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Molecular mechanisms of anoxia tolerance

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Abstract. Facultative anaerobiosis occurs widely across phylogeny and comparative studies with multiple animal models are identifying conserved molecular mechanisms that support anoxia tolerance. Our studies of freshwater turtles have analyzed metabolic, signal transduction and gene expression responses to anoxia. A key mechanism is reversible protein phosphorylation, which provides coordinate suppression of the rates of ATP-producing versus ATP-utilizing cellular processes to achieve strong metabolic rate depression (MRD). Anoxia tolerance is also supported by selective gene expression as revealed by cDNA library and cDNA array screening. Prominent groups of genes that are up-regulated under anoxia in turtle organs include mitochondrially encoded subunits of electron transport chain proteins and several serine protease inhibitors. © 2004 Elsevier B.V. All rights reserved.

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1. Introduction

Oxygen deprivation is rapidly lethal for humans and many other organisms but the realities of environment or lifestyle have lead to the evolution of facultative anaerobiosis in diverse group of animals. Among vertebrates, the premier facultative anaerobes are freshwater turtles of the *Trachemys* and *Chrysemys* genera. These endure apnoic dives of many hours and can survive for 3–4 months submerged in cold deoxygenated water, a capacity that serves winter hibernation in ice-locked ponds and lakes that can become severely hypoxic or anoxic [1]. Known molecular mechanisms of anoxia tolerance include: (a) large organ reserves of fermentable fuels, chiefly glycogen, (b) strategies for buffering or excreting end products, (c) alternative routes of anaerobic carbohydrate catabolism with higher ATP yields than are achieved from glycolysis ending in lactate, (d) well-developed antioxidant defenses to minimize oxidative stress when oxygen is

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reintroduced, (e) strong metabolic rate depression (MRD), and (f) up-regulation of selected genes whose protein products aid anoxia survival [1–3].

Of these, the most important factor overall is MRD. Maintenance of cellular energetics is the most pressing concern for anaerobic survival because fermentative pathways yield just a fraction of the ATP per mole of substrate catabolized as is available from oxygen-based catabolism; e.g. net ATP output is just 2 mol ATP/mol glucose converted to lactate versus 36 ATP/mol for conversion to CO_2 and H_2O. Hence, by strongly suppressing metabolic rate in anoxia, animals lower ATP demand and greatly extend the time that endogenous carbohydrate reserves can support survival. For example, freshwater turtles typically suppress metabolic rate to just 10–20% of the aerobic rate at the same body temperatures [1]. MRD involves coordinated controls on the rates of both ATP-producing and ATP-utilizing cell reactions so that two outcomes are achieved: (a) net ATP turnover is strongly reduced, and (b) the priorities for ATP expenditure are reorganized. Indeed, these outcomes have been well-documented in studies with isolated turtle hepatocytes [4]. Incubation under anoxia decreased ATP turnover by 94% and dramatically changed the proportion of ATP turnover devoted to five main ATP-consuming processes: ion motive ATPases, protein synthesis, protein degradation, gluconeogenesis and urea synthesis. As a result the Na^+K^+ATPase pump became the dominant energy sink in anoxic hepatocytes, consuming 62% of total ATP turnover compared with 28% in normoxia. Protein synthesis and degradation were largely shut down in anoxia (by >90%) and urea synthesis was halted.

2. Metabolic regulation via reversible protein phosphorylation

Studies by my laboratory and others have shown that the primary mechanism controlling MRD is reversible protein phosphorylation [2]. The mechanism controls ATP production via glycolysis under anoxia in both vertebrates (turtles, goldfish) and invertebrates including regulation of glycogen phosphorylase, phosphofructokinase and pyruvate kinase [2,5]. Recent work has also shown reversible phosphorylation control of various ATP-consuming reactions. Key targets of phosphorylation-mediated suppression include ion motive ATPases such Na^+K^+ATPase of the plasma membrane and Ca^{2+}-ATPase of the sarco(endo)plasmic reticulum [2]. Regulation of ion pumps as well as ion channels and membrane receptors contribute to the control of neuronal activity in brain of anoxic turtles [3,6]. Ion pumps (that move ions against concentration gradients) and ion channels (that facilitate movement down gradients) need coordinated regulation to maintain transmembrane potential difference despite a much lower ATP turnover under anoxia. Several mechanisms of ion channel arrest have been identified with reversible phosphorylation of voltage-gated channels (Na^+, Ca^{2+}, K^+) and receptor subunits (e.g. *N*-methyl-D-aspartate-type glutamate receptor) playing a substantive role [6].

