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Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate

Kevin Larade* and Kenneth B. Storey†

Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada, K1S 5B6

*Present address: Hematology Division, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA

†Author for correspondence (e-mail: kenneth_storey@carleton.ca)

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Summary

Differential screening of a *Littorina littorea* (the common periwinkle) cDNA library identified ferritin heavy chain as an anoxia-induced gene in hepatopancreas. Northern blots showed that ferritin heavy chain transcript levels were elevated twofold during anoxia exposure, although nuclear run-off assays demonstrated that ferritin heavy chain mRNAs were not transcriptionally upregulated during anoxia. Polysome analysis indicated that existing ferritin transcripts were actively translated during the anoxic period. This result was confirmed *via* western blotting, which demonstrated a twofold increase in ferritin heavy chain protein levels during anoxia, with a subsequent decrease to control levels during normoxic recovery. Organ culture experiments using hepatopancreas slices demonstrated a >50% increase in ferritin heavy chain transcript levels *in vitro* under conditions of anoxia and freezing, as well as after incubation with the second messenger cGMP. Taken together, these results suggest that ferritin heavy chain is actively regulated during anoxia exposure in the marine snail, *L. littorea*.

Key words: oxygen, gastropod, oxidative stress, transcription, translation, common periwinkle, *Littorina littorea*.

Introduction

Marine periwinkles, inhabitants of the intertidal zone, endure cyclic periods of anoxia for several hours at a time as a result of twice daily aerial exposures at low tide. Adaptations that allow snails to cope with decreased oxygen supply are discussed extensively in Larade and Storey (2002a). In addition to surviving extended periods of low oxygen, snails must also be able to cope with the large influx of oxygen that results upon their return to oxygenated water at high tide. This rapid rise in oxygen levels causes a rapid increase in metabolic rate coupled with a rapid rise in the tetravalent reduction of oxygen to H_2O by the electron transport chain. Sudden changes in oxygen availability can also result in a burst of reactive oxygen species (ROS) formation, creating conditions that are collectively known as oxidative stress. ROS species include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (·OH) that is responsible for the most oxidative damage to cellular macromolecules. *In vivo*, much of the hydroxyl radical production comes from the reduction of peroxide by superoxide (the Haber–Weiss reaction) that is, in fact, a two-step process catalyzed by transition metals (predominantly Fe^{3+}, but sometimes Cu^{3+}) and involving the Fenton reaction. Because of the central role of iron in catalyzing ROS production, iron in cells is typically strictly sequestered into storage proteins such as ferritin to keep free iron concentrations low, limiting the oxidative damage that would otherwise result.

The metal chelator, ferritin, is a multimeric heteropolymer composed of 24 subunits characterized as heavy (21 kDa) or light (19 kDa) chains, each of which performs a specific role (reviewed in Harrison and Arosio, 1996). Ferritin heavy chain subunits, which contain a catalytic ferroxidase center not present on the light chain, are responsible for the oxidation of iron and allow iron uptake/release (Lawson et al., 1989). By decreasing the intracellular free iron pool, ferritin prevents the formation of highly toxic hydroxyl radicals *via* the iron-catalyzed Fenton reaction. Ferritin has previously been demonstrated to play a major role in resistance to, and preventing damage as a result of, oxidative stress in vertebrate systems (Orino et al., 2001). This study is the first to examine expression patterns at the RNA and protein levels in a unique anoxia-tolerant model system, the marine periwinkle.

Materials and methods

Preparation of experimental animals

Marine periwinkles, *Littorina littorea* L., were acclimated with a natural light/dark cycle without feeding at ~10°C in covered tanks of artificial seawater (Coralife Scientific Grade

Marine Salt, Harbor City, CA, USA) with constant air bubbling for 1 week prior to experimentation. To prepare for anoxia exposure, a small amount of seawater (~3 cm depth) was placed in the bottom of glass incubation jars to maintain high humidity while presenting a pseudo-immersed environment. Jars were gassed with 100% nitrogen for 30 min on ice and snails were quickly transferred into the jar, which was then sealed except for the port for the N_2 gas line. Gassing was continued for a further 15 min and the jars were fully sealed. Snails were sampled after 12, 24, 48, 72 or 120 h with animals for each time point coming from a different sealed jar. Snails in other jars were given anoxia exposure for 72 or 120 h and then returned to aerobic conditions and sampled after 1 h and 6 h of recovery. Control animals were placed in jars as above (~3 cm of seawater) but were incubated without N_2 gassing and sampled after the anoxic time course was complete.

