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Gene regulation in physiological stress

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Abstract. A range of new tools in molecular biology are now available to allow the comparative biochemist to explore animal responses to environmental stress at multiple levels. In particular, new techniques of gene discovery, such as cDNA array screening, allow broad assessment of the responses of thousands of genes to a stress. This approach frequently identifies genes (and their associated metabolic functions) that have never before been associated with the stress under study and allows coordinated patterns of gene responses (e.g., by families of genes or by genes encoding multiple proteins in a metabolic pathway or a signal transduction cascade, etc.) to be elucidated. New methods for mRNA (e.g., quantitative PCR) and protein (e.g., peptide antibodies, phospho-specific antibodies) analysis, coupled with major advances in bioinformatics, also simplify the exploration of gene/protein regulation. Techniques and approaches for gene/protein discovery and regulatory analysis are discussed and illustrated with examples drawn from new studies of the responses to anoxia by the marine gastropod, *Littorina littorea*.

Keywords: Gene expression; Anoxia tolerance; Marine mollusc; cDNA array, Polysome profiles

1. Introduction

For many years, studies of biochemical adaptation to environmental stress centered primarily at the level of protein/enzyme function [1]. However, new molecular tools are now providing a huge range of opportunities for the comparative biochemist to examine adaptive responses at most, if not all, levels of metabolic organization including signal transduction, transcription, translation, kinetic and allosteric controls, post-translational modification, subcellular localization, and protein degradation [2]. Within the last decade, major advances have been made in the technology for gene screening and in our understanding of the mechanisms of gene regulation. Furthermore, the methods for gene

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and protein expression studies are no longer cumbersome and difficult technologies but have become relatively simple tools that can be put to excellent use in comparative biochemistry. Gene discovery techniques such as cDNA array screening are providing amazing opportunities for identifying the genes that are turned on in animals under different environmental stresses, frequently highlighting previously unsuspected genes and proteins (and their cell functions) that participate in adaptive response. Easy entry into the study of virtually any gene/protein found in GenBank is available by using consensus sequences to design gene primers that are then synthesized commercially and used with automated polymerase chain reaction (PCR) technology to "pluck" the mRNA for almost any identified gene from an organism. This mRNA can then be used as the starting point for studies in several directions. For example, after sequencing, species-specific primers can be made and used to quantify changes in tissue transcript levels using quantitative PCR (Q-PCR) or Northern blotting and full-length sequences can be retrieved using 5′ or 3′ RACE (rapid amplification of cDNA ends) to assess species-specific changes in gene/protein sequence. The cDNA sequences can also be used to screen genomic libraries to find complete gene sequences (introns plus exons) as well as promotor (5′ untranslated) regions. Knowledge of promotor sequences—those sites where transcription factors bind—can then unlock regulatory aspects of gene function. Bioinformatics programs can be applied to either cDNA or genomic sequences to detect regulatory motifs in both gene and protein to give hints about the signal transduction pathways that are involved in their regulation or to analyze species-specific differences in amino acid sequence that may be adaptive (e.g., amino acid substitutions that could aid stress resistance such as optimizing proteins for low-temperature function). Species-specific cDNA sequences can also be used to design peptides for antibody production to follow stress-induced changes in protein levels and phospho-specific antibodies can be used to assess the changes in relative activity of the many proteins that are modified by reversible phosphorylation. Use of phospho-specific antibodies is especially key for tracing multicomponent signal transduction cascades leading from cell surface to nuclear gene activation. Hence, we now have the molecular tools to evaluate almost any metabolic system and to search for the breadth and depth of biochemical adaptations that define the differences between stress-tolerant and stress-intolerant organisms.

