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Energetics of anoxia exposure and recovery as assessed by calorimetry and biochemical measurements

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Abstract
Some invertebrates enter quiescent metabolic states and survive extended bouts of severe hypoxia or anoxia (on the order of weeks to months). This strategy of metabolic suppression requires a coordinated arrest of both catabolic and anabolic components of energy metabolism. For embryos of the brine shrimp Artemia franciscana, calorimetry shows that energy flow under anoxia is suppressed to exceedingly low levels. Anabolic processes such as protein synthesis are also acutely arrested under anoxia in these embryos. The half-lives of macromolecules, based on evidence for cytochrome c oxidase, are extended dramatically. Recovery from anoxia is characterized by a biphasic rise in heat and oxygen consumption. Biochemical measurements suggest that aerobic processing of organic acids occurs during the first 20 min of recovery, supporting the early rise in heat dissipation and contributing to the recovery of adenylate pools.

INTRODUCTION

The phenomenon of quiescence

Quiescence is a term commonly used in the fields of cell biology and organismic biology to indicate a cell, tissue, or organism that displays a reversible transition into a state of metabolic and/or developmental arrest. The degree of the arrest and its duration can vary markedly depending on the system. At the cellular level, the term has been applied to cells at the G_0 point in the cell cycle, to eggs prior to fertilization (compared to the activated state after fertilization), to various stem cells, and to cells that display acute modulation of cellular metabolism in response to the application or removal of an exogenous insult (ischemia, hypoxia, nutrient deprivation, etc.). For example, interesting work has been published recently on hypometabolic states induced by starvation in yeast (ref. 1) and bacteria (refs. 2,3). In multicellular organisms, quiescence (variously described as environmental, facultative or exogenous quiescence) is usually induced by an unfavorable environmental condition -- desiccation, anoxia, temperature extremes, etc. Included here are states like estivation, hibernation, anaerobic dormancy, and anhydrobiosis (for review, see ref. 4).

Regardless of the various categories of hypometabolic and developmentally-suppressed states -- and the causal factors promoting each one -- there are without exception two fundamental problems that must be solved if a cell is to survive bouts of quiescence. First, there must be a *coordinated* suppression of both catabolic and anabolic processes, and second, the integrity of macromolecules must be preserved. Various molecular mechanisms including enzyme phosphorylation (refs. 5,6), reversible compartmentation of metabolic machinery (ref. 7), and transitions in intracellular pH (pH_i) (refs. 4,8,9) have been investigated as potentially contributing to the inhibition of energy-producing metabolic pathways. However, one aspect of quiescence that has received far less attention is the status of biosynthetic events (e.g., protein synthesis, membrane biosynthesis, nucleic acid synthesis). The use of metabolic suppression as a response to an exogenous insult (for example, hypoxia) would seem to require coordinated arrests of both energy-producing pathways and biosynthetic, energy-utilizing processes (refs. 10,11). Anabolic processes could simply run down during quiescence because of reduced availability of energy-rich adenylate nucleotides. Conversely, it could be argued that anabolic processes must be closely regulated upon entrance into dormancy to ensure that cellular energy levels do not reach critically low levels from which a cell or tissue might never recover. Indeed, our recent studies have shown that anabolic processes (as judged by protein synthesis) are tightly controlled during quiescence (refs. 11,12). In this paper, I will summarize recent work that

addresses the response of catabolism, anabolism and macromolecular degradation to anoxia exposure and recovery in embryos of Artemia franciscana.

Artemia embryos as a highly-connective model system

The encysted, gastrula-stage embryos of the brine shrimp, Artemia franciscana, represent an excellent system for our studies, primarily due to easy manipulation and reversibility of quiescent states in this primitive crustacean. The commercial availability of unlimited embryo tissue (kilogram quantities) allows questions to be pursued at the cellular and molecular levels, where quite commonly the isolation of organelles and macromolecular components is crucial for definitive mechanistic analyses. Furthermore, there is a wealth of physiological and biochemical data already existing for this species (ref. 13). Recently, several multi-volume series have appeared that summarize the extensive molecular, cellular and developmental information readily accessible for this embryonic system (refs. 14-17). These data not only provide a detailed framework within which to interpret the results of experiments on cellular quiescence, but the Artemia data are rapidly becoming the basis for cross reference with many developmental systems including sea urchins, Caenorhabditis elegans, Drosophila melanogaster, Xenopus, and others.

