

A Profile of the Metabolic Responses to Anoxia in Marine Invertebrates

Kevin Larade and Kenneth B. Storey¹

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

¹ Corresponding author

Tel: (613) 520-3678

Fax: (613) 520-2569

Email: kenneth_storey@carleton.ca or klarade@ccs.carleton.ca

1. Introduction

2. Metabolic response to anoxia

- 2.1. *Regulation of carbohydrate metabolism*
- 2.2. *Effects of pH on cellular metabolism*
- 2.3. *Controlling glycolytic flux*

3. Macromolecular synthesis

- 3.1. *Economics of energy conservation*
- 3.2. *mRNA and protein synthesis*
- 3.3. *Mechanisms of translational control*
- 3.4. *Polysome analysis*
- 3.5. *Ribosomal proteins*
- 3.6. *Maintaining translatable mRNA pools*

4. Gene Expression

- 4.1. *Anoxia-induced gene expression*
- 4.2. *Identification of differentially expressed genes*
- 4.3. *Pharmacology*

5. Triggering the anoxic response

- 5.1. *Oxygen sensing*
- 5.2. *Second messengers*
- 5.3. *The role of cGMP*

6. Transcription factors

7. Perspectives

8. References

1. Introduction

A capacity for long term survival without oxygen is well-developed among many invertebrate species as well as in selected ectothermic vertebrates. Anoxia tolerance has been particularly well-studied in various species of marine molluscs, including both bivalves (e.g. mussels, clams, oysters) and gastropods (e.g. littorine snails, whelks). These can encounter low environmental oxygen as a result of multiple factors: (1) gill-breathing intertidal species are deprived of oxygen when the waters retreat with every low tide, (2) burrowing and benthic species can encounter anoxic bottom sediments, (3) high silt or toxin levels in the water as well as predator harassment can force shell valve closure, leading to substantial periods of "self-imposed" anoxia, (4) animals in small tidepools can be oxygen-limited when animal and plant respiration depletes oxygen supplies in the water, and (5) freeze-tolerant intertidal species face oxygen deprivation whenever their body fluids freeze (Truchot and Duhamel-Jouve, 1980; de Zwaan and Putzer, 1985; Grieshaber et al., 1994; Loomis, 1995). Life in the intertidal zone is particularly challenging since, in addition to the cyclic availability of oxygenated water (each tide cycle lasts 12.4 h), organisms can also be challenged with multiple other stresses including desiccation, changes in salinity, and changes in temperature, sometimes including freezing; all can potentially change rapidly over the course of a single tidal cycle of immersion and emersion (Bridges, 1994; Loomis, 1995). For this reason, various residents of the intertidal zone have been used extensively as model systems of stress tolerance, the most widely studied species being the sessile bivalve, the blue mussel *Mytilus edulis*. Littoral snails that graze on rocks in the high intertidal zone are also an excellent model system for studies of both anoxia tolerance and freeze tolerance. The present chapter reviews recent advances in our understanding of the biochemistry and molecular biology of anoxia tolerance in marine molluscs. Our emphasis is on the molecular biology of the phenomenon, particularly our extensive recent work with the periwinkle snail, *Littorina littorea*, to analyze the role of gene expression in anoxia tolerance and the mechanisms that regulate anoxia-induced changes in transcription and translation.

2. Metabolic response to anoxia

2.1. Regulation of carbohydrate metabolism

Under aerobic conditions, organisms can make use of lipid, carbohydrate or amino acid fuels for respiration with considerable variation between species and between organs in the relative importance of different fuel types. Under anoxic conditions, however, carbohydrates become the primary substrate because the oxidation of hexose phosphates (derived from glucose or glycogen) to triose phosphates via glycolysis produces ATP in substrate-level phosphorylation events. Although the yield of ATP is low (2 or 3 moles ATP/mole glucose or glucosyl unit from glycogen, respectively) compared with that available from the complete oxidation to CO₂ and H₂O by the tricarboxylic acid cycle (36 or 38 moles ATP/mole, respectively), anoxia tolerant species have capitalized on this pathway with adaptations that maximize the length of time that fermentative metabolism can sustain survival. Among anoxia-tolerant molluscs, these adaptive strategies include: (1) large tissue stores of fermentable fuels (mainly glycogen but also selected amino acids), (2) coupling of glycolysis to additional substrate-level phosphorylation reactions to increase the ATP output per hexose phosphate, (3) production of alternative end products to lactic acid that are either volatile or less acidic so that cellular homeostasis is minimally perturbed by acid build-up during long term anoxia, and (4) strong metabolic rate depression that greatly lowers the rate of ATP utilization by tissues to a rate that can be sustained over the long

term by the ATP output of fermentation reactions alone. Thus, the Pasteur effect - a large increase in glycolytic rate when oxygen is limiting - is not seen in anoxia tolerant species.

Anoxia tolerant molluscs show a two-phase response to declining oxygen tension. As tissue oxygen is depleted (such as during shell valve closure or during emersion at low tide), organisms first enter a period of hypoxia. During this period, a graded increase in carbohydrate catabolism can occur that allows a compensatory increase in fermentative ATP output in order to maintain normal rates of ATP turnover. However, as hypoxia deepens, a critical low oxygen tension is exceeded and further attempts at compensation are abandoned in favour of the initiation of conservation strategies. In this phase of severe hypoxia or anoxia, the rates of ATP production and ATP utilization are strongly suppressed and net metabolic rate drops to below 10% of the corresponding aerobic metabolic rate at the same temperature (Storey and Storey, 1990). The critical pO_2 values that stimulate these transitions differ between species and contribute to the differential success of various species in hypoxic or polluted environments (de Zwaan et al., 1992). Metabolic rate depression greatly extends the time that a fixed reserve of internal fuels can support survival and many marine molluscs can survive days or weeks of anoxia exposure.

Metabolic rate depression is quantitatively the most important factor in anoxia survival. However, the use of modified pathways of fermentative metabolism substantially enhances the ATP output in anoxia and leads to the formation of non-acidic and/or volatile end products that are compatible with the maintenance of long term homeostasis in the anoxic state. The initial response to anoxia is typically the coupled fermentation of glycogen and aspartate substrates to produce the end products alanine and succinate, respectively. Glycogen is catabolized to pyruvate via glycolysis and then, instead of reduction to lactate, the pyruvate undergoes a transamination reaction to form alanine using an amino group transferred from aspartate. The product of aspartate deamination, oxaloacetate, is reduced to malate (using the NADH that would otherwise have been used by the lactate dehydrogenase reaction). This malate is converted to fumarate and then succinate in mitochondrial reactions that constitute a partial reversal of the tricarboxylic acid cycle. Fumarate conversion to succinate generates ATP and the further conversion of succinate to propionate in some species is linked with additional ATP synthesis. As aspartate pools become depleted, a metabolic shift takes place that directs the output of glycolysis, phosphoenolpyruvate (PEP), into the synthesis of oxaloacetate. This is accomplished via inhibition of the enzyme pyruvate kinase (PK) so that PEP is carboxylated instead via the PEP carboxykinase (PEPCK) reaction to produce the 4-carbon intermediate, oxaloacetate, that then feeds into the reactions of succinate synthesis. Fermentation of glucose to succinate produces 4 ATP/mol of glucose whereas the glucose to propionate conversion produces 6 ATP/mol glucose, compared with only 2 ATP/mol when lactate is the product.

The tricarboxylic acid (TCA) cycle is coupled to electron transport via the electron-transferring enzyme complex succinate dehydrogenase (complex II of the electron transport chain; ETC), which reduces malate to succinate. An extensive review of the ETC of anaerobically functioning eukaryotes has been compiled by Tielens and Van Hellemond (1998). They state that, depending on whether the system is aerobic or anaerobic, the reducing equivalents of complex II are transferred to ubiquinone or rhodoquinone, respectively. Both compounds have been detected in marine intertidal molluscs, as would be expected since molluscs are subject to aerial exposure at regular intervals (Van Hellemond et al., 1995). During immersion, molluscs rely on aerobic energy metabolism via the Krebs cycle, with electrons being transferred from succinate to ubiquinone through complex II in the ETC. During

immersion, molluscs function anaerobically, with electron transfer likely occurring through rholoquinone to fumarate producing succinate (Tielens and Van Hellemond, 1998). Fumarate acts as the electron acceptor when oxygen is not available, producing succinate. Increased succinate levels during anaerobiosis allow mitochondria to remain metabolically active and intact until oxygen again becomes available, initiating respiration (Bacchiocchi and Principato, 2000).