cDNA library synthesis and differential screening

A hepatopancreas cDNA library was synthesized as outlined previously (Larade et al., 2001). Briefly, mRNA from hepatopancreas was isolated from animals from each of three anoxia exposure times (1, 12 and 24 h) and an equal amount (1 μg) from each time point was combined to construct a composite cDNA library using a Uni-ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Differential screening was performed using two sets of radiolabeled probe synthesized from (a) anoxia exposed animals (1, 12, 24 h) or (b) control snails held under aerated conditions at 10°C. Radiolabeled single-stranded cDNA probe was synthesized using random priming and [α-^{32}P]dCTP (3000 Ci mol^{-1}; Amersham, Cleveland, OH, USA) and hybridized to plaque-lift membranes in Church's buffer (0.25 mol l^{-1} Na_2HPO_4, 0.25 mol l^{-1} NaH_2PO_4, pH 7.5, 1 mmol l^{-1} EDTA, 7% w/v SDS) at 44°C overnight (~16 h). Blots were washed in 6× SSC (1× SSC=0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate, pH 7.6) at room temperature for 15 min or until background was eliminated. Blots were then exposed to Kodak XAR5 X-ray film at –80°C for an appropriate length of time and developed manually using Kodak chemicals (Rochester, NY, USA). Putative anoxia-responsive clones were re-plated on separate circular (10 cm diameter) agar plates to 50–100 p.f.u. per plate. Secondary screening with radiolabeled control and anoxia-exposed probes was carried out as described above for primary screening. Putative positive clones representing genes that were upregulated by anoxia-exposure were selected upon examination of the resulting autoradiograms. Clones were bidirectionally sequenced by Canadian Molecular Research Services (Ottawa, ON, USA) and sequence analysis was performed.

Northern blotting

Total RNA was isolated from hepatopancreas of control and experimental snails (anoxia exposed or aerobic recovery) using Trizol reagent (Gibco BRL, Burlington, ON, Canada), according to the manufacturer's instructions, and separated on a 1.2% agarose formaldehyde denaturing gel (20 μg per lane). Gels were blotted onto Hybond 0.45 μm nylon membranes (Amersham) that were then UV cross-linked and baked in a BioRad (Hercules, CA, USA) Model 583 gel dryer for 2 h at 80°C. Pre-hybridization of blots was carried out at 44°C with Church's buffer for at least 30 min. Radiolabeled single-stranded cDNA probe was synthesized using random priming with [α-^{32}P]dCTP (3000 Ci mol^{-1}, Amersham), hybridized to blots, with subsequent autoradiography, as outlined above. Densitometric analysis of the developed autoradiograms was performed using a Scan Jet3C scanner in conjunction with Deskscan II v2.2 software (Hewlett-Packard, Palo Alto, CA, USA), producing high-resolution computer generated images that were then analyzed with Imagequant v3.22 (Innovative Optical Systems Research, Sunnyvale, CA, USA).

Nuclear isolation and transcriptional run-off assays

Nuclear isolation and run-off assays were performed as described previously (Larade and Storey, 2002b). Briefly, fresh hepatopancreas samples were homogenized in ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 50 mmol l^{-1} Hepes, pH 7.5, 25 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA), filtered through two layers of cheesecloth, and loaded on top of a sucrose cushion (2.0 mol l^{-1} sucrose, 10% v/v glycerol, 50 mmol l^{-1} Hepes, pH 7.5, 25 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA). Nuclei were pelleted with centrifugation at 13,000 ***g*** for 30 min at 4°C and resuspended in a glycerol storage buffer (40% v/v glycerol, 75 mmol l^{-1} Hepes, pH 7.5, 60 mmol l^{-1} KCl, 15 mmol l^{-1} NaCl, 0.5 mmol l^{-1} dithiothreitol, 0.1 mmol l^{-1} EGTA, 0.1 mmol l^{-1} EDTA, 0.125 mmol l^{-1} phenyl methyl sulphonyl fluoride). Nuclear run-off assays were incubated for 30 min at 26°C in a final volume of 800 μl containing 0.1 mol l^{-1} Tris-HCl, pH 7.4, 200 mmol l^{-1} NaCl, 4 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} $MnCl_2$, 1.2 mmol l^{-1} dithiothreitol, 0.4 mmol l^{-1} EDTA, 1.25 mmol l^{-1} GTP, 1.25 mmol l^{-1} CTP, 1.25 mmol l^{-1} ATP, 500 μCi [α-^{32}P]UTP (Amersham), 10 mmol l^{-1} creatine phosphate, 2 μg of creatine phosphokinase, 20 units ml^{-1} RNasin, 10 μl of heparin stock (1/10 dilution), 20% v/v glycerol and nuclei equivalent to approximately 20 μg of protein. Total RNA was isolated from nuclei and used to probe dot blots prepared using clones isolated from differential screening of the hepatopancreas cDNA library. Blots were exposed to X-ray film, developed and analyzed as above.