EXPERIMENTAL OBSERVATIONS

Catabolic arrest during anoxia

The Artemia embryo is perhaps the quintessential example of an animal capable of acute metabolic quiescence in response to anoxia (ref. 11). Anoxia is common in the hypersaline and often high-temperature lakes into which these embryos are released, and the survivorship of these embryos under anoxia is truly remarkable. In the laboratory cysts can survive up to nine months of anoxia in aqueous medium (ref. 18). Furthermore, we are unaware of any other euryoxic animal with the capacity to reduce cellular energy flow under anoxia to the levels seen for Artemia. Energy flow is acutely depressed within minutes and reaches 2.4% of aerobic values in the first hours of anoxia (refs. 19,20; Figure 1); after several days energy flow approaches levels suggestive of an ametabolic state (ref. 20). The intracellular pH of embryos declines over the first few minutes to pH 6.8 and to as low as pH 6.3 over the next several hours (ref. 21).

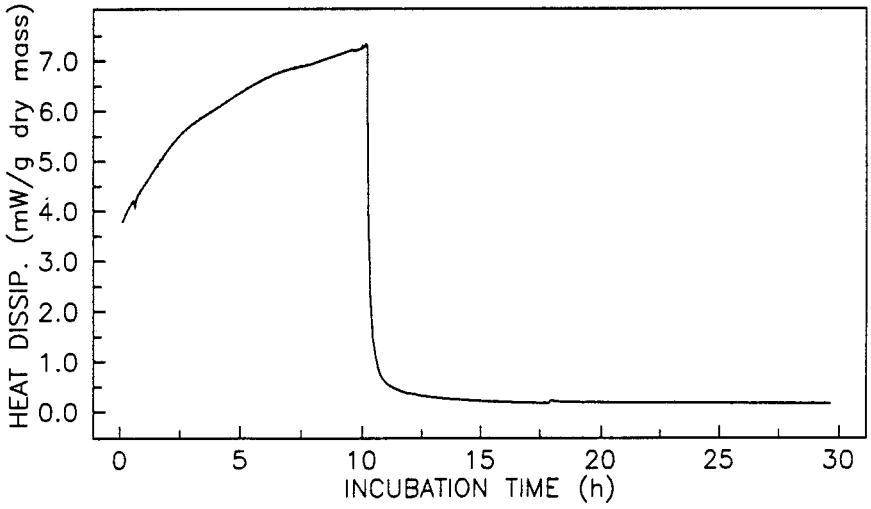

Figure 1. Heat dissipation from Artemia embryos during transition into anoxia. Perfusion with nitrogen-saturated medium initiated at hour 10. Modified after ref. 20.

A qualitatively and quantitatively similar state of metabolic arrest can be promoted simply by exposing the embryos to elevated levels of CO_2 in the presence of oxygen (aerobic acidosis) (refs. 8,19,22). The pH_i of embryos under the artificial condition of aerobic acidosis is 6.8 (ref. 8), and the biochemical features describing the shutdown of metabolism are virtually identical to anoxia (ref. 22). This latter observation provides compelling support for pH_i as a key cellular signal in the metabolic and developmental switching.