2.2. *Effects of pH on cellular metabolism*

The precise relationship between pH and metabolic rate depression in molluscs has yet to be established and has been reviewed by a number of researchers (Storey, 1993; Guppy et al., 1994; Hand and Hardewig, 1996). Both intracellular and extracellular pH decrease during anaerobiosis in marine molluscs (Ellington, 1983; Walsh, et al., 1984), but it has yet to be determined whether changes in pH play a role in initiating metabolic suppression. The change in pH during anoxia is typically a steady decline over a long time, sometimes extending to days, whereas the transition into the hypometabolic state during anaerobiosis occurs shortly after anoxia begins (Storey and Storey, 1990 and references therein). Since pH change takes place gradually, it is unlikely to play a role in signaling metabolic suppression during anaerobiosis. Furthermore, anoxia-tolerant molluscs use strategies to minimize the acid load during anaerobiosis (accumulating neutral or volatile products, using shell bicarbonate for buffering) (de Zwaan, 1977; Storey and Storey, 1990), which argues against a signaling role for low pH. However, a moderate decrease in pH during anoxia probably helps to create a metabolic context that favors metabolic depression (Busa and Nuccitelli, 1984). For instance, a lower pH environment favours the catabolism of phosphoenolpyruvate (PEP) via the PEP carboxykinase (PEPCK) reaction rather than the pyruvate kinase (PK) reaction and, therefore, contributes to the diversion of glycolytic carbon into the reactions of succinate synthesis (Hochachka and Somero, 1984). A lower pH environment during anoxia may also facilitate other actions such as enzyme binding with subcellular structural elements, enzyme reaction rates, the relative activities of protein kinases versus protein phosphatases, and changing protein stability (Storey, 1988; Hand and Hardewig, 1996; Schmidt and Kamp, 1996; Sokolova et al., 2000).

2.3. *Controlling glycolytic flux*

Several mechanisms contribute to the anoxia-induced regulation of glycolytic rate in marine molluscs. Allosteric controls by metabolites affect specific enzyme loci during anoxia; for example, reduced levels of fructose-2,5-bisphosphate, a potent activator of 6-phosphofructo-1-kinase (PFK-1), and increased levels of the L-alanine, a strong inhibitor of pyruvate kinase (PK) contribute to anoxia-induced inhibition of these two enzymes. Reduced cellular pH in anoxia also favours PEP processing via PEPCK versus PK. Seasonality also affects the response to anoxia. The effects of anoxia exposure on the maximal activities of selected glycolytic enzymes were generally stronger in winter versus summer in oyster tissues (Greenway and Storey, 1999) and for PK this appeared to be due to seasonal changes in PK isoform present, the winter isoform showing much more pronounced changes in kinetic properties in response to anoxia than the summer form (Greenway and Storey, 2000). For example, winter PK in oyster gill showed a 4-fold increase in the K_m for PEP and a 7-fold increase in the K_a for the activator, fructose-1,6-bisphosphate after anoxia exposure (both changes that would reduce PK activity under anoxia) whereas these parameters were unaffected by anoxia exposure of summer animals (Greenway and Storey, 2000). Anoxia exposure had similar strong effects on the kinetic

properties of PK from hepatopancreas of winter *L. littorea* (also including a strong increase in enzyme sensitivity to L-alanine inhibition) but again had little effect on the summer enzyme (Greenway and Storey, 2001). However, *L. littorea* muscle PK showed similar sensitivity to anoxia in both seasons (Greenway and Storey, 200). Seasonal changes in isoform and in anoxia effects on enzymes may aid prolonged periods of cold-induced inactivity with closed valves or opercula and self-imposed hypoxia/anoxia that triggers strong metabolic rate depression.

Although the above mechanisms contribute to glycolytic control during anoxia, the primary mechanism of overriding importance appears to be the reversible phosphorylation of enzymes catalyzed by the actions of protein kinases and protein phosphatases (Storey, 1992). Anoxia-induced covalent modification of enzymes strongly reduces maximal activities, alters kinetic properties and strongly suppresses overall glycolytic rate. The major kinases involved in the reversible control of enzymes of intermediary metabolism are cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and Ca^{2+} and phospholipid-dependent protein kinase (PKC), their actions mediated by their respective second messengers, adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3',5'-cyclic phosphate (cGMP), and Ca^{2+} and phospholipids (Fig. 1). Several glycolytic enzymes are subject to anoxia-induced phosphorylation that suppresses their activity. These include glycogen phosphorylase (GP), PFK-1, 6-phosphofructo-2-kinase (PFK-2), and PK (Storey, 1993). Control over GP regulates the availability of the primary fuel, glycogen, which is available in high quantities in all tissues of anoxia-tolerant marine molluscs. PFK-1 activity is a major determinant of glycolytic flux and inhibitory control of PFK-2 (that synthesizes fructose-2,6- P_2 , a potent activator of PFK-1) relieves PFK-1 from responding to anabolic demands during anoxia by suppressing levels of the activator (F2,6 P_2) that normally regulates the anabolic use of carbohydrate reserves. As mentioned above, regulation of PK controls the catabolism of PEP, directing glycolytic flux away from aerobic routes (via PK and into mitochondrial oxidation reactions) and into anaerobic routes (via PEPCK and into the synthesis of succinate or propionate) (de Zwaan, 1977). In molluscs, anoxia-induced PK phosphorylation appears to be stimulated by cGMP, implying a key role for PKG in the aerobic/anoxic transition (Brooks and Storey, 1990).

Anoxia-induced phosphorylation produces major changes in PK kinetic properties that converts it to a much less active enzyme. For example, anoxia-induced phosphorylation of PK from radular retractor muscle of *Busycon canaliculatum* results in a 12-fold increase in the K_m for PEP, a 24-fold increase in the K_a for fructose-1,6-bisphosphate, and a 490-fold decrease in the K_i for alanine (Plaxton and Storey, 1984). Similarly, phosphorylation of PFK-1 alters enzyme kinetic properties to convert the enzyme to a less active form and in the anterior byssus retractor muscle (ABRM) of *M. edulis* this again appears to be mediated via cGMP (Michaelidis and Storey, 1990). Interestingly, an increase in cGMP occurs in the ABRM in response to acetylcholine, which is the neurotransmitter that stimulates the contraction of this catch muscle to close the shell (Kohler and Lindl, 1980). For bivalve molluscs, shell valve closure is the proximal event that soon leads to internal oxygen depletion and necessitates the induction of anaerobic adaptations. Further assessment of PKG control of PK found that it is indirect with PKG apparently stimulating a specific PK-kinase that, in turn, phosphorylates and inactivates PK (Brooks and Storey, 1991). The potential for wide-ranging effects by cGMP and PKG in the control of other metabolic responses to anoxia is high but remains an unexplored area.

3. Macromolecular synthesis

3.1. Economics of energy conservation

Until recently, the majority of research on molluscan anaerobiosis has focused on the regulation of intermediary energy metabolism and very little was known about the molecular biology of anoxia tolerance. Of the variety of biochemical mechanisms involved in molluscan anaerobiosis (Grieshaber et al., 1994), the capacity for metabolic rate suppression stands out as crucial (Storey, 1993; Guppy and Withers, 1999). Metabolic rate is reduced to a level that can be sustained over the long term by fermentative ATP production alone and in species with well-developed anoxia tolerance, anoxic metabolic rate is only 1-10% of the normoxic value at the same temperature (de Zwaan and Putzer, 1985). Metabolic rate is a measure of net ATP turnover and it is obvious, then, that to maintain long term homeostasis under anoxic conditions that the rates of all ATP utilizing reactions in cells must be strongly suppressed to a level that matches the anoxic rate of ATP production. A hierarchy of energy-consuming processes exists in cells, with pathways of macromolecule biosynthesis (protein/RNA/DNA synthesis) being the most sensitive to ATP supply (Buttgereit and Brand, 1995). It can be proposed, therefore, that these biosynthetic pathways are obvious targets for strong suppression during molluscan anaerobiosis and, indeed, recent research (described below) is confirming this.

3.2. mRNA and protein synthesis

Stress-induced suppression of macromolecular synthesis has been examined recently in several invertebrate systems (Hand, 1998; Marsh et al., 2001; Robertson et al., 2001), including one anoxia-tolerant marine mollusc, the periwinkle *L. littorea* (Larade and Storey, 2002a). In *L. littorea* hepatopancreas, a ~50% reduction in the rate of protein synthesis (measured as ³H-leucine incorporation into protein) was evident within 30 minutes when snails were placed under an N₂ gas atmosphere and was sustained over 48 h of anoxia exposure (Larade and Storey, 2002a). Following a return to oxygenated conditions, protein synthesis was restored to control values within hours (Fig. 2). Complementing these findings, *L. littorea* hepatopancreas also showed a dramatic reduction in nuclear transcription rates during anoxia. The rate of mRNA elongation (measured as ³²P-UTP incorporation into nascent mRNAs by isolated nuclei) dropped to less than one-third of the normoxic rate (Larade and Storey, unpublished data). Hence, the mRNA substrate available for translation may be reduced under anoxic conditions. Gene expression and protein synthesis are costly processes that require many resources, not only large amounts of ATP for energy but supplies of nucleotide and amino acids substrates and sustained amounts of the transcriptional and translational machinery (enzymes, ribosomes, tRNAs). ATP limitation during anoxia is probably the first and strongest reason for the suppression of nuclear transcription rates but other components of the transcriptional and translational machinery may also become limiting, particularly if anoxia exposure is prolonged. It is understandable, therefore, that cells and organisms would conserve their resources during anaerobiosis by strongly suppressing the rates of transcription and translation of most genes. Against a background of generally reduced gene transcription, those genes whose transcription is specifically up-regulated during anoxia stand out as genes whose protein products are likely to play very important roles in anoxia. We explore this idea more in sections 4.5 and 5 with new research on anoxia-induced gene expression.