Recent studies in my lab have used the technologies described above to analyze stress-responsive gene and protein expression underlying animal adaptive strategies such as anoxia tolerance, freeze tolerance, hibernation and estivation as well as to identify the universal mechanisms of metabolic rate depression (MRD) that are a part of each of these strategies [2–5]. In the remainder of this article, I will review the main mechanisms of gene/protein regulation, highlight some advances in our understanding of the role of these mechanisms in MRD, and illustrate some of these mechanisms from recent studies by my lab of anoxia tolerance in the marine gastropod, *Littorina littorea.*

2. Review of transcriptional and translational control

For most cellular proteins, the initiation of gene transcription is the principle point at which their expression is regulated [6]. Hence, many regulatory controls, both positive and negative, global and specific, are applied to transcription. Global controls include

regulation of the assembly and binding of RNA polymerase II and a group of general transcription factors at the gene promoter as well as mechanisms of chromatin remodelling and histone modification to allow polymerase to gain access to the DNA template. Such controls often alter overall transcriptional activity in cells in response to factors such as environmental stress, growth and developmental timetables, and nutrient availability. Global activation of transcription has been extensively studied whereas global repression is less well understood [6] but is an area of active interest in my lab due to the obvious need to suppress this energy-expensive activity in animals that exhibit stress-induced MRD [2]. More specific controls on individual genes or groups of genes are applied via the actions of gene-specific transcription factors and their cofactors that bind at more distant sites (generally in the 5′ untranslated region) to stimulate or repress transcription in response to specific signals [7]. Activation of transcription factors is often a result of protein phosphorylation mediated by protein kinases that are often at the terminus of long signal transduction cascades [8]. Identification of the transcription factor response elements present in different genes as well as of the protein kinases that phosphorylate each transcription factor provide the clues to the signal transduction cascades that regulate different genes. In addition, by using phospho-specific antibodies, the relative levels of active kinases and transcription factors can be quantified to help confirm the signal transduction cascade operating in each situation of stress-responsive gene expression. Other controls also regulate transcript elongation, capping and splicing, polyadenylation and export of the completed mRNA to the ribosomes [6,7].

The stability of mRNA transcripts, both pre-translation and during translation, is another level at which control can be exerted. Most mRNA transcripts proceed directly to the ribosomes where they are translated a variable number of times with half-lives for transcript stability ranging from seconds to hours. However, transcripts of some genes are not immediately translated but are maintained in an untranslatable state by bound inhibitor proteins that are only released by the binding of specific ligands. Hence, primary control over the expression of these genes is actually at the translational level. Other layers of regulatory controls are applied to translation. Because protein synthesis is energy-expensive (needing ~5 ATP equivalents per peptide bond formed), the rate of protein synthesis must be closely matched with the cell's ability to generate ATP. Not surprisingly, then, protein synthesis is a main target of MRD; strong global suppression of translation occurs with only a few stress responsive genes that are up-regulated and translated [2]. Two main mechanisms of global protein synthesis control are (a) the state of ribosome assembly, and (b) reversible phosphorylation that modifies the activities of multiple ribosomal initiation and elongation factors. Active translation takes place on polysomes whereas monosomes are translationally silent. The proportion of ribosomes in polysomes can vary widely depending on the cellular demand for protein biosynthesis and/or the availability of ATP and amino acids. Tight regulation is required and the dissociation of polysomes and sequestering of mRNA into monosome and ribonuclear protein fractions is one of the major events in cells entering hypometabolic states [2]. Phosphorylation of key ribosomal proteins also modifies global translational activity. Inhibitory controls on translation include phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which halts delivery of the initiating methionine residue to the ribosome and several types of inhibitory and fragmentation events affect subunits of eIF4 to block the

entry of m^7G-capped mRNAs (the majority of transcripts) onto the 40S ribosomal subunit [9]. However, some transcripts can circumvent this mode of transcription initiation due to the presence in their mRNA sequence of an internal ribosome entry signal (IRES). This provides the means for selected translation of stress-responsive proteins under conditions where global translation is strongly suppressed. For example, this is how the alpha subunit of the hypoxia-inducible factor 1 (HIF-1α) can be translated under low oxygen conditions [10]. Similar methods using an IRES or other novel method of translation initiation may be involved in the selective up-regulation of the stress-responsive genes that support animal adaptation to other challenges (e.g., high or low temperature, freezing, dehydration, heavy metals, etc.). Other controls target the elongation stage of peptide synthesis. For example, signals acting through a variety of protein kinases regulate the eukaryotic elongation factor 2 (eEF2), another key site for global inhibition of translation during hypometabolism [2].

