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QUIESCENCE IN *ARTEMIA FRANCISCANA* EMBRYOS: REVERSIBLE ARREST OF METABOLISM AND GENE EXPRESSION AT LOW OXYGEN LEVELS

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Summary

Depression of the production and consumption of cellular energy appears to be a prerequisite for the survival of prolonged bouts of anoxia. A correlation exists between the degree of metabolic depression under anoxia and the duration of anoxia tolerance. In the case of brine shrimp (*Artemia franciscana*) embryos, oxygen deprivation induces a reversible quiescent state that can be tolerated for several years with substantial survivorship. A global arrest of cytoplasmic translation accompanies the transition into anoxia, and rates of protein synthesis in mitochondria from these embryos appears to be markedly reduced in response to anoxia. Previous evidence suggests that the acute acidification of intracellular pH (pH_i) by over 1.0 unit during the transition into anoxia contributes to the depression of biosynthesis, but message limitation does not appear to play a role in the down-regulation in either cellular compartment. The ontogenetic increase in mRNA levels for a mitochondrial-encoded subunit of cytochrome *c* oxidase (COX I) and for nuclear-encoded actin is blocked by anoxia and aerobic acidosis (artificial quiescence imposed by intracellular acidification under aerobic conditions). Further, the levels of COX I and actin mRNA do not decline appreciably during 6 h bouts of quiescence, even though protein synthesis is acutely arrested across this same period. Thus, the constancy of mRNA levels during quiescence indicates that reduced protein synthesis is not caused by message limitation but, instead, is probably controlled at the translational level. This apparent stabilization of mRNA under anoxia is mirrored in an extension of protein half-life. The ubiquitin-dependent pathway for protein degradation is depressed under anoxia and aerobic acidosis, as judged by the acute drop in levels of ubiquitin-conjugated proteins.

Mitochondrial protein synthesis is responsive to both acidification of pH_i and removal of oxygen *per se*. Matrix pH declines in parallel with pH_i, and evidence from experiments with nigericin indicates that mitochondrial protein synthesis is depressed directly by acidification of matrix pH. The oxygen dependency of organellar protein synthesis is not explained by blockage of the electron transport chain or by the increased redox state. Rather, this cyanide- and antimycin-insensitive, but hypoxia-sensitive, inhibitory signature for the arrest of protein synthesis suggests the presence of a molecular oxygen sensor within the mitochondrion.

Key words: *Artemia franciscana*, anoxia, oxygen-sensing protein, protein synthesis, ubiquitin, intracellular pH, macromolecular stability.

Introduction

For numerous aquatic invertebrates, exposure to extended periods of severe hypoxia is a common occurrence. The duration of oxygen limitation can range from a momentary disruption of electron transport function to a protracted bout of oxygen deprivation that may extend for many years. For example, at the extremes of hypoxia tolerance, studies of sediment cores from coastal marine habitats (Marcus *et al.* 1994) and freshwater lakes (Hairston *et al.* 1995) have revealed viable rotifer and copepod embryos that have apparently survived oxygen limitation for decades. These buried developmental stages presumably serve as egg banks for future generations. Major bioenergetic readjustments must occur to allow survival under these and other conditions where the capacity for generating cellular energy has been compromised. It is now becoming clear that a carefully orchestrated suite of molecular and metabolic events is brought into play that serves to preserve both the energy stores and the existing macromolecular components (i.e. biological structure) of cells during these energy-limited states. In this short review, I will try to highlight issues that appear central to the survival of organisms during chronic hypoxia and will focus largely on findings from studies with a useful model species, embryos of the brine shrimp *Artemia franciscana*.

Principles governing survival during oxygen limitation

Conservation of cellular energy stores

To the extent that they are currently understood, several requirements must be met if an organism is to enter, survive and exit bouts of prolonged oxygen limitation. First, studies of the physiological responses to environmental insult have underscored the importance of suppressing both energy production (e.g. oxidative pathways) and energy consumption (e.g. transcription, translation, ion pumping) in a coordinated fashion (Hochachka and Guppy, 1987; Storey and Storey, 1990; Lutz, 1992; Hand, 1991, 1993; Guppy *et al.* 1994; Hand and Hardewig, 1996; Krumschnabel *et al.* 1996; Schwarzbaum *et al.* 1996; Buck and Hochachka, 1993). Otherwise, cellular energy reserves would be depleted and organisms would probably reach an energetic state from which recovery would become impossible (Hofmann and Hand, 1990). In fact, the predominating pattern seen among anoxia-tolerant animals from aquatic environments is that the duration of anoxia tolerance is correlated with the degree of metabolic depression promoted by oxygen removal. Fig. 1 indicates that, for a variety of species, as the level of ATP turnover declines from 30 % of the aerobic rate to values approaching zero, the tolerance to anoxia increases by more than three orders of magnitude. As pointed out in the legend to Fig. 1, these data were collected at a variety of different temperatures, a complicating factor that probably explains some of the scatter in the plot (an inverse, exponential relationship exists between anoxia tolerance and temperature; see, for example, Musacchia, 1959). Still, it would seem that conservation of the onboard energy stores is a fundamental prerequisite for long-term survival under anoxia.