3.3. *Mechanisms of translational control*

Protein synthesis is controlled by the efficiency of the translational apparatus, which is determined by the factors that influence translation initiation (Kaufman, 1994). Initiation of translation involves consecutive recruitment of the small and large subunits of ribosomes to specific mRNAs, with the formation of an active ribosome at the initiation site. The predominant mechanism for control of protein synthesis appears to be reversible phosphorylation, under the control of selected protein kinases and protein phosphatases. The targets of covalent modification in this case are translational components, specifically the initiation and elongation factors (Hershey, 1991). The eukaryotic Initiation Factor 2 alpha (eIF-2 α), which promotes the binding of initiator tRNA to the 40S ribosomal subunit, is an example of a factor that is regulated in this manner. Phosphorylation of eIF-2 α is correlated with inhibition of protein synthesis in a range of eukaryotes (Rhoads, 1993) and our recent studies with *L. littorea* concur (Larade and Storey, 2002a). In *L. littorea* hepatopancreas, the total content of eIF-2 α was constant in three groups: aerobic control snails, snails exposed to 24 h under a N₂ gas atmosphere, and snails given 1 h aerated recovery after 24 h anoxia exposure. However, in response to anoxia exposure, the content of phosphorylated eIF-2 α rose ~15-fold compared with aerobic controls (Fig. 3) (Larade and Storey, 2002a). This was reversed rapidly during aerobic recovery with phosphoprotein content reduced again to control levels within 1 h post-anoxia. These data support the concept that anoxia exposure in *L. littorea*, and likely also in other anoxia-tolerant molluscs, stimulates a substantial suppression of protein synthesis, a proposal that is further supported by the direct measurements of the protein biosynthesis rates (discussed earlier) and by the changes in the distribution of ribosomes between polysomes versus monosomes (discussed below).

3.4. *Polysome analysis*

Protein translation requires the sequential accumulation of specific aminoacyl-tRNAs by active ribosomes. This recruits corresponding amino acids to the peptidyl transferase site where protein elongation occurs through the formation of peptide bonds. The ribosome travels down the message, liberating previously occupied codons in the process and allowing additional ribosomes to initiate translation on the 5' end of the message. Depending on the length of a particular mRNA, transcripts have the capacity to retain several ribosomes, creating a structure known as a polyribosome or polysome. When not translationally active, these polysome aggregates dissociate again into monosomes. In general, the activity state of the protein-synthesizing machinery in a cell/tissue can be inferred from the state of ribosomal assembly. Hence, an effective way of gauging the effects of a stress on cellular protein synthesis is to assess the relative proportions of polysomes versus monosomes in control versus stressed states, these two ribosomal states being readily separable on a sucrose gradient (Surks and Berkowitz, 1971). Indeed, several recent studies have documented a strong reduction in polysome content and increase in monosomes in other situations of facultative metabolic rate depression (e.g. in hibernating mammals, Frerichs et al., 1998; Martin et al., 2000; Hittel and Storey, 2002).

In an aerobic environment, mRNAs are generally associated with polysomes indicating active protein synthesis. When ribosome distribution patterns were examined in extracts of *L. littorea* hepatopancreas, a high proportion of ribosomes were found associated with polysomes in extracts from normoxic control snails (Fig. 2). This is consistent with active translation in the normoxic state. After anoxia exposure, however, there was little evidence of polysomes remaining in hepatopancreas and most of the ribosomal RNA occurred in the monosome peak.

This indicates a significant suppression of the activity of the protein synthesizing machinery during anoxia, consistent with the other lines of evidence discussed above. Such a decrease in polysome size (ie. the number of ribosomes attached to a mRNA transcript) could result from either a decrease in initiation or stimulation of elongation and termination (discussed in Mathews et al., 1996). To determine which is the trigger for polysome breakdown, the overall rate of protein synthesis must also be examined. Suppression of protein synthesis, combined with a decrease in polysome size, is indicative of blocked initiation. The observed decrease in the polysome population and rate of protein synthesis in *L. littorea* during anoxia indicates regulation of translation at the level of initiation. This is consistent with observations by Guppy et al. (1994) who suggested that regulation of protein synthesis is at the level of initiation for systems where the rate of protein synthesis is down- and up-regulated in a global manner. When 24 h anoxic snails were returned to aerobic conditions, a strong shift back towards polysomes was observed, with the polysome peak appearing similar to that in normoxic control profiles (Fig. 2). This corresponds well with the results from the *in vitro* protein synthesis experiments, described earlier, which showed that the rate of protein synthesis returned to normoxic control values during aerobic recovery.

3.5. Ribosomal proteins

Ribosomes are composed of four types of ribosomal RNAs and over 80 ribosomal proteins. The expression of genes encoding ribosomal protein and the synthesis of the proteins is tightly regulated and coordinated and has been linked with various physiological conditions suggesting an active role for ribosomal proteins during adaptation and cellular responses to stress (Teem et al., 1984; Mager, 1988; Li et al., 1999; Meyuhas, 2000; Larade et al., 2001; Mitsumoto et al., 2002). Many ribosomal proteins play a major role in the activity and stability of ribosomes, but few have been examined in marine invertebrates. Those that have been studied were isolated from marine invertebrates in various developmental stages (Rhodes and Van Beneden, 1997; Watanabe, 1998; Snyder, 1999) or stresses (Larade et al., 2001). Of particular interest are those ribosomal proteins located at the interface of the large and small subunit, since this site represents the active center of the ribosome. Proteins found here often play a role in stabilizing the mature ribosome through interactions with other ribosomal proteins on the adjacent ribosomal subunit, thereby allowing translation to occur. Ribosomal proteins located at the peptidyl transferase center, specifically ribosomal protein L26 (Villarreal and Lee, 1998; Marzouki et al., 1990), can regulate initiation of translation via interaction with elongation factor-2 (Nygard et al., 1987). Lee and Horowitz (1992) suggested that L26 is likely to be involved in subunit interactions, based on the observation that it undergoes structural rearrangement as the ribosomal subunits associate. Other authors have implicated L26 as a protein involved in forming the region that binds EF-2 to the 60S ribosomal subunit preceding translocation of peptidyl-tRNA from the A to the P site during peptide bond formation (Yeh et al., 1986; Nygard et al., 1987). In either role, L26 is central to the formation and function of the intact ribosome. Analysis of L26 gene expression during anoxia in *L. littorea* hepatopancreas revealed a steady increase in transcript levels over the course of anoxia exposure, reaching about 5-fold higher than in controls and remaining high during the early hours of aerobic recovery (Fig. 3). Similarly, L26 transcripts in foot muscle rose by about 3-fold over the course of anoxia (Larade et al., 2001). These increases in L26 transcript levels suggest that a similar increase in the synthesis of L26 protein may occur during anoxia and/or recovery.

It has been demonstrated that during stress, cells are able to suppress the biosynthesis of their translational apparatus, regulating gene expression at the translational level (for review, see Meyuhas, 2000). Therefore, ribosomal protein transcripts may increase during anoxic exposure and be sequestered or stored in some manner, in preparation for normoxic recovery, which for *L. littorea* in a natural environment would occur during re-immersion of the snails in water at the end of a low tide cycle. High levels of transcript would then be in position to be translated once oxygen was re-introduced into the system. Translation of L26 when aerobic conditions are re-established, likely in concert with various other ribosomal proteins because these are coordinately expressed, will provide a quick increase in the capacity of the translational apparatus to cope with a demand for increased protein synthesis upon the re-introduction of oxygen.

3.6. *Maintaining translatable mRNA pools*

In addition to its role as a translatable message, mRNA also appears to function as a regulator of protein translation. It accomplishes this via specific nucleotide sequences (and in some cases secondary structures) that are recognized by either general or specific factors. To be translated, mRNA must be exported from the nucleus, a process generally mediated by specific mRNA binding factors known as heterogeneous nuclear ribonucleoproteins (hnRNPs). Specific hnRNPs bind to mRNA and help to export it from the nucleus and, in some cases, transport mRNA to its final destination. The majority of mRNAs in a cell are usually present in mRNP (messenger ribonucleoprotein) complexes, often localized as a stress granule, representing a stable intermediary for untranslated messages consisting of a core of both mRNA and RNA-binding proteins such as hnRNPs. Specific RNA binding domains, including those of hnRNPs, are described in detail by Derrigo et al. (2000). In most cases, hnRNPs bind to pre-mRNAs as they are synthesized in the nucleus, although some associate later as a consequence of mRNA processing reactions (Dreyfuss et al, 2002). It has been reported that hnRNPs regulate mRNA localization, translation, and turnover (reviewed by van der Houven van Oordt et al., 2000). Kedersha et al. (1999) report that additional RNA-binding proteins, TIA-1 and TIAR, associate with stress granules in the cytoplasm, an assembly triggered by the phosphorylation of eIF-2 α . As discussed in section 3.3, this leads to inhibition of translation at the initiation stage; active transcripts continue to be processed until all ribosomes “fall off”, resulting in a corresponding decrease in polysome number. Studies in plant cells have demonstrated the existence of stress granules that store untranslated mRNAs during induced stress (Nover et al., 1983; Scharf et al., 1998), while supporting the hypothesis that stress-induced mRNAs are kept liberated (Collier et al., 1988; Nover et al., 1989).