Once synthesized, proteins are then subject to multiple controls on their actions and activities within cells. This is a massive subject of its own and such controls include substrate/ligand availability, allosteric regulation, binding with regulatory proteins, subcellular compartmentation, and many kinds of covalent modification [1,11]. A final set of controls on the overall expression level of proteins is at the level of proteolysis. Global controls can be exerted by inhibition of the proteasome or by inhibition of the ubiquitination reactions that target proteins for recognition by the proteasome. Both can participate in MRD [2]. Selected proteins are also regulated at the level of degradation via specifically tailored mechanisms. For example, the oxygen-dependent hydroxylation of two proline residues on HIF-1α targets the protein for attack by an E3 ubiquitin-protein ligase and subsequent rapid degradation by the proteasome under aerobic conditions. However, under hypoxia, hydroxylation is inhibited and HIF-1α is stabilized.

3. Transcriptional and translational control in a model of anoxia tolerance

A primary interest of my laboratory is anoxia tolerance—mechanisms and adaptations used by animals to survive long-term oxygen deprivation. We study both vertebrate [5] and invertebrate models, the latter focused mainly on marine molluscs [12,13]. Studies have examined different aspects of biochemical control including alternative routes of anaerobic energy production, regulation of glycolytic enzymes by multiple mechanisms (e.g., allosteric regulation, reversible phosphorylation, subcellular enzyme binding), and antioxidant defenses that aid recovery when oxygen is reintroduced [12–15]. Our recent work [16–26] has focused on the periwinkle, *L. littorea*, an inhabitant of the intertidal zone around the north Atlantic, examining both anoxia tolerance and freezing survival.

A key component of anoxia tolerance in all systems where it has been studied is MRD; for marine molluscs, metabolic rate under anoxia is typically <10% of the aerobic rate [2]. The strong reduction in ATP turnover results from coordinated repression of ATP-utilizing and ATP-producing reactions so that vital processes can be supported over the long-term by the ATP output of fermentative metabolism. A critical mechanism of MRD is reversible protein phosphorylation that produces major changes in the activity states of many enzymes and functional proteins with consequences for all areas of metabolism. As in other anoxia-tolerant molluscs, reversible phosphorylation of

selected enzymes of glycolysis in *L. littorea* is critical for redirecting carbon flow into anaerobic routes of fermentative metabolism as well as suppression of glycolytic rate [2,14]. For example, changes in the properties of phosphofructokinase and pyruvate kinase were consistent with a conversion of the enzymes to less active forms under anoxia [16,20]. Anoxia also reduced the percentage of cAMP-dependent protein kinase (PKA) present as the active catalytic subunit from ~30% in normoxia to just 1–3% in anoxia [19]. Hence, metabolic functions that are PKA-stimulated would be suppressed during anaerobiosis; indeed, general suppression of signal transduction cascades seems to be a part of MRD [2].

It is estimated that gene transcription consumes 1–10% of a cell's ATP budget whereas protein synthesis can utilize as much as 40% [2]. Not surprisingly, studies have shown that both are crucial targets of MRD during anaerobiosis. We used the nuclear run-on technique to evaluate transcription in *L. littorea*; this method measures the rate of mRNA elongation in isolated nuclei and we found that the overall rate of ^{32}P-UTP incorporation into nascent mRNAs in hepatopancreas nuclei fell to less than one-third of the normoxic rate [13]. Similarly, the rate of ^{3}H-leucine incorporation into protein in hepatopancreas extracts was reduced by 50% within 30 min of anoxic exposure and remained low over a 48 h anoxia exposure [23]. Interestingly, these events occurred without signs of energy stress in the snails (ATP levels remain high for several days