Western blotting

For protein analysis, hepatopancreas samples were gently homogenized (1:3 w/v) with a ground glass homogenizer in homogenization buffer (25 mmol l^{-1} Tris, pH 7.6, 25 mmol l^{-1} NaCl, 100 mmol l^{-1} sucrose, 1% w/v SDS). Protein samples (20 μg) were separated on 10% SDS-polyacrylamide gels and a standard western blotting protocol (Sambrook et al., 1989) was used to determine ferritin protein levels. Membranes were blocked for 2 h at room temperature using MTBST (1% w/v powdered skim milk, 150 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris-HCl pH 6.8, 0.05% v/v Tween 20), incubated with primary antibody (1:3000 dilution) in 10 ml of MTBST overnight at

4°C. Bound antibody was detected with horse radish peroxidase-linked anti-rabbit IgG secondary antibody (1:2000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized using enhanced chemiluminescence (Renaissance, Perkin Elmer Life Sciences, Inc.). Blots were exposed to X-ray film for an appropriate length of time and film was developed as previously described, with subsequent densitometric analysis. The ferritin antibody was a gift from the laboratory of Oivind Andersen (Akvaforsk, Institute of Aquaculture Research; Andersen et al., 1995).

Polysome analysis and northern blotting

An individual fresh hepatopancreas (~0.5 g) was gently homogenized with a ground glass homogenizer in 2 ml of STSM buffer [300 mmol l^{-1} sucrose, 250 mmol l^{-1} Tris-HCl, pH 7.6, 25 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 0.1% diethyl pyrocarbonate (DEPC)]. The homogenate was centrifuged at 13,000 ***g*** for 15 min at 4°C. The supernatant was removed, Triton X-100 was added to a concentration of 0.5% v/v, and then the sample was loaded onto a 5 ml sucrose gradient (15–30%) prepared in 250 mmol l^{-1} Tris-HCl, pH 7.6, 250 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 0.1% DEPC. Gradients were centrifuged at 40 000 ***g*** for 2 h and then recovered in 0.5 ml fractions. The RNA content of each fraction was monitored spectrophotometrically at 254 nm. The transcript distribution of ferritin heavy chain mRNA and alpha tubulin mRNA within the polysome gradient was analyzed by northern blotting and densitometric analysis as described above.

Tissue explants

Incubations of tissue explants were performed as previously described (Larade and Storey, 2002b). Briefly, fresh hepatopancreas from individual snails was chopped into cubes of ~0.5 cm^3. Paired samples from each hepatopancreas were placed into separate sterile 15 ml tubes (control and experimental) containing 4 ml of explant medium (artificial seawater filtered through a 0.2 μm filter, 100 μg ml^{-1} streptomycin sulfate, 100 μg ml^{-1} benzylpenicillin, 10 μg ml^{-1} fungizone) held at 4°C for the duration of each experiment. Anoxia was imposed by bubbling nitrogen gas through the explant medium for 15 min prior to the addition of tissue, followed by continuous gassing over the 12 h anoxia exposure time. Control tissues were bubbled in an identical manner with air. Freezing stress was produced by placing tissues in their explant medium in a freezer set at –7°C for 12 h. For the second messenger experiments, tissues were pre-incubated in aerobic culture medium at 4°C for 30 min followed by addition of db-cGMP (N^2-2′-O-dibutyrylguanosine-3′:5′-cyclic monophosphate; 10 μl ml^{-1} of medium; final concentration 0.2 mmol l^{-1}) to one of each pair of samples with 10 μl ml^{-1} of the vehicle added to control samples. All samples were then incubated for 2 h. After incubation, tissues were removed from the medium, immediately frozen in liquid nitrogen and then stored at –80°C until used for total RNA isolation using Trizol reagent (Gibco BRL). RNA samples were resuspended in 50 μl of DEPC treated water.

Results

Anoxic induction of ferritin heavy chain mRNA

Hepatopancreas of anoxic *L. littorea* was used to create a cDNA library that was differentially screened using ^{32}P-labeled cDNA probes made from mRNA isolated from aerobic *versus* anoxic snails. Screening revealed several clones that were upregulated during anoxia (Larade et al., 2001; Larade

A

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1    CATCAAGGAGCTCGGTGACCACATCACCAACCTCAAGCGTGTGGGCAGCGGCCTGGGAGA
1      I  K  E  L  G  D  H  I  T  N  L  K  R  V  G  S  G  L  G  E