Recent studies have supported the idea that untranslated mRNA transcripts (sometimes described as latent mRNA) are maintained in cells when metabolic rate is depressed. These mRNA transcripts are sequestered into mRNP complexes, where they are hidden from the translational apparatus (Spirin, 1996; Ruan et al., 1997) and remain untranslated until the stress is removed and cells return to normal metabolic function. The mRNA transcripts involved may include those produced prior to metabolic depression as well as some transcript types that are up-regulated by the stress. Indeed, Laine et al. (1994) suggest that mRNA transcription under stress conditions can be an anticipatory response that is important for the ultimate recovery after stress is removed. Thus, transcripts can continue to accumulate during the period of metabolic arrest but their translation is prevented by sequestering them into mRNPs, which lowers the number of active polysomes. When stress conditions are reversed (e.g. a return to aerobic life), these stored

mRNAs are immediately available for translation so synthesis of their protein products can be very rapidly reinstated. This model was verified for ribosomal protein S25 by Adilakshmi and Laine (2002).

4. Gene Expression

4.1. *Anoxia-induced gene expression*

Most energy-consuming processes are strongly suppressed in organisms that show long term endurance of oxygen deprivation and this includes general protein synthesis, as described above. However, selected specific RNA transcripts are up-regulated by anoxia exposure, presumably providing for the synthesis of specific protein products that enhance survival. Recent work from our laboratory has described anoxia-induced up-regulation of several genes in *L. littorea* (Larade et al., 2001; Larade and Storey, 2002b; Larade and Storey, unpublished data) and also in organs of anoxia-tolerant turtles (Cai and Storey, 1996; Willmore et al., 2001).

Gene expression responses to hypoxia (low oxygen) have been extensively explored for the last ten years or more and are typically mediated via the hypoxia-inducible transcription factor, HIF-1. Hypoxia-responsive genes are typically those whose gene products mediate compensatory responses to overcome low oxygen stress. For example, HIF-1 mediates the increased synthesis of glycolytic enzymes to enhance the capacity for fermentative ATP production while at the same time stimulating red blood cell production (via induction of erythropoietin) and the proliferation of capillaries (via induction of vascular endothelial growth factor) (Semenza, 2000).

Gene expression responses to anoxia by anoxia-tolerant animals are different. They are not compensatory in nature but rather are involved in altering metabolism to ensure long-term survival in the absence of oxygen. Furthermore, the transcripts of some anoxia-induced genes may be accumulated during anoxia but not immediately translated. Rather they may be stored to allow translation to be rapidly re-initiated when oxygenated conditions return.

4.2. *Identification of differentially expressed genes*

To identify genes that are up-regulated during anoxia exposure, a number of options are available. Numerous methods, including differential display PCR, cDNA libraries screening (normalized or subtracted), SAGE (Serial Analysis of Gene Expression), and more recently cDNA array technology, have been used to screen for differentially expressed genes, with each method offering advantages and disadvantages. Due to the large number of methods and even larger range of applications, this review will not discuss all of the available methods, since usage often depends on the project of interest or the preferences of the particular researcher. However, we will highlight our recent experiences with cDNA array technology, in particular the value of heterologous screening as a tool for the initial screening and putative identification of stress-specific genes up-regulated in unusual animal model systems.

Array technology provides the opportunity to simultaneously screen the expression profiles of hundreds of genes, representing multiple protein families and many kinds of cellular functions. The data provides broad insight into the coordinated genetic responses by cells to specific stresses, identifying responses that may or may not have been anticipated. At present, commercially-available arrays have been produced for several of the major animal model systems (e.g. human, rat, *Drosophila*, *Caenorhabditis elegans*) and many labs are using these for homologous screening to identify cell and tissue specific gene expression profiles associated with multiple metabolic events (e.g. growth, development, stress, response to hormones,

carcinogenesis). Our lab has become interested in the potential for using arrays in heterologous or "cross-species" screening and we have found excellent cross-reactivity that has allowed us to use human and rat cDNA arrays to effectively assess hibernation-specific gene expression in ground squirrels and bats (Hittel and Storey, 2001; Eddy and Storey, 2002). Success with cross-species screening between different mammal species is perhaps not unexpected as there is upwards of 80% sequence identity between homologous genes in placental mammals. Use of mammalian cDNA arrays for screening a molluscan system has a much greater chance of failure due to the probability that sequence identity between mammalian and molluscan gene homologues could be very low. Nonetheless, for those genes that do show significant cross-reaction, the potential benefit is enormous, allowing a one-step survey of the responses to anoxia stress by hundreds of genes, and the potential identification of many new genes that respond to anoxia, including many whose role in anoxia survival may never before have been suspected.

Human 19K cDNA arrays were screened with cDNA probe made from mRNA isolated from hepatopancreas of control versus anoxic (12 h) *L. littorea*. In general, cross-reactivity was low; *L. littorea* cDNA showed significant reactivity with only 18.35% of the human cDNA sequences (Fig. 4). This can be compared with the ~85% cross-reactivity seen when the same arrays were screened with cDNA made from mRNA of hibernating bats (Eddy and Storey, 2002). This points out the obvious limitations of heterologous probing and is perhaps not surprising given the phylogenetic distance between gastropods and humans. Nonetheless, of those genes that did show significant cross-reaction due to high sequence identity, a number of very interesting results were observed. Despite the metabolic rate depression of the anoxic state, nearly 11% of the cross-reacting genes were apparently up-regulated by 2-fold or more during anoxia. These included selected protein phosphatases and kinases, MAP kinase-interacting factors, translation factors, antioxidant enzymes, and nuclear receptors (Larade and Storey, unpublished data). Virtually all of these represented proteins that have never before been implicated in anoxia adaptation and, hence, we now have a much broader view of the potential gene expression responses that may play significant roles in anoxia survival. Hence, this use of heterologous probing of cDNA arrays has provided multiple new avenues of research to pursue. Although all of these candidates must be confirmed as anoxia up-regulated in mollusc tissues via RT-PCR or Northern blotting, and some may not hold up to this scrutiny, this heterologous screening technique is sure to lead to novel discoveries about molluscan anoxia tolerance.

An interesting outcome of the array analysis was the distribution of responses by snail genes to anoxia exposure. Whereas 10.6 % of those that cross-reacted were up-regulated by anoxia, only 0.6% of genes showed significant down-regulation (by more than 2-fold) in anoxia and mRNA levels of all the rest were unchanged under anoxia exposure. Recalling that the rate of protein synthesis is suppressed by about 50% during anoxia in *L. littorea* hepatopancreas (Larade and Storey, 2002a), it is obvious that a suppression of mRNA transcript levels is not the mechanism by which this is achieved. More likely, as discussed earlier, mRNA transcripts are retained throughout the anoxic episode but sequestered in translationally inactive pools due to the break-up of active polysomes.

Current commercially-available cDNA arrays are made from the genomes of anoxia-intolerant organisms (e.g. humans) and, therefore, one of the key limitations in their use with anoxia-tolerant organisms is that they may not be able to detect novel genes that are specific for anoxia survival. This problem might be remedied by using a *C. elegans* array or one produced from another invertebrate species, but until that time, differential screening of a cDNA library provides the best way to scan for genes that are differentially expressed in response to anoxia or

any other stress. Indeed, using a cDNA library constructed from hepatopancreas of anoxic *L. littorea*, we have identified a number of genes that are up-regulated during anoxia in the snails, including both identifiable genes and novel genes.

Genes that are up-regulated during anoxia exposure in *L. littorina* include ribosomal protein L26 (Larade et al., 2001), ferritin heavy chain, cytochrome c oxidase subunit II (COII), granulin/epithelin, a novel gene that we have named *kvn* (Larade and Storey, 2002b), and various unidentified genes (Larade and Storey, unpublished data). It is not difficult to draw a parallel for some of these genes between their known roles in other organisms and their possible roles in anoxia tolerance. For example, ferritin sequesters iron and is actively regulated during hypoxia in many organisms. COII plays an integral role in the electron transport chain and may play a role in oxygen signaling. However, the rationale for the up-regulation of granulin (a growth factor) is a mystery at present. Other clones encoded novel proteins that could not be identified by sequence searches in BLASTX or tBLASTX (Larade and Storey, 2002b; Larade and Storey, unpublished data). These may encode a suite of unique proteins that confer anoxia tolerance and that are not found in the genomes of anoxia intolerant species. Novel stress-specific genes have been identified in a range of stress-tolerant invertebrates (Bogdanov et al., 1994; Ma et al., 1999; Balaban et al., 2001; Goto, 2001; Larade and Storey, 2002b) and often prove to perform unique functions. In this case, it is likely that genes transcribed during anoxia exposure help the organism to survive without oxygen, or prepare it for the inevitable return of oxygen into the system.