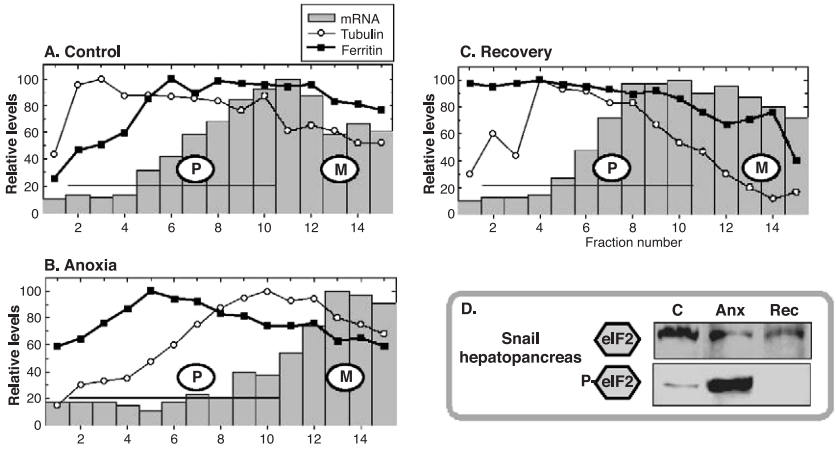

Fig. 1. Controls on protein synthesis in hepatopancreas of marine snails *L. littorea.* Panels A through C show the distribution of rRNA and of mRNA transcripts of alpha-tubulin (a constitutive gene) and ferritin (an anoxia up-regulated gene) in hepatopancreas of (A) control, (B) 72 h anoxic, and (C) 6 h aerobic recovered snails. Ribosomes were separated on a 15–30% continuous sucrose gradient and fractions were collected with highest sucrose (30%) in fraction 1 and lowest sucrose (15%) in fraction 15. Each fraction was assessed for rRNA content by absorbance at 254 nm and mRNA levels via Northern blotting. Data are expressed relative to the fraction containing the highest rRNA or mRNA content. The horizontal line running between fractions 2 and 10 shows the general position of polysome fractions (labeled with a P), whereas the higher fractions contained monosomes (labeled with an M). (D) Western blots show the relative levels of eukaryotic initiation factor 2 alpha (eIF2α) in hepatopancreas from control, anoxic and recovered snails. The upper panel shows total eIF2α protein whereas the lower panel shows the amount of phosphorylated eIF2α that is the inactive form. Data are reworked from Larade and Storey [23,25].

under anoxia) [17] showing that suppression of transcription and translation are actively regulated components of MRD, not reactions to energy limitation. The mechanisms of translational regulation were those mentioned earlier: polysome dissociation and phosphorylation of ribosomal proteins (Fig. 1). The latter was documented by Western blotting, which found no change in total eIF2α protein during anoxia/recovery whereas the use of phospho-specific antibodies revealed that the amount of phosphorylated eIF2α rose by ~15-fold in hepatopancreas of anoxic *L. littorea*, compared with aerobic controls (Fig. 1D) [23].

Changes in polysome content were revealed by separating polysomes and monosomes on a sucrose gradient and tracking the distribution of rRNA, the mRNA for α-tubulin (a constitutively active gene) and the mRNA for ferritin heavy chain (an anoxia-responsive gene) [23–25]. Under aerobic conditions, rRNA distribution showed