61   GTACGAGTACGACAAGAACCTCTCCTCCTAGACGCTTCACCAGCCTCCACAGCACAGYWG
21     Y  E  Y  D  K  N  L  S  S  *

121  CAAGAATATCCATAGGATCCTGGAATCCCTTTCTTGAAATCAACGGCTGTGTAGTGTTCT
181  CCTTGTCTTTGACGACGAACGCTGTGTCCACGGTTTTGTGCGGTCTGGCCTCGGCTAGTT
241  TCAACGGCTGAGTTTTACTTTTTCTTTCTTTCTCTTTTCTTAGCGTTGTGGTGATCGAAC
301  TGTCTGATTTGTTAGCATCGAAAAACAAAAATCTTCGAACCGTATTTTGGTTGTGCAAGT
361  AGGGAAGTGAAAGGAAATGAAAAGAACTAAAAAGAAATTTTAGGGAAACAATAATAAAAA
421  TTGCAAAAAAACACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
481  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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B

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Littorina  IKELGDHITNLKRVGS...GLGEYEYDK.NLSS....
Lymnaea    ----s-y--------p...-----if--et---ss..
Chiton     ----s-y--i---t-d...-----lf--d.-d-ky..
Starfish   ---------q-t---p...-----t---e--ged...
Salmon     --k--------tkmdavknkma--lf--ht-ggqs..
Human      -------v---rkm-apes--a--lf--ht-gdsdne
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Fig. 1. Sequence and partial alignment of ferritin heavy chain from *L. littorea*. (A) Nucleotide (upper) and deduced amino acid (lower) sequences of the cDNA encoding the C-terminal end of ferritin heavy chain from the *L. littorea* library. The nucleotide sequence has the GenBank accession number AY090096. (B) Alignment of the C-terminal amino acid sequence of ferritin heavy chain from *L. littorea* with sequences from *Lymnaea* (pond snail; accession number P42577), chiton (BAA21810), starfish (AAB60883), salmon (P49946), and human (P02794). Identical residues are indicated by (–); stops indicate gaps.

and Storey, 2002b, 2004), including a 513 bp cDNA identified as ferritin heavy chain *via* BLASTX searching. The nucleotide sequence of the *L. littorea* ferritin heavy chain (GenBank accession number AY090096) is similar to previously identified sequences of this protein from other molluscs. The sequence isolated was partial and represented the terminal 91 bp of the 3′ end of the established open reading frame. Translation of this putative protein yielded the 29 C-terminal amino acids of the *L. littorea* ferritin heavy chain protein, which demonstrated a high degree of identity with existing ferritin heavy chain sequences (Fig. 1). This cDNA sequence was used as a probe for expression analysis.

Ferritin mRNA transcriptional status

To confirm that ferritin heavy chain was an anoxia-responsive gene in *L. littorea* hepatopancreas, changes in ferritin transcript levels were monitored over a time course of anoxia and aerobic recovery. Northern blotting revealed that ferritin transcript levels increased significantly (by twofold) after 24 h of anoxia exposure and stayed at this level for the remainder of the 5-day anoxia exposure (Fig. 2). However, within 1 h of oxygenated recovery, transcript levels were reduced again to near control levels. Since cellular mRNA levels are governed by the rates of both mRNA production and decay, the observed increase in ferritin heavy chain transcripts may be due to increased transcription of the gene. To determine if the transcription of the ferritin heavy chain gene was actually increased during anoxia, the rate of transcription was measured directly using the nuclear run-off assay. Dot blots using ferritin heavy chain cDNA were used to determine the extent of labeling of ferritin heavy chain mRNA transcripts after incubation of nuclei isolated from normoxic *versus* 48 h anoxic hepatopancreas. The ratio (anoxic:aerobic) of radiolabeled transcripts encoding ferritin heavy chain showed similar levels of ^{32}P-dUTP incorporation into newly synthesized transcripts from nuclei of anoxic snails relative to levels of incorporation into ‘housekeeping’ messages (Fig. 3).

Ferritin mRNA translational status

To assess qualitatively whether mRNA shifted its association from polysomes to monosomes during anoxia, northern blots of the total RNA isolated from each fraction of a polysome profile were assayed for the presence of ferritin heavy chain transcripts and alpha tubulin. The densitometric profiles in Fig. 4 show that ferritin heavy chain message (line graph) remained associated with polysome fractions [P] during normoxia (Fig. 4A), 72 h anoxia exposure (Fig. 4B) and 6 h aerobic recovery (Fig. 4C), regardless of overall polysome aggregation state as indicated by total RNA profile (Fig. 4, bar graphs). The absorbance at 254 nm of total RNA profiles showed a distinct shift of ribosomal RNA into the monosome fractions (M) in the 72 h anoxic animals and a reversal of this effect when aerobic conditions were re-established. The