4.3. Pharmacology

L. littorea has proven to be a suitable organism for performing organ culture experiments, allowing tissues to be maintained *in vitro* for short periods of time in order to analyze influences on anoxia-induced gene expression. For example, this analysis was applied to evaluate the influences of various protein kinases on the expression of the ribosomal protein, L26, that is anoxia-induced in *L. littorea* hepatopancreas and foot muscle (Larade et al., 2001). Hepatopancreas explants were incubated *in vitro* under aerobic conditions with various second messengers including dibutyryl cAMP, dibutyryl cGMP, calcium ionophore (A23187), and phorbol 12-myristate 13-acetate (PMA), second messengers that should stimulate protein kinases A, G, B and C, respectively (Fig. 1). The expression of several genes (each up-regulated by anoxia exposure *in vivo* in *L. littorea*) was assessed after tissues were exposed to each of the second messengers *in vitro* in 2 h incubations. For comparison, the effects of *in vitro* anoxia (incubation in N₂ bubbled medium for 12 h) and *in vitro* freezing (at -7°C for 12 h; *L. littorea* is a freeze tolerant species) were tested. Transcript levels of all genes up-regulated by anoxia exposure *in vivo* in *L. littorea* also increased under anoxia exposure *in vitro*, and most also responded to freezing, an ischemic stress that imposes an oxygen limitation and a strong osmotic stress on tissues. L26 transcript levels were strongly induced by anoxia exposure *in vitro* and also by incubation with db-cGMP but did not respond to incubation with cAMP, PMA or Ca²⁺ ionophore/Ca²⁺ (Larade et al., 2001). A novel gene of unknown function, *kvn*, was also up-regulated by anoxia and freezing exposure *in vitro* and this was mimicked by incubation with db-cGMP (Larade and Storey, 2002b). Indeed, all of the *L. littorea* anoxia-responsive genes tested were up-regulated by cGMP but showed little or no response to the other second messenger treatments (Larade and Storey, unpublished results). This implicates a cGMP-mediated signaling cascade in the gene expression response to anoxia in this marine mollusc. Little can be concluded at this time about other elements of the signal transduction pathway involved in the up-regulation

of these genes but additional evidence for the role of a cGMP-mediated process is discussed in section 5.3 below.

5. Triggering the anoxic response

5.1. Oxygen sensing

A topic of interest to many labs is the specific signal that initiates and/or modulates the metabolic responses to hypoxic or anoxic conditions. One obvious signal is the decrease in pO_2 . The level of available oxygen is gauged by an as yet unknown mechanism, with organisms, cells, and pathways responding accordingly. The hunt for a cellular oxygen sensor encompasses a wide range of approaches and model systems that is beyond the scope of this review. Bunn and Poyton (1996) pieced together an exhaustive summary of the research in this area. The most recent research suggests that the oxygen sensor is a prolyl hydroxylase enzyme, which oxidatively modifies (hydroxylates) a highly conserved proline residue found in the oxygen-dependent degradation domain of HIF-1, the central transcription factor involved in the regulation of gene transcription by oxygen (Ivan et al., 2001; Jaakkola et al., 2001). Whether or not this prolyl hydroxylase sensor is universal remains to be determined, although it is likely to be conserved across most phyla (Zhu and Bunn, 2001). By contrast, the secondary target(s) of the oxygen sensor, HIF-1 or others, are most likely different between hypoxia-sensitive cells/tissues and anoxia-tolerant ones. HIF-1 is well known in hypoxia-sensitive mammalian systems for its actions in triggering various compensatory responses that increase oxygen delivery to tissues and improve their glycolytic capacity. Anoxia-tolerant animals do none of these things but instead they initiate a series of actions that suppress metabolic rate and reversibly shut down multiple energy-expensive metabolic processes for the duration of the anoxic excursion. It is likely, therefore, that a transcription factor different to HIF-1 is involved in mediating any necessary gene expression responses to oxygen deprivation in anoxia tolerant species. Indeed, using antibodies to mammalian HIF-1, we have been unable to detect HIF-1 in either anoxia-tolerant vertebrates (freshwater turtles) or in invertebrates (*L. littorea*) (Storey, unpublished data).

5.2. Second messengers

So how exactly is the low oxygen signal mediated in molluscs? The parallel responses of tissues to anoxia exposure *in vivo* vs. *in vitro* (both metabolic and gene responses) argues against hormonal or nervous mediation of signals (Storey, 1993; Larade et al., 2001). It is probable, therefore, that each cell detects the low oxygen signal by itself and from this triggers one or more intracellular signal transduction cascades. These, in turn, initiate adaptive responses that suppress metabolic rate, redirect carbohydrate flux into fermentative pathways, slow overall protein biosynthesis, and initiate the expression of selected genes. A candidate for a primary role in anoxia-responsive signal transduction is cyclic guanosine 3',5' monophosphate (cGMP) and its protein kinase (cGMP-dependent protein kinase; PKG). PKG is now known to mediate both the anoxia-induced phosphorylation of selected enzymes (e.g. pyruvate kinase) involved in carbohydrate fermentation in many molluscan species (Brooks and Storey, 1990, 1997) and the expression of anoxia-responsive genes (Larade et al., 2001; Larade and Storey, 2002b). The PKG I pathway has also been implicated in the control of gene expression of various promoter response elements (Gudi et al., 2000).

5.3. *The role of cGMP*

The accumulation of cGMP, via stimulation of guanylyl cyclases, regulates complex signaling cascades through downstream effectors (Fig. 1). Specific guanylyl cyclases, which are activated through the actions of coupled receptors and/or associated cofactors, convert GTP to cGMP, which is presumed to propagate the anoxic signal. It has been demonstrated that cGMP activates various protein kinases, directly gates specific ion channels, and alters intracellular concentrations of cyclic nucleotides through regulation of phosphodiesterases (for a recent review, see Lucas et al., 2000). A well-known activator of guanylyl cyclases, the diffusible signal molecule nitric oxide (NO) exerts its effects through direct binding, association or interaction with target proteins (Stamler et al., 1992). Little is known about the exact mode of action for NO and NO-related metabolites. In molluscs, intracellular cGMP levels have been shown to increase in the nervous system of various species due to activation of soluble guanylyl cyclase by NO and nitric oxide synthase (NOS) activity has been confirmed in a number of gastropod species, with putative NOS activity localized in ~30 molluscan genera (Moroz, 2000). It is interesting to note that the reaction that produces NO, that is catalyzed by the NOS family of enzymes, requires molecular oxygen (Griffin and Stuehr, 1995). Recent studies have shown that NO is involved in low oxygen signaling in *Drosophila* (Wingrove and O'Farrell, 1999) and this, coupled with demonstrations of cGMP involvement in anoxia-induced protein phosphorylation and gene responses by anoxia-tolerant molluscs (Brooks and Storey, 1997; Larade et al., 2001; Larade and Storey, 2002b) suggests that the nitric oxide/cGMP signaling pathway may be centrally involved in the response to oxygen deprivation in anoxia-tolerant species. Preliminary results are promising, but the mechanism by which cGMP up-regulates genes in molluscs remains unknown at present. A number of questions remain unanswered: How does cGMP operate? Is NO involved? Are cGMP protein kinases involved? Are transcription factors involved? If so, which ones? To date, however, there is little information on the intracellular levels of cGMP in marine molluscs and how these respond to anoxia (Higgins and Greenberg, 1974; Kohler and Lindl, 1980; Holwerda et al., 1981), so much remains to be investigated.

6. Transcription factors

Different model systems are now being explored using the wide variety of commercial antibodies available, in the hopes of determining which protein kinases mediate adaptive responses to environmental stress. The initial step in a signaling cascade generally involves the activation of a target protein that has either kinase activity or activates protein kinases in the cytoplasm. This signal then travels to the nucleus to activate transcription factors that regulate gene expression. It has been previously reported that environmental stresses activate MAP kinases (Raigneaud et al., 1995; Karin and Hunter, 1995), which function primarily to regulate gene expression. This suggests that links may exist between the actions of these kinases and the stress-induced expression of selected genes, including those that are responsive to anoxia signals.