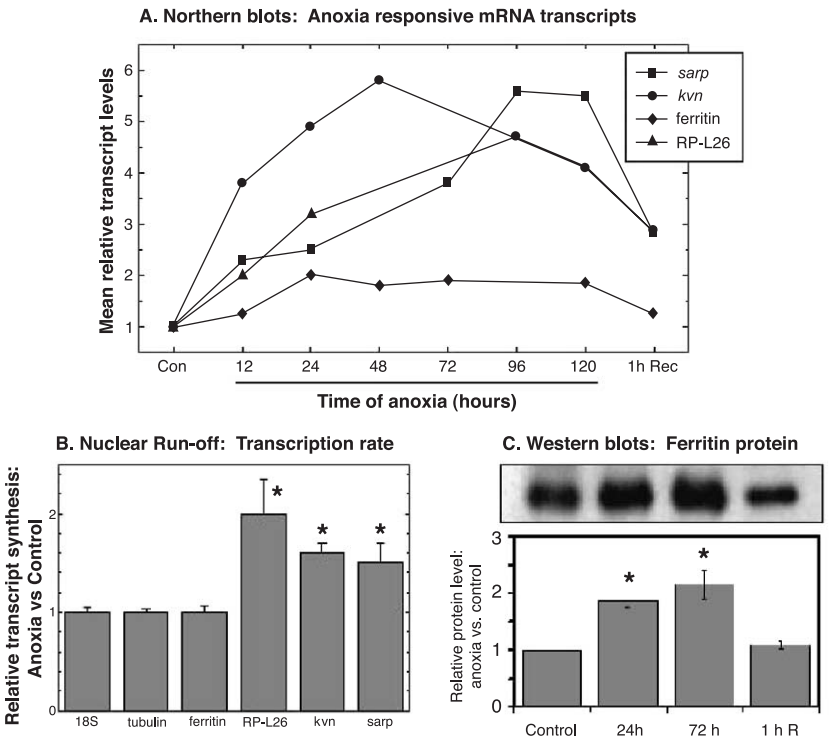

Fig. 2. Anoxia responsive gene expression in hepatopancreas of marine snails *L. littorea.* (A) Northern blots show changes in mRNA transcript levels over a time course of anoxia exposure followed by 1 h of aerobic recovery for four genes identified as anoxia responsive from cDNA library screening. Con-aerobic control; RP-L26-ribosomal protein L26. (B) The nuclear run-off technique monitored ^{32}P-UTP incorporation into mRNA in vitro to measure the rate of transcription in isolated nuclei from aerobic control versus 48 h anoxic snails. Transcription rates of two housekeeping genes, 18S ribosomal RNA and alpha-tubulin, were also measured. (C) Western blotting shows ferritin heavy chain protein levels in hepatopancreas during anoxia and after 1 h of aerobic recovery. Upper panel shows a representative blot. Histograms in both B and C show means±S.E.M. for n=3 independent trials. *Significantly different from the control value, P<0.05. Data are reworked from Refs. [22,24–26].

that most ribosomes were in the high-density polysome fractions (Fig. 1A). The mRNA for α-tubulin was also found predominantly with the polysomes; both results are consistent with a state of active translation in the control state. However, after 24 h of anoxia, rRNA began to shift towards lower densities indicating polysome disaggregation [23] and by 72 h there was little evidence of polysomes remaining in hepatopancreas (Fig. 1B); α-tubulin transcripts also moved into monosome fractions. These data are consistent with the idea that most mRNA species are maintained and but not translated in the hypometabolism state. However, within 6 h of the return to oxygenated conditions, both parameters indicated reassembly of polysomes and the return of mRNA for the constitutively active gene to the polysome fractions (Fig. 1C) [23]. The mRNA transcripts of anoxia up-regulated genes, *kvn* and ferritin, behaved very differently [24,25]. Fig. 2C illustrates this for ferritin showing that a higher proportion of ferritin transcripts were found in the polysome fractions under anoxia than in the aerobic control situation. This suggests preferred translation of ferritin by the remaining polysomes during anoxia. This result was supported by a measured ~2-fold increase in ferritin protein in hepatopancreas of anoxic snails (Fig. 2C) [25].

4. Anoxia-induced gene expression in the marine gastropod *L. littorea*

A variety of modern techniques can be used for gene discovery and recent studies in our lab have used two of these (cDNA library screening, cDNA microarrays) to reveal anoxia responsive genes in *L. littorea* [13,21–26]. We constructed and screened cDNA libraries from both foot [21] and hepatopancreas [22]. This method is time-consuming and expensive and favors the discovery of mRNA species that are in abundant supply but its key advantage is the ability to find novel species-specific transcripts that have no homologues in gene banks. We found two such genes in periwinkle hepatopancreas [24,26] that may have novel functions in anaerobiosis (see below). For the comparative biochemist, this ability to seek out novel genes/proteins that may be species- or stress-specific is of great importance for understanding biochemical adaptation. In the past, stress-specific genes and proteins were generally detected by an obvious functional footprint, such as the action of the antifreeze proteins of cold-water fishes in creating a thermal hysteresis between freezing and melting points of blood plasma. However, many important stress-specific proteins may, in fact, have no easily detectable physiological action. Hence, species-specific gene screening is invaluable.