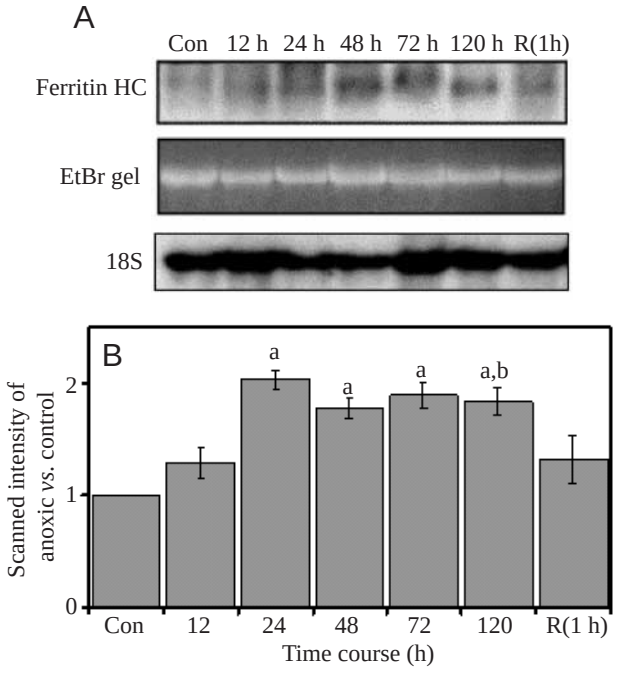

Fig. 2. (A) Expression of ferritin heavy chain (HC) transcripts in hepatopancreas of *L. littorea* over a time course of anoxia exposure (12–120 h) and aerobic recovery (1 h after 120 h anoxia) as shown by northern blots (top). Scanned intensity of anoxic samples was normalized against the ethidium bromide (EtBr) stained gel (middle) and the 18S ribosomal RNA band (bottom) and then plotted relative to the intensity of the control band. (B) Histograms show means ± S.E.M. for N=3 independent blots using RNA extracted from different snails. [a]Significantly different from the normoxic control as determined by the Dunnett’s test, P<0.01. [b]Significantly different from 1 h aerobic recovery, P<0.05. Con, control; R, recovery.

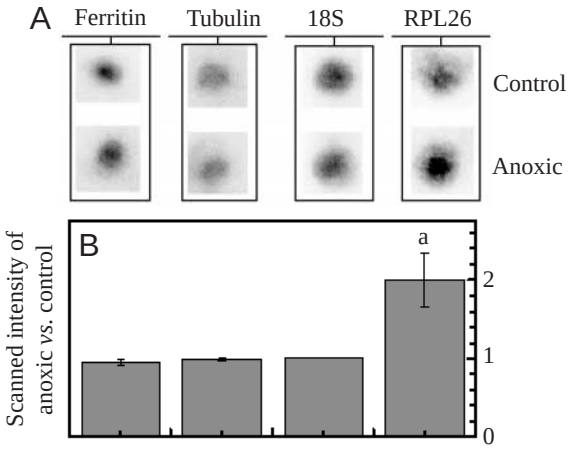

Fig. 3. Nuclear run-off assays examining transcriptional regulation of ferritin heavy chain. (A) Assays were performed using nuclei prepared from the hepatopancreas of normoxic and 48 h anoxic *L. littorea*. Transcripts labeled with [^{32}P]-UTP were hybridized to nylon membranes carrying immobilized inserts coding for the clones indicated. Ribosomal protein L26 was included as a positive control (Larade et al., 2001). (B) Histograms show the ratio of transcript levels in anoxic *versus* control samples, means ± S.E.M. for N=3 independent trials, each consisting of nuclei isolated from the hepatopancreas of five snails.