One class of MAPKs is the stress-activated protein kinases (SAPKs), which includes the p38 MAPK. These MAPKs are regulated by tyrosine and threonine phosphorylation mediated by MKKs (MAP Kinase Kinases), which are, in turn, phosphorylated and activated by specific MKKKs (MKK Kinases). This cascade has been outlined in numerous review articles (Cohen, 1997a; Martin-Blanco, 2000). Target substrates of p38 MAPK include various transcription factors (Cohen, 1997b) and protein kinases, such as the serine/threonine specific kinase MAPKAP2, which phosphorylates the small heat shock protein HSP27 (Rouse et al., 1994), and MNK (Fukunaga and Hunter, 1997), which phosphorylates the translation initiation factor eIF-

4E (Waskiewicz et al., 1997; Pyronnet et al., 1999). Canesi et al. (2000) have suggested a relationship between cellular redox balance and tyrosine kinase-mediated cell signaling in molluscs, specifically involving MAPK activation. Such a relationship has been demonstrated in mammalian cells, involving the mitogen and stress-activated protein kinase, MSK-1. This kinase is activated by oxidative stress and its effects are mediated via the p38 and ERK pathways (Deak et al., 1998). Although it has not yet been proven, it appears that NO and cGMP may also play a role in transcriptional activation in these systems. Browning et al. (2000) found that nitric oxide activated p38 MAPK via PKG, whereas Gudi et al. (2000) demonstrated that the cAMP-response element binding protein (CREB) is phosphorylated by PKG both *in vitro* and *in vivo*, when PKG is activated by cGMP or by NO. This data must be interpreted cautiously since the response of any given pathway can be highly tissue- or cell-specific.

p38 MAPK signaling pathways, known to play a role in regulating transcription and translation, are currently being investigated in *L. littorea* (Fig. 5) (Larade and Storey, unpublished results). These studies use two types of antibodies (a polyclonal to provide an estimate of total protein and a specific antibody raised against the phosphopeptide to estimate the amount of phosphoprotein) to evaluate the effect of stress on both the total amount of a given protein and the relative level of protein phosphorylation. Preliminary results on the effects of 12 h anoxia exposure on hepatopancreas indicate an increase in the amount of phosphorylated p38, CREB and HSP27 with little or no change in the total content of these proteins (Fig. 5). These data, although preliminary, suggest that the p38 MAPK pathway may play a role in the response to anoxia by marine molluscs. A great deal of research remains to be completed to confirm any of the pathways involved.

7. Perspectives

The material covered in this chapter demonstrates the value of basic science and attempts to link genomic and cellular responses with physiological adaptation. Molluscan model systems promote both *in vivo* and *in vitro* research at the level of cell, organ, and whole organism, allowing experimental results to be confirmed at various levels. The use of molluscan models with new molecular techniques (e.g. cDNA arrays), standard molecular techniques (e.g. cDNA library screening), and the development and adaptation of techniques (e.g. organ culture with pharmacological agents; polysome profiling) have provided new information on the effects of hypoxia and anoxia on metabolic response and regulation of gene expression.

Metabolic depression can be examined from a variety of viewpoints, with each new development extending in many different directions (Figure 6). Identifying the role of anoxia-induced genes and proteins (genomics and proteomics) is in vogue at the present time. In particular, there is high interest in genes that are activated during anoxia, producing proteins responsible for generating (or regulating) many of the observed adaptations. Studies of gene-protein expression during anoxia are particularly interesting because of the high energetic cost associated with biosynthetic activities. Genes that are actively transcribed and translated when energy supply is restricted should, predictably, be only those whose protein products perform important roles in anaerobiosis, or that facilitate metabolic recovery when oxygen is again available.

Expressed genes that represent known homologues can often fit into an existing scheme, or be justified based on the established function of the gene. Novel genes, however, pose a considerable challenge, since little is known about them other than the nucleotide sequence. This “small” amount of information does, however, allow the protein sequence to be decoded which

permits study of the protein itself via homology analysis, domain searching and evaluation with specific antibodies. Future research should involve not only the actual production of proteins, but also the modification to proteins, both of which are proving to be exciting and dynamic fields of research. Examination of promoter regions of novel genes may uncover conserved promoter elements in the suite of anoxia-induced genes, which will help determine the transcription factors involved in adaptive responses to anoxia. The exact function of cGMP during anoxia is not yet known, although this classical second messenger appears to play a significant role in triggering metabolic depression at the level of transcription, translation, and glycolysis. Future research on marine molluscs will hopefully elucidate functional relationships that exist between anoxia-induced genes and proteins, providing insight into their mechanism of regulation and involvement in metabolic depression.

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Figure legends

Figure 1. Pathways used to generate a cellular response. The major mechanism for regulating protein phosphorylation is via protein kinases, which are mediated by their respective second messengers. The general pathway involves a “message” that is sensed by a receptor and this signal is transmitted to the cytoplasm in the form of a second messenger. The second messenger activates the protein kinase, which triggers a cellular response.

Figure 2. Examination of translation and protein synthesis in the marine mollusc *L. littorea*. Polysome profiles of hepatopancreas extracts from normoxic, anoxic (72 h) and aerobic recovered (72 h anoxia followed by 3 h recovery) snails are shown on the left side of the figure. Post-mitochondrial supernatants were centrifuged on 15-30% continuous sucrose density gradients and fractions were collected with RNA detection by two methods: absorbance monitored at 254 nm (upper panels) and (b) agarose gel electrophoresis of samples from each fraction followed by ethidium bromide staining (lower panels). Polysome region [P]; Monosome peak [M]. The right side of the figure shows the relative rates of protein synthesis in *L. Littorea* hepatopancreas measured as [³H] leucine incorporation into trichloroacetic acid-precipitable material in cell-free-lysates sampled from normoxic, anoxic (0.5 h), and aerobic recovered (12 h) animals. Modified from Larade and Storey (2002b).

Figure 3. Analysis of ribosomal components during anoxia exposure in *L. littorea*. The top left panel shows an increase in the expression of ribosomal protein L26 mRNA in hepatopancreas over the course of anoxia exposure (4 lanes representing 12, 24, 96 and 120 h anoxia, respectively) and aerobic recovery (1 h recovery after 120 h anoxia). The bottom left panel shows changes in the phosphorylation of eIF-2 α after 24 h anoxia or 1 h recovery after anoxia. Both L26 and eIF-2 play roles in efficient translation; the right panel is a schematic diagram that displays the location of both components on the intact ribosome. L26 is located at the subunit interface and functions during the transfer from the A (aminoacyl) site to the P (peptidyl) site, while eIF-2, which performs a role in initiation, is associated with the A site. Modified from Larade et al. (2001) and Larade and Storey (2002).

Figure 4. Cross-reactivity of genes on human 19k cDNA arrays screened with mRNA isolated from normoxic and anoxic *L. littorea* hepatopancreas. The left side of the graph shows the percentage of *L. littorea* cDNA clones that showed significant reactivity with the human cDNA sequences on the array. The right side of the graph evaluates changes in mRNA transcript levels in response to anoxia exposure by those snail genes that showed cross-reactivity with the human array. Levels of mRNA transcripts were compared in aerobic control versus 12 h anoxic snails to identify genes that showed greater than 2-fold up-regulation during anoxia, greater than 2-fold down-regulation in anoxia, or no change.

Figure 5. Stress-induced signaling in *L. littorea*. MAPK signaling pathways, previously reported to play a role in regulating transcription and translation, have been investigated in the marine snail. The relative amount and phosphorylation state of various target substrates,

including transcription factors and protein kinases, was assessed in *L. littorea* hepatopancreas and preliminary results suggest that the p38 MAPK pathway may play a role in the response to anoxia in molluscs. Con = control; 12 h = 12 h anoxic exposure; T = total; P = phosphorylated.

Figure 6. Summary of the different cellular mechanisms that are differentially regulated as a response to anoxia exposure in molluscs.