Giant advances in gene discovery are also coming from the use of cDNA array screening, which allows simultaneous screening of the expression of thousands of named genes for their responses to an imposed stress or treatment. To date, arrays are available for only a few species but we have had excellent results using human 19,000 cDNA arrays (from the Ontario Cancer Institute, Toronto) for heterologous probing for stress-responsive genes in frozen frogs [3], hibernating ground squirrels and bats [4], and anoxic turtles [5]. The percentage of genes that cross-react falls off with phylogenetic distance but, nonetheless, can still provide dozens or even hundreds of "hits" for follow-up studies. We used the human arrays to search for anoxia-responsive gene expression in *L. littorea* hepatopancreas. Not unexpectedly, cross-reactivity was low, only 18.35%, and of the genes that did cross-react, most showed no change in transcript levels in anoxia (88.8%)

whereas 0.6% showed reduced transcripts in anoxia. This finding is consistent with the idea that most transcripts are simply maintained/sequestered during anoxia so that they are available when the reintroduction of oxygen allows MRD to be lifted and normal translational activity to be resumed. However, 10.6% of the cross-reacting genes were putatively up-regulated by twofold or more in anoxia and this represented over 300 genes. These included protein kinases and phosphatases, mitogen-activated protein kinase interacting factors, translation factors, antioxidant enzymes, and nuclear receptors [13]. Virtually all of these are proteins that have never before been implicated in anoxia tolerance and, hence, we now have a much broader view of the potential gene expression responses that may play significant roles in anaerobiosis. Results from heterologous array screening must be confirmed by other methods and we are analyzing several of these candidate genes. The current methods for follow-up with any given gene are: (1) design of DNA primers based on a consensus sequence put together from available sequences for the gene in GenBank; (2) use of the primers to retrieve the species-specific cDNA via PCR; (3) nucleotide sequencing of the retrieved cDNA for confirmation of identity and then synthesis of species-specific probe to evaluate organ-, time-, and stress-specific gene expression via Q-PCR; (4) as needed, use of 3′ or 5′ RACE to retrieve the full sequence for analysis of possible adaptive changes in amino acid sequence compared with other species; and (5) use of the putative amino acid sequence to design and synthesize peptide antibodies for quantifying patterns of protein expression in anoxia.

Our first studies using cDNA library screening revealed several anoxia-responsive genes. Libraries were constructed from *L. littorea* foot and hepatopancreas using equal amounts of mRNA from snails given 1, 12, or 24 h N_2 gas exposure. By combining mRNA from multiple stress points, the screening (against normoxic controls) can detect genes that are up-regulated at any point in the anoxic time course. Later, the time-dependent expression of individual genes can be characterized using methods such as Northern blotting or Q-PCR to quantify mRNA levels at each sampling time. Screening revealed several genes that were putatively up-regulated in anoxia; these included metallothionein (MT) from the foot library [21] and ferritin, the L26 ribosomal protein, and two novel genes from the hepatopancreas library [22,24–26]. Each showed independent patterns of transcript elevation over a time course of anoxia exposure and transcripts of all four genes were significantly reduced again within 1 h of the return to aerobic conditions (Fig. 2A).

The novel genes are particularly intriguing and were named *kvn* and *sarp-19* [24,26]. The clone *kvn* contained 525 bp with a full open reading frame that encoded 99 residues of the KVN protein. The predicted molecular weight of KVN was 12 kDa and it showed an N-terminal hydrophobic signal sequence. Such sequences typically direct proteins to the endoplasmic reticulum where they are processed and secreted to a final destination; this suggests that KVN has an extracellular function. Features of its sequence such as the spacing of cysteine clusters suggest that KVN may be an iron–sulfur protein that binds iron and is related to the ferredoxin family [24]. It may function similar to other ferredoxin-like proteins, possibly mediating electron transfer reactions during anoxia or recovery. *Sarp-19* (snail anoxia-responsive protein 19 kDa) codes for a different protein type [26]. The open reading frame encoded 168 amino acids with an N-terminal signal sequence and two putative EF-hand domains. The common function of EF-hand domains