Mechanisms of anabolic arrest: control of protein synthesis

In addition to suppressing hatching rates (ref. 8) and blocking subcellular differentiation (refs. 12,23), the experimental regimes of anoxia and aerobic acidosis both promote the arrest of protein synthesis in Artemia embryos (refs. 11,12). Specifically, by following the in vivo incorporation of radiolabeled amino acids into the enzyme cytochrome c oxidase (COX), followed by purification if the enzyme from embryos at various time points, it was possible to show that substantial biosynthesis of the enzyme occurs during aerobic development. Synthesis of COX is blocked under anoxia and aerobic acidosis. When the specific radioactivities of COX were adjusted for differences in amino acid pool radioactivity between the two treatments, it was clear that the inhibition was virtually identical in each case (ref. 11). These data strongly implicated pH_i, directly or indirectly, as a regulator of protein synthesis in these embryos during anoxia. The choice of COX as a model enzyme for our studies of protein synthesis was based on several considerations. The activity of this enzyme is crucial for the recovery of Artemia embryos from anoxia. Also COX is a multimeric enzyme with subunits encoded by both the nuclear and mitochondrial genomes. Subunits I-III (the catalytic core of the protein) are encoded and synthesized within the mitochondrion, whereas subunits IV-VII are encoded by the nuclear genome and synthesized on cytoplasmic ribosomes. Consequently, one can assess the coordination of these genomes during quiescence. Both the synthesis of mitochondrial and cytoplasmic subunits appear to be suppressed to the same degree during anoxia and aerobic acidosis, based on the distribution of radioactivity among COX subunits in gel slices (ref. 11).

A key point to be made is that the ATP/ADP ratio (as well as adenylate energy charge) remains high during aerobic acidosis but decreases during anoxia (ref. 22; Table 1). Since both treatments effectively block the increases in COX activity during preemergence development, it appears that the influence of pH_i on protein synthesis is independent of the state of ATP stores in Artemia embryos. This difference in ATP levels between anoxic and aerobic acidotic cysts suggested to us that there must be simultaneous but independent suppression of biosynthetic and energy-producing pathways -- i.e., low levels of ATP alone could not explain the arrest of biosynthesis (ref. 22).

TABLE 1. Intracellular pH (pH_i) and metabolite concentrations during active and quiescent metabolic conditions in Artemia embryos. Intracellular pH was measured by ^{31}P NMR (refs. 8,21). Other metabolite values were measured after 4 h of aerobic development, followed by either an additional 4 h of aerobic acidosis or anoxia. Values are expressed as mM ± 1 S.E (ref. 24).

	Aerobic Development	Aerobic Acidosis	Anoxia
pH_i	$\geq$ 7.9	6.8	6.3
ATP	1.17 ± 0.06	1.29 ± 0.02	0.34 ± 0.01
ADP	0.35 ± 0.02	0.37 ± 0.02	0.41 ± 0.01
AMP	0.09 ± 0.05	0.16 ± 0.03	1.16 ± 0.20
phosphate	12.2 ± 0.3	11.4 ± 0.1	15.0 ± 0.3

While the mechanisms responsible for the arrest of protein synthesis during quiescence are not fully understood, two independent lines of evidence suggest that there is an element of translational control operative in dormant embryos. First, as determined by in vitro translation techniques, there were no significant quantitative differences in mRNA pools in dormant as compared to actively developing embryos (ref. 25; Table 2). However, direct information on the status of the mRNA specifically encoding for cytochrome c oxidase subunits is needed if one is to assess whether or not the decreased expression of COX during quiescence reflects a reduction in the steady-state level of message. Second, polysome profiles showed that dormant embryos possessed reduced levels of polysomes relative to those found in cells of active embryos (Table 3). This drop in polysomes is an indication that the initiation step in protein synthesis is disrupted.

Degradation of macromolecules during quiescence

A second crucial problem that requires attention is *how the integrity and viability of macromolecules are to be preserved* during cellular quiescence. In light of our evidence that both energy producing pathways and protein synthetic processes are arrested during

TABLE 2. In vitro translation of polyadenylated mRNA isolated from Artemia franciscana embryos with oligo(dT) cellulose chromatography. Modified from ref. 25.

Treatment	Translatable mRNA (10^{-6} X cpm ^{35}S-Met. incorporated per 3 g embryo)
Anoxia	28.5 ± 1.56
Aerobic control	28.0 ± 0.19
Aerobic acidosis	36.4 ± 3.34
Aerobic control	39.9 ± 1.72

Values are means ± S.E. for triplicate determinations on one batch of isolated polyadenylated mRNA.