The rate of gene transcription is also suppressed in hypometabolic states although this is done with little change in global mRNA transcript levels. For example, total RNA levels were unaffected in turtle organs and mRNA content was constant in four out of five organs over 16 h of submergence anoxia [7]. In addition, mRNA remains largely intact during hypometabolism, creating an effective life-extension of transcripts so that these are immediately available when aerobic conditions return. However, instances of specific transcriptional repression are known; for example, mRNA transcripts of voltage-dependent potassium channels in turtle brain were reduced to 18.5% of normoxic levels after 4 h of

anoxia but rebounded after reoxygenation [8]. Mechanisms of transcriptional control can be global (e.g. inhibition of RNA polymerase) or specific to families of genes sharing a common response element. Both mechanisms can involve reversible phosphorylation; for example, the former can arise from the phosphorylation of core subunits of RNA polymerase II [9,10] and the latter from suppression of signal transduction pathways involving mitogen-activated protein kinases (MAPKs) and transcription factors, most of which are regulated by reversible protein phosphorylation [11]. Still unstudied in comparative systems, mechanisms of global transcriptional repression are obviously a key area that needs future study in facultative anaerobes and other systems of MRD. A little more is known about gene-specific repression. For example, extracellular signal regulated kinases (ERKs) showed no response to anoxia in turtles or freezing in freeze tolerant frogs and turtles [11–13] suggesting that the genes that they control are not up-regulated under these stresses. This is not surprising since the ERK module primarily transduces signals that stimulate growth and proliferation, cellular activities that would be curtailed under anoxic conditions. However, the c-Jun N-terminal kinases (JNKs) appear to play a role in the response to anoxia. JNK activity rose during survivable anoxia exposure in tissues of both adult and hatchling turtles; in both cases, JNK activity peaked after 5 h of anoxic submergence but fell with longer exposure [11–13]. This suggests a role for JNK activation in the hypoxia transition period during the early hours of submergence with JNK suppressed again when metabolic arrest is fully developed in support of long-term anoxia survival.

Protein synthesis is major energy expense in cells and is strongly suppressed during anaerobiosis [4,14,15] and in other hypometabolic states (e.g. mammalian hibernation) [2]. Two mechanisms that contribute to protein synthesis arrest have been studied: (1) reversible phosphorylation control of the translational machinery, and (2) the state of ribosome assembly. Both have been seen during MRD in multiple systems, e.g. anoxia exposure in *Artemia* and the marine snail *Littorina littorea* and mammalian hibernation [2]. Reversible phosphorylation control is directed at key ribosomal regulatory proteins, such as the alpha subunit of the eukaryotic initiation factor 2 (eIF-2α) and the eukaryotic elongation factor-2 (eEF-2) [2,16,17]. Translation is also suppressed by the stress-induced dissociation of polysomes (that conduct active translation) into monosomes (translationally silent) with the movement of a high proportion of total mRNA into the monosome fraction [2]. Both mechanisms aid global suppression of protein synthesis but leave open ways to achieve selective expression of specific anoxia-responsive genes. For example, under stress conditions (including hypoxia, ischemia, starvation, etc.) message selection for translation changes to favour only those messages that contain an internal ribosome entry site (IRES) [16] whereas the vast majority of cellular mRNAs (m^7G-capped mRNAs) cannot bind to the small ribosomal subunit due to stress-induced fragmentation of eIF4G [17]. Of note for studies of anoxia-responsive protein translation is the fact that an IRES is present in the mRNA of the hypoxia-inducible factor-1α (HIF-1α) [18] and this provides a way to elevate HIF-1α protein which, in turn, mediates a variety of gene expression responses to hypoxia. Interestingly, the mRNA of vascular endothelial growth factor (VEGF), one of the targets of HIF-1 action, also has an IRES [19]. It will be interesting to explore anoxia-responsive genes in facultative anaerobes to determine if they also possess an IRES (the IRES is typically found within a long and GC-rich 5′-

untranslated region) that permits their translation under the ATP-restricted conditions of the anoxic state.