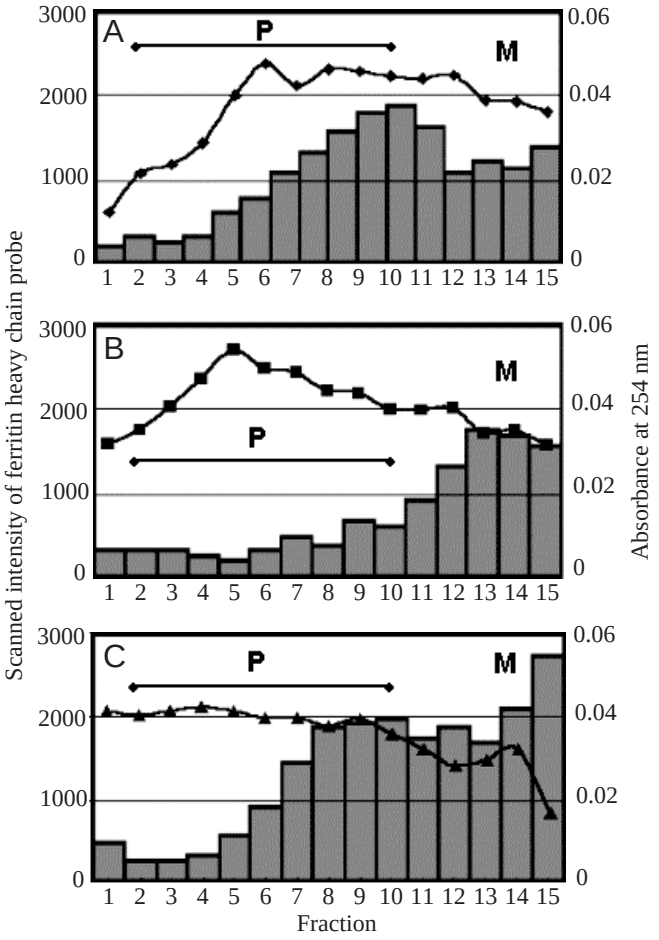

Fig. 4. Ferritin heavy chain translation status measured using polysome profiles of *L. littorea* hepatopancreas extracts prepared using post-mitochondrial supernatants centrifuged on 15%–30% continuous sucrose density gradients. Fractions were collected with high sucrose (30%) in fraction 1, decreasing to 15% in fraction 15. Bar graphs show absorbance at 254 nm, representing the relative amount of total RNA in each fraction. P, polysomes; M, monosomes. Line graphs show ferritin heavy chain transcript levels as determined from northern blots of total RNA isolated from each fraction. (A) normoxia; (B) 72 h anoxia; (C) 6 h aerobic recovery following 72 h anoxia.

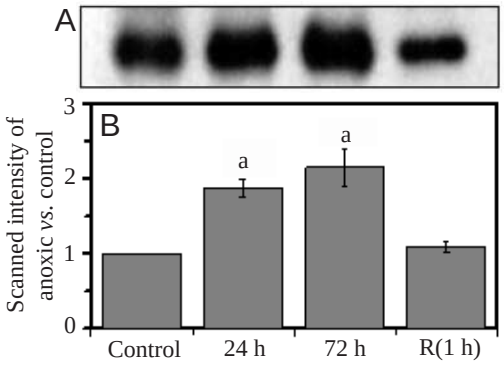

Fig. 5. Ferritin heavy chain protein levels during anoxia and aerobic recovery. (A) Western blots of hepatopancreas protein samples (20 μg) from normoxic, 24 h anoxic, 72 h anoxic and 1 h recovered (after 72 h anoxia) snails probed with an anti-ferritin antibody (Andersen et al., 1995). (B) Histograms show means ± S.E.M. for N=3 samples. [a]Significantly different from the control value as determined by the Dunnett's t-test, P<0.01.

Ratio of experimental *vs*. control ferritin transcript levels

Control	1.01 ± 0.052
Anoxia	1.54 ± 0.082[a]
Freezing	1.66 ± 0.23[a]
db-cGMP	1.58 ± 0.072[a]

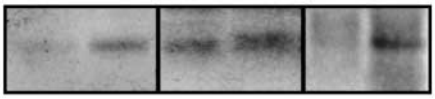

Fig. 6. Expression of ferritin heavy chain transcripts in hepatopancreas exposed to various conditions *in vitro*. Total RNA was isolated from the explanted hepatopancreas incubated under each condition and resolved on a 1.5% formaldehyde gel, blotted onto nitrocellulose and hybridized at 45°C to ^{32}P-labeled probes produced from the ferritin heavy chain cDNA clone. Levels of mRNA transcripts are illustrated during normoxia (Con) and stress or second messenger exposure for paired hepatopancreas samples. The scanned intensity of experimental samples listed in the table is relative to the corresponding normoxic control. Values are means ± S.E.M. for N=3 independent hepatopancreas samples. [a]Significantly different from control values as determined by the Student's t-test, P<0.01.

polysome northern blots were stripped and re-probed with alpha tubulin (not shown), resulting in a virtually identical profile to that of the ethidium bromide stained RNA, where the transcript being translated (in this case, alpha tubulin) shifts towards the monosome fraction. These results suggest that ferritin heavy chain is actively, and possibly preferentially, translated under anoxic conditions.

These results are supported by data obtained *via* western blotting. Relative levels of ferritin protein were measured during normoxia, after 24 h anoxia exposure, 72 h anoxia exposure and after 72 h anoxia exposure followed by 1 h oxygenated recovery (Fig. 5). Ferritin heavy chain protein increased over twofold (relative to aerobic controls) following 72 h anoxic exposure. Within 1 h of oxygenated recovery, however, ferritin protein levels were reduced and not significantly different from normoxic levels.