is calcium binding, typically inducing a conformational change causing activation (or inactivation) of target proteins [27]. The function of SARP-19 in anaerobiosis may include calcium-activated signaling or calcium sequestering. The latter might be a key physiological function for SARP-19 in extracellular spaces for the following reason. The shift from aerobic to anaerobic life in shelled molluscs is accompanied by a significant dissolution of the calcium carbonate shell with the bicarbonate released buffering acidic products of fermentative metabolism [28]. Aerial exposure stimulates bivalve molluscs to close their valves and gastropods to "seal themselves in" by covering the shell opening with the operculum. Hence, they become closed systems, a feature that aids water and osmotic balance but means that hemolymph calcium levels rise substantially during anaerobiosis [29]. Calcium is a key signaling molecule and intracellular levels are strictly regulated at very low values in all cells by sarcoplasmic reticulum and plasma membrane Ca^{2+} pumps. Hence, elevated amounts of a Ca^{2+}-binding protein under anoxia could help to minimize free Ca^{2+} levels, particularly under hypometabolic conditions when ATP expenditure on Ca^{2+} pumping should be minimized.

The identifiable genes revealed from screening of *L. littorea* libraries also revealed prominent up-regulation of ion-binding proteins under anoxia, suggesting that this may be a principle of marine invertebrate anoxia tolerance. Screening of the foot library showed anoxia-responsive up-regulation of MT [21]. Periwinkle MT shared ~50% identity with the copper- and cadmium-binding MT isoforms from the land snail, *Helix pomatia* and ~45% identity with marine bivalve MTs and contained the mollusc-specific C-terminal motif: Cys-X-Cys-X(3)-Cys-Thr-Gly-X(3)-Cys-X-Cys-X(3)-Cys-X-Cys-Lys [30]. Northern blots showed up-regulation of MT within 1 h and transcripts rose by 3–3.5-fold in foot and 5–6-fold in hepatopancreas within 12–24 h of either anoxia or freezing exposure [21]. Hence, MT responds to changing oxygen levels within the time frame of normal tidal cycles and that adds support to the idea that variation in MT protein aids anoxia tolerance in the natural environment. Although MTs are typically thought of as metal-binding proteins and are widely used as bioindicators of heavy metal pollution in the marine environment [31], recent evidence from both mammalian and marine invertebrate systems suggests that MTs also function in antioxidant defense. In mammals, ischemia–reperfusion injury was lessened by MT overexpression but enhanced by MT knock-out [32,33]. In molluscs, MT induction via pre-exposure to cadmium greatly increased the survival of mussels that were then exposed to iron in an anoxic environment [34]. Although MT could contribute to antioxidant defense by binding copper and thereby limit copper-induced reactive oxygen species (ROS) generation via the Fenton reaction, MT is poor at binding iron, the main metal involved in Fenton chemistry. Hence, the antioxidant effect of MT does not appear to derive from iron binding but, instead, there is evidence that MT can scavenge ROS (including hydroxyl radicals and superoxide) via thiolate oxidation of its cysteine residues [35,36].

Another metal-binding protein, ferritin heavy chain, was identified as up-regulated from screening the *L. littorea* hepatopancreas library [25]. Northern blots showed that transcript levels rose twofold during anoxia exposure and Western blots showed a comparable rise in protein content followed by a return to control levels within 1 hour of normoxic recovery (Fig. 2A,C). However, when nuclear run-off assays were used to evaluate the rate of transcription of anoxia-responsive genes, the results showed that the