3. Anoxia-induced gene expression in freshwater turtles

Although anoxia survival is aided by global suppression of transcription and translation, recent studies have documented several instances of anoxia-responsive up-regulation of selected protein types. For example, selected heat shock proteins are up-regulated in an organ-specific manner under anoxia in turtles [20–22]; their chaperone actions may aid long-term stability of other proteins over extended periods of hypometabolism. Recent work in my laboratory has explored anoxia-induced gene expression in the red-eared slider turtle, *Trachemys scripta elegans*. Initial studies used differential screening of cDNA libraries and, interestingly, revealed multiple examples of the up-regulation of genes encoded on the mitochondrial genome under anoxia. Screening of a heart library made from adult turtles submerged for 20 h in N_2-bubbled water at 7 °C showed anoxia-responsive up-regulation of *Cox1* that encodes cytochrome *c* oxidase subunit 1 (COX1), *Nad5* that encodes subunit 5 of NADH-ubiquinone oxidoreductase (ND5), and the mitochondrial WANCY (tryptophan, alanine, asparagine, cysteine, tyrosine) tRNA gene cluster [23,24]. Transcripts of all three rose within 1 h of anoxia exposure to levels that were 4.5-, 3- and 3.5-fold higher than controls for *Cox1*, *Nad5* and WANCY, respectively. Levels remained high after 20 h of anoxia and then declined during aerobic recovery. *Cox1* and *Nad5* also responded to anoxia in red muscle, brain and kidney [23]. Other mitochondrially encoded genes were anoxia responsive in liver: transcripts of *Cytb*, encoding cytochrome *b*, and *Nad4*, encoding subunit 4 of ND, rose by 5- and 13-fold, respectively, within 1 h of anoxia exposure [25].

The reason for mitochondrial gene up-regulation in anoxia is not yet known but we have also seen the phenomenon in other animals under low oxygen. For example, *Cox2* transcripts rose 6–7-fold in *L. littorea* under an N_2 atmosphere (K. Larade, unpublished) and freezing (which causes ischemia) triggered the up-regulation of mitochondrially encoded genes in freeze tolerant turtles (*Chrysemys picta*) and frogs (*Rana sylvatica*). *Cox1* and *Nad5* were freeze-responsive in turtle liver [23] as was *Nad4* in frog liver and subunits 6 and 8 of F_0F_1 ATPase in frog brain (S. Wu and K.B. Storey, unpublished). Furthermore, four genes on the mitochondrial genome (*Nad2*, *Cox1*, ATPase subunits 6 and 8) were also up-regulated during mammalian hibernation [2] which suggests that the phenomenon is a general principle of hypometabolism. The proteins encoded by these mitochondrial genes all belong to large complexes on the mitochondrial inner membrane that are made up of multiple subunits coded on both the nuclear and mitochondrial genomes (e.g. 3 of the 13 subunits of COX and 6 of the 41 subunits of ND are on the mitochondrial genome) [26,27]. However, we have found no case to date where a nuclear-encoded subunit of these proteins was up-regulated in a hypometabolic state (anoxia, freezing, hibernation). Mitochondrial DNA has only one promoter on each of the L and H strands and genes are transcribed as one RNA precursor from the same initiation site (except for rRNA genes) [28]. Long polycistronic messages are then cleaved to give individual RNA species. This mode of transcription explains the parallel increases in *Cox1*, *Nad5* and WANCY transcripts in anoxic turtle heart but not the tissue-specific differences in gene up-regulation. For example, transcripts of

Cox1 and *Nad5* rose in anoxic turtle heart but not in liver whereas *Cytb* transcripts rose in liver but not in heart [23,25]. Further study of the responses to anoxia by the mitochondrial genome in tissues of a single anoxia-tolerant species may resolve the question.