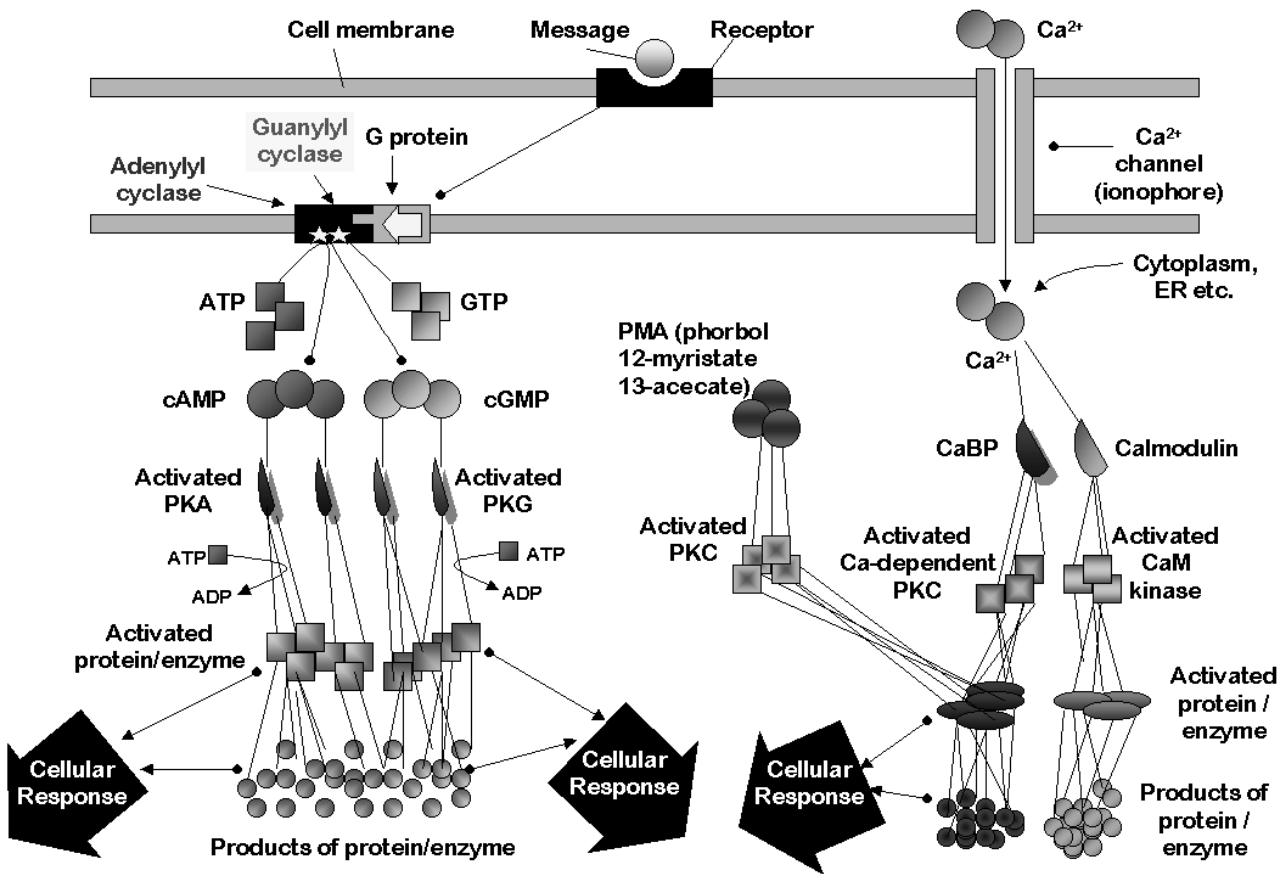


Figure 1 Larade & Storey

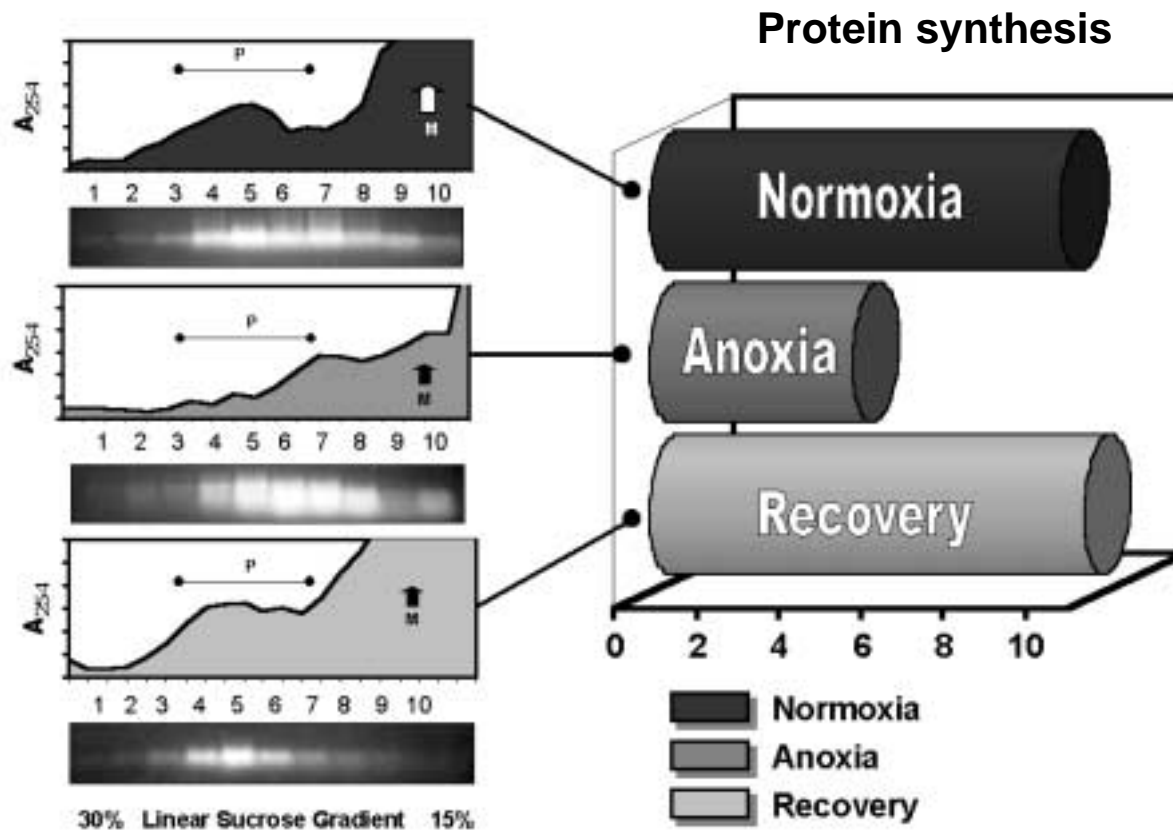


Figure 2 Larade & Storey

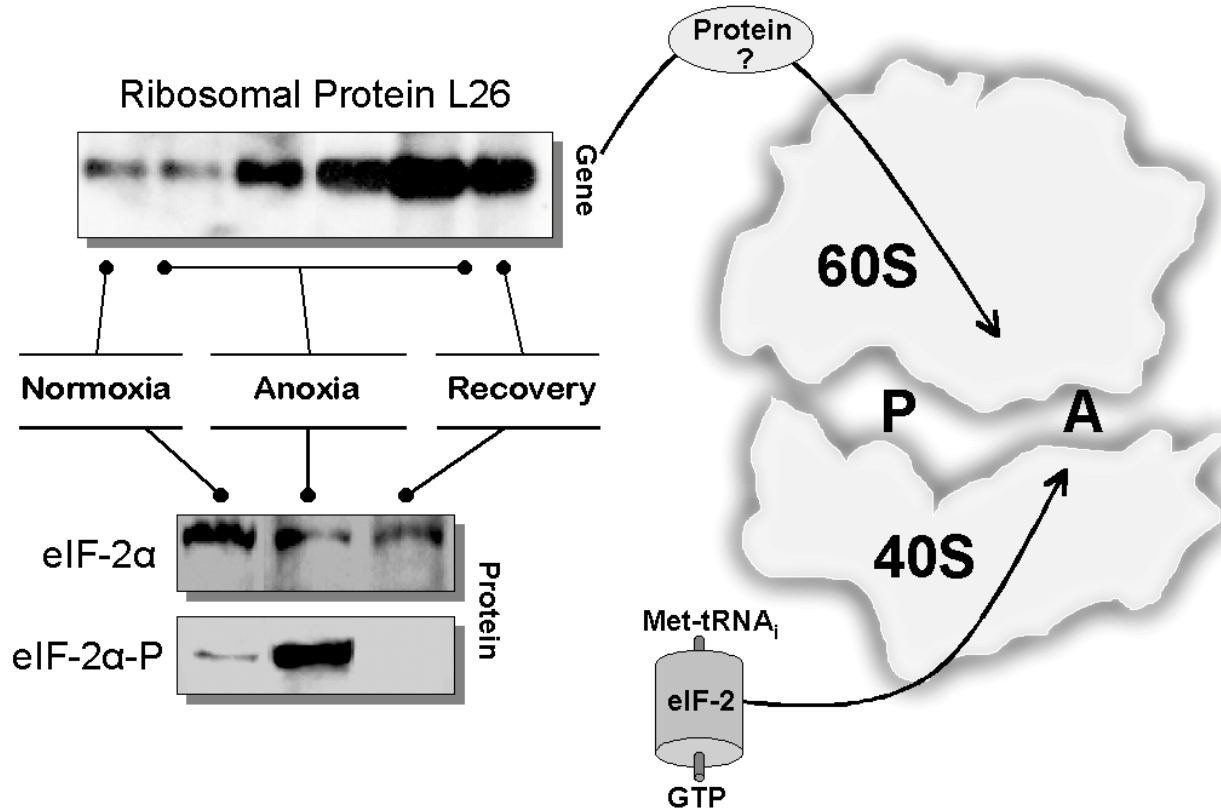
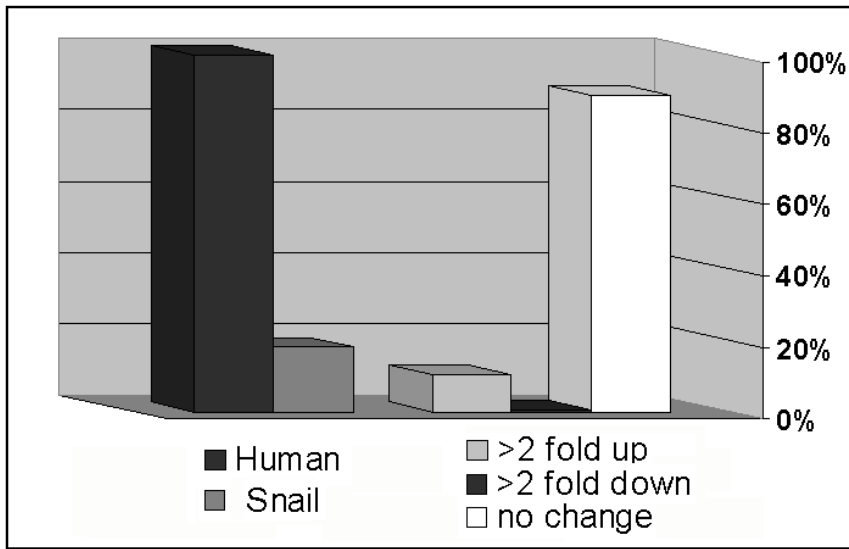


