gels were stained with Coomassie blue to confirm equal loading; a section of the gel showing the 40–55 kDa protein region is shown. Histograms show means ± SEM for $n = 3$ independent trials. [a] Significantly different from the corresponding control value after analysis of variance (ANOVA) followed by the Student Newman Keuls test, $P < 0.01$; [b] Significantly different from the corresponding 8 hr frozen or the 20% dehydrated values, $P < 0.01$.

PGK1 protein response to dehydration was less pronounced than it was to anoxia. Transcripts of *pgk1* were also elevated in wood frog liver in response to freezing, anoxia, and dehydration stresses. A response to all three stresses has only been seen previously for two novel genes, *fr47* and *li16*, cloned from wood frog liver; their functions are not yet known (McNally et al., 2002, 2003). It should be noted that at higher values of water loss, dehydration stress does have a hypoxic component to it because the increased viscosity and reduced blood volume in dehydrated animals makes adequate oxygenation of organs increasingly difficult. Hence, it is possible that the *pgk1* expression responses seen under both freezing and dehydration are the result of a very sensitive response by this gene to hypoxia. Studies have also shown that subpopulations of PGK1 and several other glycolytic enzymes are found in the nucleus where they are known to bind DNA and could have regulatory functions (Ronai, '93; Brice et al., 2004). It would be interesting to determine whether this also occurs in frog organs and whether a rapid early up-regulation of PGK1 in response to stress would lead to the accumulation of PGK1 protein in the nucleus of wood frog brain cells where it could affect transcriptional regulation of other genes. Notably, under anoxia, *pgk1* transcript levels in brain were elevated within 1 hr and protein levels were increased by > eight-fold within 4 hr (Figs. 4 and 6).

The amino acid sequence of PGK1 appears to be highly conserved across vertebrate phylogeny. Wood frog PGK1 showed a very high identity (92.5%) with the *Xenopus laevis* sequence and 86–88% identity with fish, avian, and mammalian sequences. There were four instances where unique amino acid substitutions occurred in the wood frog protein, which were fully conserved in the other species. Given that the other four species were either tropical ectotherms (*Xenopus*, zebrafish) or endotherms (chicken, human), it is possible that these substitutions may provide the wood frog protein with a greater flexibility to maintain an active conformation over the wide range of body temperatures (from >30°C to <0°C), which this northern frog would encounter. In particular, substitutions that sustained enzyme conformation and catalytic function at near 0°C body temperatures would be required for winter life.

In summary, PGK1 is one of two ATP-generating reactions in glycolysis and, hence, has an essential role to play in cellular energy production

under oxygen-limiting conditions. Increased amounts of this enzyme in response to stresses that cause low oxygen conditions (e.g. freezing) enhance glycolytic capacity and contribute to maintaining cellular homeostasis and energy levels under stress conditions. The present study shows that wood frogs that are both freeze tolerant and anoxia tolerant elevate PGK1 transcripts and protein in response to hypoxia/ischemia in the same manner that anoxia-intolerant species (e.g. mammals) do, arguing for a conserved vertebrate response to low oxygen, including gene transcription responses mediated via HIF-1. However, recent studies have also indicated that PGK1 can have multiple roles, acting in the cytoplasm as an enzyme of glycolysis but in the nucleus as a DNA binding protein that may have regulatory effects (Ronai, '93).

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