ferritin gene was not actually transcriptionally activated under anoxia, unlike the L26, *kvn* and *sarp* genes that showed enhanced transcript synthesis under anoxia (Fig. 2B). This result for ferritin concurs with data on the regulation of the protein in other systems. Ferritin transcripts contain *cis*-acting nucleotide sequences in the 5′ -UTR called iron regulatory elements (IREs) that are recognized by cytosolic RNA-binding iron-regulatory proteins (IRPs). When bound, IRPs prevent the transcript from associating with ribosomes but, when iron levels are high, iron binds to the IRPs and triggers their dissociation from ferritin transcripts [37]. Hence, ferritin protein levels are controlled at the transcript level. Oxygen is another of several signals that regulates the system; in mammals, hypoxia reduces the RNA binding activity of IRPs and this is reversed by reoxygenation [38]. Anoxia exposure in littorines may similarly reduce the number of IRP-blocked ferritin transcripts and, thereby, promote translation of ferritin mRNA. Furthermore, as noted earlier, ferritin mRNA transcripts remain associated with polysomes during anoxia, another factor that would promote their translation [25].

5. Regulation of anoxia-induced gene expression

The signal transduction pathways that regulate anoxia-induced gene expression in *L. littorea* are another area of great interest. We have begun to explore this by incubating hepatopancreas explants in vitro with various second messengers and stimulators including dibutyryl cAMP, calcium ionophore A23187, phorbol 12 myristate 13 acetate (PMA), and dibutyryl cGMP to stimulate protein kinases A, B, C and G, respectively [22,24–26]. Transcript levels of ferritin, L26 ribosomal protein, *kvn* and *sarp 19* all increased when tissues were incubated in a medium bubbled with nitrogen gas as compared with aerobic control samples; this confirmed that anoxia exposure stimulated gene up-regulation both in vivo and in vitro. Tissue samples were then incubated under aerobic conditions with each of the protein kinase stimulators. Analysis via Northern blotting showed that transcript levels of the four anoxia responsive genes all increased by 1.5–2.5-fold when aerobic tissues were incubated with dibutyryl cGMP. However, the genes did not respond to dibutyryl cAMP. Transcripts of *sarp-19* also rose in response to calcium ionophore and PMA which suggests that the gene responds to Ca^{2+} levels, in line with the structural information that indicates that SARP-19 is a Ca^{2+} binding protein, as discussed earlier [26]. The common response by all four genes to cGMP suggests a central role for cGMP and protein kinase G (PKG) in the regulation of gene expression responses to anoxia in *L. littorea*. Indeed, previous studies have implicated PKG in the control of intermediary metabolism during anaerobiosis in other marine molluscs. For example, PKG mediates the anoxia-induced phosphorylation of enzymes (in particular, pyruvate kinase) as part of glycolytic rate depression [39]. Incubation with cGMP also mimicked the effect of anoxia on the kinetic properties of phosphofructokinase from the anterior byssus retractor muscle of *Mytilus edulis* whereas cAMP incubation had the opposite effect [40]. A well-known activator of guanylyl cyclases is the diffusible signal molecule, nitric oxide (NO) [41]. Recent studies have shown that NO is involved in low oxygen signaling in *Drosophila melanogaster* [42] and this, coupled with the evidence cited above of cGMP mediation of anoxia-induced events in molluscs, suggests that the NO/cGMP signaling pathway may be central in the response

to oxygen deprivation in anoxia-tolerant invertebrates, coordinating both metabolic and gene expression responses to anoxia. In line with this, NO up-regulates herritin transcripts in land snail neurons [43].

Much remains to be determined about the mechanisms of oxygen sensing, the signal transduction cascade(s) involved in transmitting low oxygen signals, and the metabolic and gene expression responses to low oxygen by anoxia tolerant species. Continuing explorations will not only solve the mysteries of life without oxygen but will help to highlight the key differences in sensing, signaling and responding to low oxygen that differentiate anoxia tolerant and intolerant species, and aid in the search for applied treatments in medicine that can prevent or correct hypoxic/ischemic damage. New technologies for screening and analyzing gene expression will play a primary role in this search, allowing researchers to view broad patterns of response by hundreds of genes across a broad spectrum of species, using the power of comparative biochemistry to identify the critical gene/protein responses that impart stress tolerance on organisms.

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