4. cDNA array screening

One of the hottest new technologies in biology is cDNA array screening. Microarrays spotted with thousands of unique cDNAs on a single glass slide provide researchers with the opportunity to screen for changes in the expression genes representing hundreds of different cell functions. We have made extensive use of human 19,000 gene cDNA arrays produced by the Ontario Cancer Institute (University of Toronto) to provide a comprehensive overview of gene responses to environmental stress in multiple animal systems including hibernating mammals, freeze tolerant frogs, and anoxia tolerant turtles and snails (Refs. [2,29,30] and unpublished). Cross-reaction is never 100% with heterologous probing and therefore cannot give a full picture of all gene changes under stress, but the method still allows thousands of genes to be screened and many putatively up-regulated genes to be highlighted. Moreover, cDNA array screening provides the key advantage of allowing the researcher to look for coordinated responses by groups of genes, e.g. genes representing families of proteins, pathways, signal transduction cascades, etc.

Indeed, one outstanding result from our screening of turtle liver, heart, skeletal muscle and brain was the repeated identification of members of one such family as putatively up-regulated in anoxia. These are the serine protease inhibitors (serpins). Several serpins, as well as tissue factor pathway inhibitor (TFPI), were putatively up-regulated (by 2-fold or more) in organs from anoxic turtles, compared with aerobic controls (Table 1). Array screening indicated that four proteinase inhibitors were up-regulated in liver, a main site of synthesis of these proteins, three in heart, two in muscle and one in brain.

Serpins are a superfamily of proteins with 16 clades. All show a common core domain of three β-sheets and 8-9 α-helices and most are glycoproteins of 40–60 kDa [31]. The majority of serpins are extracellular (except those in clade B) and most exhibit serine or cysteine proteinase inhibition although some have evolved to take on other tasks such as hormone transport (e.g. corticosteroid and thyroxin binding globulins) or blood pressure regulation (angiotensinogen). Serpins are irreversible covalent inhibitors of proteases that cleave specific proteins. Many are specific inhibitors of proteases that act as critical checkpoints in self-perpetuating proteolytic cascades such as the proteases involved in blood coagulation (e.g. thrombin, factor Xa and XIa), fibrinolysis (e.g. plasmin, tissue

Table 1
Proteinase inhibitors identified from cDNA array screening as putatively up-regulated during anoxia in turtle organs: aerobic control versus 4 h of anoxic submergence in nitrogen-bubbled water

Inhibitor	Full name	Organ	Function
SERPINA1	α_1-antitrypsin	Brain, muscle	inhibits elastase, trypsin, chymotrypsin, thrombin, plasmin, kallikrein, collagenase
SERPINC1	antithrombin	Liver, heart	inhibits thrombin, factor Xa, IXa
SERPIND1	heparin cofactor II	Liver, muscle	inhibits thrombin
SERPINF1	pigment epithelium derived factor (PEDF)	Liver, heart	noninhibitory towards proteinases; acts as an anti-angiogenic factor
TFP1	tissue factor pathway inhibitor	Liver, heart	TF–FVIIa complex inhibitor

plasminogen activator, urokinase plasminogen activator), inflammation (e.g. elastase, cathepsin G), and complement activation [31].

Up-regulation of selected serpins under anoxia could be important for inhibiting proteolytic reactions and cascades that could otherwise cause cumulative damage to tissues over the long term in an energy-restricted state. This suggests that another key aspect of MRD is the suppression of proteolytic cascades. SERPIN1A (α_1-proteinase inhibitor or α_1-antitrypsin), the most abundant of the circulating serpins, has a primary physiological role in protecting the lower respiratory tract from proteolytic destruction by neutrophil elastase which hydrolyzes structural proteins as part of its attack on bacterial infections [32]. Low circulating SERPIN1A is associated with chronic obstructive pulmonary emphysema caused by excessive elastase attack on lung tissue. Anoxia-responsive up-regulation of SERPINA1 may act to suppress the action of these circulating proteases involved in inflammation during periods of breath-hold (apnea) when the intake of air-borne pathogens is interrupted. This would help to avoid nonspecific damage to tissues over what could be days or weeks of anoxia exposure, a time when cellular repair mechanisms that require de novo protein synthesis to replace damaged proteins would be suppressed as part of MRD.