TABLE 3. Comparison of monosome and polysome regions in extracts of Artemia embryos. Extracts were applied to a 15%-35% sucrose gradient and centrifuged for 4 h at 60,000 g. Modified from ref. 25.

Treatment	Monosomes (% of total area)	Polysomes (% of total area)
Aerobic (4 h)	38.3 ± 3.66	61.7 ± 3.66
Anoxic (1 h)	59.0 ± 4.93*	41.0 ± 4.93*
Aerobic acidosis (1 h)	68.3 ± 2.03*	31.7 ± 2.03*

Each value represents the mean ± S.E. for three separate experiments. *Significantly different from aerobic controls in a *t*-test ($P<0.05$).

quiescence, we anticipated that protein turnover must also be suppressed in order to avoid depletion of macromolecules during quiescence. In terms of recovery from quiescence, insurmountable difficulties could arise if key metabolic enzymes have to be resynthesized de novo prior to resuming the energy flows characteristic of developing embryos. Our recent data from pulse-chase experiments and enzyme activity measurements during anoxia confirmed a marked increase in half-life of COX relative to control aerobic embryos (ref. 26). If one estimates from semi-logarithmic plots the half-life of COX under anoxia, the value obtained is approximately 101 days (relative to 31 hours under conditions of aerobic development). Equally interesting are the data obtained under conditions of aerobic acidosis. The estimated half-life under this condition is 9.7 days. While degradation under aerobic acidosis is clearly slower than under control conditions (aerobic development), the rate is not suppressed to the extent seen during anoxia. We believe that these results reflect the marked difference in ATP levels in the embryo under aerobic acidosis versus anoxia (Table 1). Specifically, ATP levels early in aerobic acidosis are identical to control values, in striking contrast to the low levels seen under anoxia.

What, then, are the molecular mechanisms that explain this dramatic change in macromolecular stability during quiescence? Our current hypothesis (ref. 26) is that the conditions existing during quiescence effectively shutdown the ubiquitin-dependent proteolysis pathway in the embryo. The process of ubiquination is known to be a key event in the degradation of short-lived proteins like regulatory metabolic enzymes (refs. 27, 28). A key feature of the ubiquitin-mediated pathway is that it is strongly ATP dependent. Specifically, the activation of the ubiquitin molecule, which is essential if the molecule is to bind to a protein and identify it for subsequent degradation, is catalyzed by the enzyme E1 (ubiquitin activating enzyme). The enzyme has an absolute requirement for ATP (refs. 29-32). The ubiquinated proteins are then degraded by a large (26S), ATP-dependent protease (ref. 33). Further, Rapoport and colleagues have shown the ubiquitin-dependent pathway to be acutely pH dependent, with greater than 90% inhibition occurring below pH 7.0 for reticulocytes (refs. 34,35). Again, the drop in ATP levels during quiescence, as well as the decrease in intracellular pH, may be key features in suppressing the ubiquitin pathway for protein degradation. We suggest it is likely that macromolecular degradation is a key feature that sets the ultimate survival time of cells during quiescence.

Metabolic aspects of recovery

It is clear that at the initial point where oxygen is returned to Artemia embryos and metabolic recovery begins, the pH_i of embryos is still low and the trehalose pathway (the primary energy-producing processes in these embryos) is blocked (refs. 22,24,36). Currently, features of this metabolic recovery are not fully understood. We have suggested that the immediate rise in heat dissipation under these conditions is a result of mitochondrial metabolism of organic acids (ref. 19). The mitochondrial-based metabolism would serve to elevate pH_i due to elimination of organic acids and the formation of ATP from ADP, P_i and H^+. After this initial phase, trehalose catabolism would be reactivated due to the elevated pH, and normal energy metabolism during