Figure 3 Larade & Storey



Human	100%			
Snail	18.35%	→	>2 fold up	10.61%
		→	>2 fold down	0.62%
		→	No change	88.76%

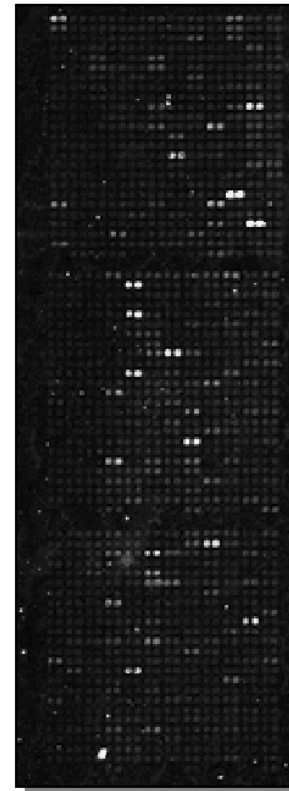


Figure 4 Larade & Storey

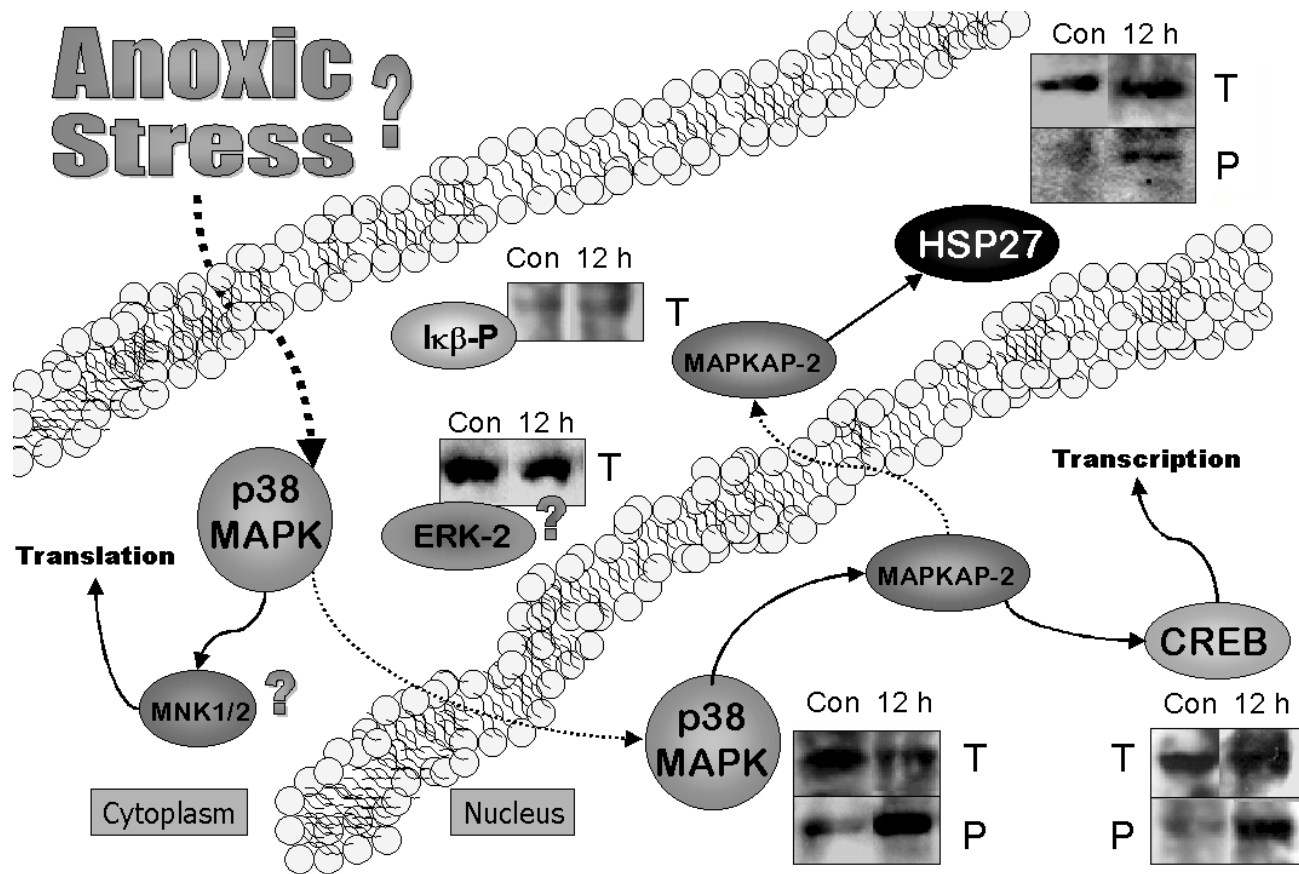


Figure 5 Larade & Storey

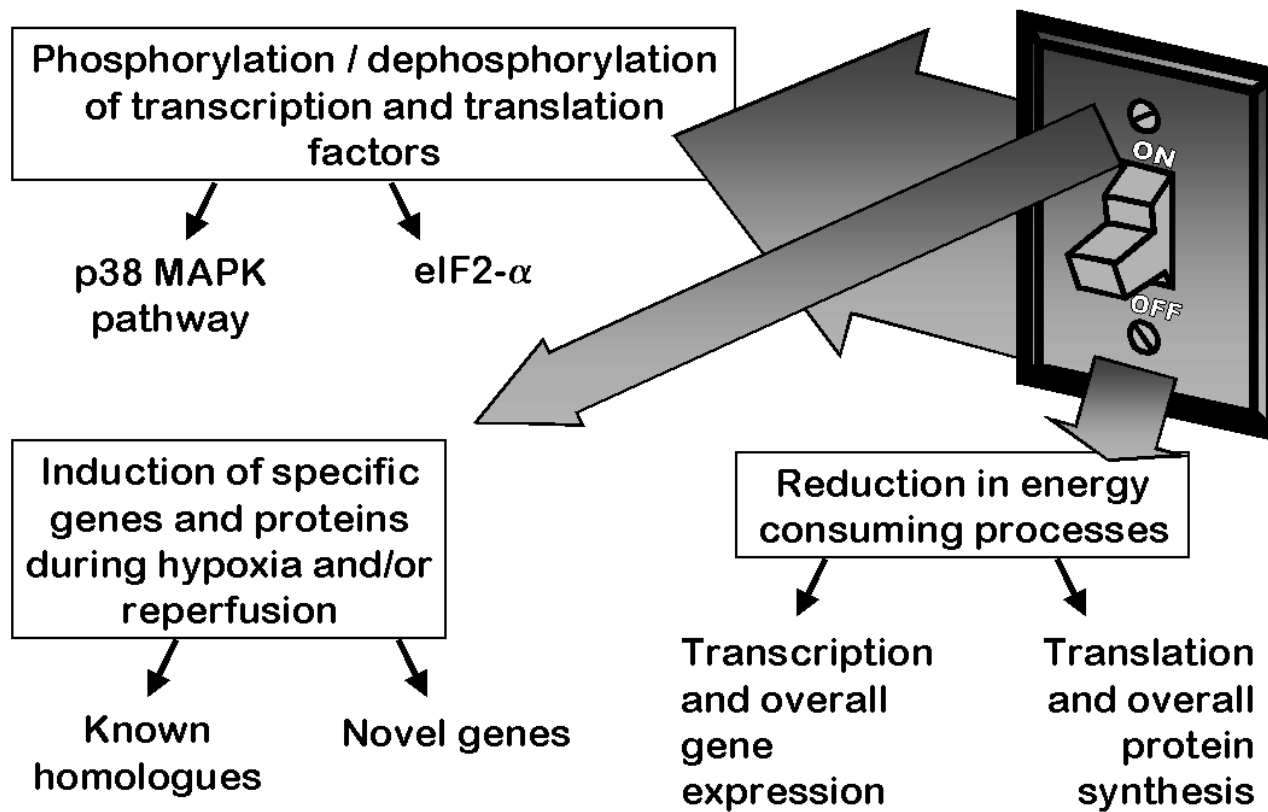


Figure 6 Larade & Storey