Other serpins target proteinases associated with the clotting cascade. SERPINC1 (antithrombin) inhibits thrombin and, thereby, also blocks feedback activation of the cascade by thrombin. SERPIND1 (heparin cofactor II) also inhibits thrombin. Both are activated by binding to heparin or other glycosaminoglycans by one of two main ways: (1) the glycosaminoglycan can simultaneously bind both serpin and proteinase to bring them together, or (2) glycosaminoglycan binding to a serpin can alter its conformation to one that is more reactive towards the proteinase [31]. Clotting capacity could be reduced during anaerobiosis to minimize the risk of thrombosis in the microvasculature under the low blood flow conditions caused by bradycardia during hypometabolism. Indeed, reduced clotting capacity is a component of another form of hypometabolism that is also associated with profound bradycardia—mammalian hibernation. Using array screening, we have similarly noted putative up-regulation of SERPINC1 (and SERPINA1) in liver of hibernating ground squirrels [33] and other mechanisms for clotting inhibition during hibernation have been previously reported including up-regulation in liver and export of α_2-macroglobulin (a non-serpin general inhibitor of clotting cascade proteases) [34] and reduced circulating levels of platelets and several clotting factors [35].

Inhibition of clotting under low blood flow conditions during hypometabolism can also be aided by up-regulation of TFPI, another protein targeted by our array screening. Tissue factor (TF) is an initiator of coagulation. Injury to blood vessel walls exposes TF to circulating Factor VII and TF forms a complex with the active (a) form of FVII (TF–FVIIa) that induces a conformational change in the protease domain of Factor VIIa to activate it [36]. This allows the protease to activate Factors IX and X which then go on to stimulate the conversion of prothrombin to thrombin. TFPI is an inhibitor of the TF–FVIIa complex and the main regulator of the tissue factor pathway [36]. Hence, TFPI has an important anticoagulant action and its up-regulation under anoxia in turtles may further suppress spontaneous clot formation during hypometabolism.

SERPINF1, also known as pigment epithelium-derived factor (PEDF), is a serpin that does not inhibit a proteinase. Instead, PEDF has potent anti-angiogenic and neurotrophic

actions and counterbalances the angiogenic effects of VEGF [37,38]. PEDF was first described as a factor that inhibited aberrant blood vessel growth in models of ischemia-induced retinopathy; PEDF acts by inducing apoptosis in actively dividing endothelial cells [39]. New work has shown that PEDF also inhibits vascular growth and tissue mass in prostate and pancreas [40]. Circulating levels of PEDF in humans are high enough to be physiologically relevant indicating that systemic delivery of PEDF could affect angiogenesis throughout the body [38]. Although no research has yet been done, the putative up-regulation of PEDF in anoxia-tolerant turtles is highly intriguing. Low oxygen typically activates HIF-1 that, in turn, up-regulates a variety of genes whose protein products address two main goals: (1) improvement of oxygen delivery to tissues (e.g. up-regulation of VEGF and erythropoietin), and (2) improvement of glycolytic ATP output (e.g. up-regulation of glycolytic enzymes and glucose transporters). For facultative anaerobes, enhanced capillary growth during natural hypoxia/anoxia excursions is counterintuitive as it is energy-expensive at a time when energy savings are crucial and unproductive since low tissue oxygen levels cannot be improved by enhanced oxygen delivery under apnoic conditions. Hence, up-regulation of PEDF could serve to counteract an angiogenic response to low oxygen under conditions (e.g. breath-hold diving, submerged hibernation) that cannot be aided by enhanced capillary growth.

In conclusion, much is already known about intermediary energy metabolism and the mechanisms of metabolic arrest under anoxia but new explorations of anoxia-induced gene expression are producing many new leads into areas of metabolic function that that have never before been considered as contributing to facultative anaerobiosis. For the comparative biochemist, the opportunities for significant new discoveries are exciting.

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