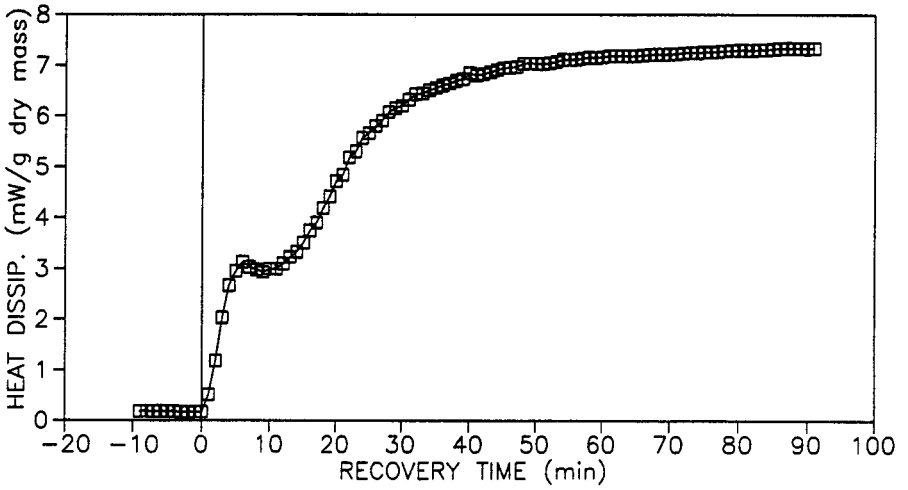

Figure 2. Heat dissipation from Artemia during recovery from anoxia (modified after ref. 19).

preemergence development would be resumed. Consistent with this notion, the heat dissipation profile observed during recovery is biphasic (Fig. 2, ref. 19) -- characterized by a rapid burst, a short lag, and then a second period of increase. One interpretation of this pattern is that the first phase is a result of aerobic processing of organic acids and the second phase represents the delayed mobilization of trehalose (ref. 19). A delayed reinitiation of trehalose catabolism is consistent with the kinetic properties of the enzyme trehalase, which show a slow reactivation in response to increasing pH in vitro (ref. 36).

Our preliminary data are consistent with the above predictions (ref. 37). Adenylate energy charge (AEC) increased from 0.62 ± 0.022 to 0.86 ± 0.001 (mean ± SE) during the first 20 minutes of recovery from a 12 hour bout of anoxia. The low levels of organic acids that accumulated during anoxia declined rapidly during this same period of recovery. Lactate declined from 27.0 ± 1.63 to 12.9 ± 1.73 nmol/mg protein, and propionate dropped from 54.1 ± 3.79 to 34.9 ± 1.99 nmol/mg protein. If even 5% of this total decline in organic acid levels were due to catabolism by the TCA cycle, this processing would account for the heat dissipation measured during the early phase of recovery (first 20 minutes). Trehalose levels did not change during this early time interval, but declined by 14% after 2 h, consistent with the reinitiation of carbohydrate metabolism. These data suggest that acid processing is a component of the early recovery phase and may explain the rapid burst of heat dissipation. Burning of organic acid may contribute to the recovery of adenylate pools by supporting oxidative phosphorylation. Concomitantly, during this 20 minute period, there is a rapid alkalinization of intracellular pH from 6.8 to above 7.7, as measured by ^{31}P-NMR (Hand, Rees and Shapiro, unpublished observations).

IMPLICATIONS

Results of our studies with Artemia may have general implications for tissues or cells where acute modulation of biosynthesis and energy metabolism are known to occur. Understanding the mechanisms responsible for *coordinated* arrest of catabolic and anabolic processes, and for reductions in rates of macromolecular degradation during quiescence, could be important for biological systems where survival depends on successful recovery from physical and chemical insults, as well as for tissue and organ transplantation procedures, where improved tissue viability is a primary concern. For example, Lemasters and associates have recently reported intriguing data which suggests that acidification of cardiac myocytes and liver greatly enhances the survivorship of these cells during hypoxia, ischemia, and reperfusion (refs. 38,39). In Artemia embryos the decrease in intracellular pH that accompanies entry into quiescence is the largest ever reported for a living tissue (1.6 pH units, ref. 21). We believe that the features of quiescence discussed above are intimately linked with this profound cellular acidification. Certainly, the mechanisms underlying this extreme tolerance to anoxia should have strong implications for other cells and tissues confronting even transient hypoxic insults.

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