In vitro *induction of ferritin mRNA*

Tissue slices from *L. littorea* hepatopancreas were incubated under anoxic conditions, freezing conditions (frozen hemolymph causes ischemic conditions), or exposed to db-cGMP (which stimulates protein kinase G) to determine if any of these factors would affect the levels of ferritin heavy chain

mRNA transcripts *in vitro*. As assessed by northern blotting, transcript levels increased by >1.5-fold under anoxia (Fig. 6), which corresponded well with the response seen *in vivo* (Fig. 1). An increase of 1.7-fold and 1.6-fold was also observed in tissues frozen for 12 h and after 2 h exposure to cGMP, respectively (Fig. 6).

Discussion

Increased levels of ferritin heavy chain mRNA and production of the resulting protein during anoxia in *L. littorea* suggest that ferritin may have an important role to play in animal survival during the cyclic periods of hypoxia/anoxia that are a reality of life in the intertidal zone. Although ferritin sequences have been identified and characterized from a variety of molluscs, including snails, the expression patterns of ferritin heavy chain mRNA and protein during oxygen deprivation have never before been examined. The present data show an increase in ferritin heavy chain mRNA in the hepatopancreas of *L. littorea* during anoxia exposure. Ferritin transcript levels were in greater abundance after 24 h of anoxia exposure and remained at this level until oxygen was returned to the system. The observed increase in ferritin heavy chain transcripts may be partially due to increased transcription of the gene. However, transcriptional run-off assays using nuclei from *L. littorea* hepatopancreas demonstrated that ferritin heavy chain was not transcriptionally regulated during anoxia. Analysis of ferritin heavy chain mRNA distribution during anoxia using polysome profiles provided a qualitative measure of the translation status of the ferritin heavy chain protein. It has previously been demonstrated in snail hepatopancreas that polysomes are present during normoxia, diminish during anoxia exposure (shifting to the monosome fraction), and then re-aggregate to form active polysomes upon return to oxygenated conditions (Larade and Storey, 2002c). This behavior is also demonstrated in the present study by the shift in the ribosomal RNA absorbance profile to a peak in the low-density (monosome) fractions during anoxia (Fig. 4). Transcripts of most *L. littorea* genes that have been examined to date also shift their association from the polysome to the monosomes fractions under anoxic conditions (Larade and Storey, 2002a,b). In the case of ferritin heavy chain, however, transcripts encoding this subunit remained associated with the polysome fraction after 72 h of anoxia exposure (Fig. 4B) suggesting that the protein continues to be actively transcribed throughout anoxia, in contrast to most other proteins. To determine whether translation of ferritin heavy chain subunits increased in *L. littorea* during anoxia exposure, ferritin protein levels were assessed directly *via* western blotting, which demonstrated a gradual increase in ferritin protein content over the course of anoxia exposure, with a sharp decrease during normoxic recovery (Fig. 5). This response suggests that ferritin translation is positively regulated during anoxia exposure.

Organ culture experiments, previously optimized by the authors (Larade and Storey, 2002b; Larade et al., 2001), have produced results consistent with those observed in whole animal studies (reviewed in Larade and Storey, 2002a). These short-term tissue incubations were used to examine the effects of various conditions on ferritin expression in an attempt to identify factors involved in transcript accumulation during anoxia. Anoxia tolerance is a critical factor that complements the ability of *L. littorea* to survive freezing temperatures. Such tolerance allows the animal to endure the ischemic conditions associated with hemolymph freezing. It has been demonstrated previously that gene expression, metabolic and enzymatic responses to anoxia *versus* freezing in *L. littorea* share a number of similarities (Churchill and Storey, 1996; Russell and Storey, 1995; MacDonald and Storey, 1999; Larade and Storey, 2002a,c; English and Storey, 2003). Analysis of ferritin heavy chain transcript levels in hepatopancreas samples that were frozen *in vitro* showed a 66% increase as compared with controls (Fig. 6). Incubation with the cyclic nucleotide db-cGMP also resulted in increased levels of ferritin mRNA transcripts relative to control values. The effect of cGMP on ferritin transcription has received little attention, although Oberle et al. (1999) reported that both db-cGMP and 8-bromo cGMP had no effect on ferritin protein synthesis in porcine aortic endothelial cells. It is possible that cGMP induces or stabilizes ferritin transcripts through activation of protein kinase G (PKG). A number of anoxia-induced responses in marine molluscs are cGMP-mediated (Larade and Storey, 2002a); therefore, the observation that ferritin heavy chain is also cGMP-responsive suggests that elevated ferritin may have a significant role to play in anoxia survival.

Why are ferritin levels elevated during anoxia?

The data presented here, suggesting that ferritin heavy chain transcripts accumulate during anoxia, promoting translation of the protein, are supported by the results of previous studies, that report an increase in ferritin mRNA levels in other systems during hypoxia exposure (Kuriyama-Matsumura et al., 1998; Schneider and Leibold, 2003). Since increased transcription of the ferritin heavy chain cannot account for the observed increase in mRNA, it is probable that the transcript is stabilized in an as yet unknown manner. Ai and Chau (1999) have reported the ‘up-regulation’ of ferritin heavy chain in human THP-1 cells through stabilization of its message *via* a pyrimidine-rich sequence in the 3′-UTR. They state that the stability of ferritin transcripts is regulated by an unidentified protein factor that binds in this region. A similar pyrimidine-rich sequence is present in the 3′-UTR of the *L. littorea* sequence and could potentially be responsible for the stabilization and ‘up-regulation’ of this transcript in the anoxic period. Virtually all ferritin heavy chain mRNA transcripts, including snails (Von Darl et al., 1994; Xie et al., 2001) and other invertebrates (Dunkov et al., 1995; Charlesworth et al., 1997; Huang et al., 1999; Chen et al., 2003) contain *cis*-acting nucleotide sequences in the 5′-UTR known as iron regulatory elements (IREs), similar to that characterized in mammals (Addess et al., 1997). IREs form a stem loop structure that is recognized by *trans*-acting cytosolic RNA-binding proteins known as iron-regulatory proteins (IRPs). Specific IRPs bind

to the IRE of ferritin mRNA to prevent association of the transcript with translating ribosomes, effectively blocking translation of the ferritin protein. This elegant system allows for the regulation of cellular iron levels at the stage of uptake and storage (reviewed in Theil, 2000). Homologues of IRP1 have been identified in various invertebrates (Muckenthaler, 1998), which implies that a similar IRP should exist in *L. littorea*. The authors' attempts to isolate a snail IRP using a homologous sequence as a probe were unsuccessful, although this may simply be a result of low cross-species homology. Hanson and Leibold (1998) demonstrated that hypoxia promoted a decrease in the RNA binding activity of IRP1 in a rat hepatoma cell line, which was reversed by reoxygenation. A decrease in the binding capacity of IRP1 would reduce the number of 'blocked' ferritin transcripts and, in turn, promote translation of ferritin mRNA. This hypothesis has received support from a recent study by Schneider and Leibold (2003). Kuriyama-Matsumura et al. (2001) also demonstrated a hypoxic inactivation of IRP1 in mouse peritoneal macrophages, resulting in an increase in ferritin synthesis. This regulatory mechanism may also explain the observed increase in ferritin heavy chain protein in anoxic snails.

Since ferritin is generally thought of as a 'protective' protein that functions to protect cells from oxidative stress, it was unexpected to observe a decrease in the protein when pro-oxidants are likely to be at their peak early in the aerobic recovery after anoxia. This may be a testament to the adaptability of the *L. littorea* antioxidant defenses. It has previously been demonstrated that *L. littorea* has an excellent antioxidant defense system that protects them from oxidative stress during reoxygenation after anoxia (Pannunzio and Storey, 1998). In addition, *L. littorea* are capable of regulating their metabolic rate depending upon levels of available oxygen. Therefore, accumulation of the ferritin heavy chain transcript during the anoxic period is probably a method to ensure that ample template is available for subsequent protein production. Similar to existing mammalian models of hypoxia/anoxia stress (reviewed in Torti and Torti, 2002), marine snails show increases in ferritin (relative to normoxic levels) at both the mRNA transcript and protein levels in response to anoxia. A potential motive for ferritin production during anoxia is to control free iron levels in the cell, which may increase as a result of the anoxic stress (Khan and O'Brien, 1995). An increase in free iron can lead to production of the extremely toxic hydroxyl radical created from a series of reactions involving oxygen and intracellular iron, known as the Fenton reaction. These 'activated' oxygen molecules promote the production of additional free radicals, which often lead to irreversible cellular damage. Therefore, by sequestering intracellular iron during the anoxic period, the production of toxic free radicals may be diminished during anoxia, and probably also during the aerobic recovery period, since iron is stored in new and existing ferritin molecules where it is unable to interact with available oxygen. A recent publication by Berberat et al. (2003) documents a role for heavy chain ferritin in preventing ischemia-reperfusion injury in rat liver by limiting the role of iron in free radical production as described above.

In summary, the marine intertidal snail, *L. littorea*, has developed a number of remarkable mechanisms to survive anoxic exposure, including regulation of metabolic processes, transcription and translation (Larade and Storey, 2002a). This study reports accumulation of ferritin heavy chain transcripts during anoxic exposure, with a subsequent increase in ferritin heavy chain protein. Ferritin appears to play a significant role in the anoxia survival of *L. littorea*, presumably as a metal chelator, preventing the production of potentially damaging oxygen free radicals.

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