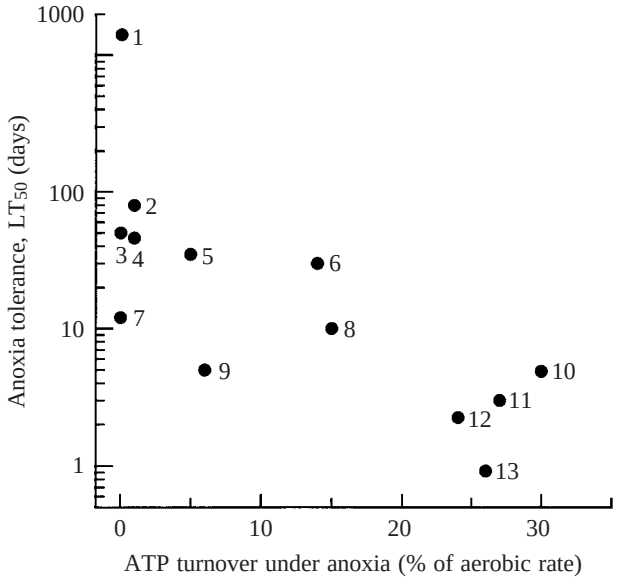

Fig. 1. Anoxia tolerance of representative aquatic animals as a function of the degree of metabolic depression under anoxia. Survival time is expressed as the LT_{50} (days required under anoxia for 50 % mortality). Metabolic depression is calculated as the anoxic ATP turnover rate divided by the aerobic rate. When data were available, ATP turnover under anoxia was calculated from the accumulation rates of biochemical end products. When only heat dissipation data were available, the mean values of $-80\,kJ\,mol^{-1}$ ATP and $-40\,kJ\,mol^{-1}$ ATP were used for the calorific equivalent for ATP turnover for aerobic and anoxic metabolism (Gnaiger, 1983), respectively, to obtain a first approximation. Aerobic ATP turnover was calculated from respiration rate or from the rates of utilization of carbon substrates. Species identities, temperatures of measurement and references are as follows: 1, *Artemia franciscana* embryos (brine shrimp), 21–25 °C, Clegg (1997), Hand (1995), Hontoria *et al.* (1993); 2, *Arstarte borealis* (infaunal bivalve), 10 °C, Oeschger (1990), Theede (1984); 3, *Nephelopsis obscura* (leech, large), 5 °C, Davies *et al.* (1987), Reddy and Davies (1993); 4, *Artica islandica* (infaunal bivalve), 10 °C, Oeschger (1990), Theede (1984); 5, *Mytilus edulis* (bay mussel), 13 °C for ATP turnover and 10 °C for LT_{50} at $0.15\,ml\,O_2\,l^{-1}$, deZwaan and Wijsmann (1976), Theede *et al.* (1969); 6, *Actinia equina* (sea anemone), 15 °C, Shick (1981); 7, *Nephelopsis obscura* (leech, small), 5 °C, Davies *et al.* (1987), Reddy and Davies (1993); 8, *Sphaerium* sp. (freshwater clam), 10 °C, Holopainen and Penttinen (1993), McKee and Mackie (1983); 9, *Cardium tuberculatum* (cockle), 18 °C for ATP turnover and 10 °C for LT_{50}, Meinardus-Hager *et al.* (1989), Theede (1984); 10, *Hirudo medicinalis* (medicinal leech), 20 °C, Schmidt and Zerbst-Boroffka (1993), Schmidt *et al.* (1996); 11, *Sipunculus nudus* (marine coelomate worm), 15 °C, Hardewig *et al.* (1991), Hand and Hardewig, 1996; 12, *Chrysemys picta* and *Trachemys scripta* (turtles), 24 °C, Jackson (1968), Musacchia (1959); 13, *Carassius auratus* (goldfish), 20 °C, van Waversveld *et al.* (1989), van den Thillart *et al.* (1983).

Preservation of existing macromolecules

Owing to restricted biosynthetic capacity, the half-lives of pre-existing macromolecules (e.g. mRNA, proteins) must be extended under anoxia. Otherwise, if macromolecular turnover proceeded unabated, recovery from the anoxic bout could be delayed or precluded because of the compromised status of the metabolic machinery. The evidence available thus far indicates that degradative processes are acutely arrested under anoxia (e.g. the ubiquitin-dependent pathway) and that direct molecular stabilization by molecular chaperones and/or solutes of low molecular mass may contribute to the extension of macromolecular half-lives.

Oxygen sensing and signalling

Mechanisms must be in place for sensing the environmental stress (i.e. oxygen limitation) and for signalling the appropriate physiological response. From the foregoing information, it is clear that many biochemical events must be triggered in response to entry into quiescent states. Questions arise as to how these environmental perturbations are sensed and how these signals are transduced to initiate the down-regulation processes. Considerable effort is currently being directed at elucidating mechanisms of cellular oxygen sensing and the subsequent effects on gene expression. Evidence indicates that, in many cells, free heme and/or hemoproteins are intimately involved in oxygen sensing (Goldberg *et al.* 1988; Padmanaban *et al.* 1989; Zitomer and Lowry, 1992; Bunn and Poyton, 1996; Hochachka *et al.* 1996; Hand and Hardewig, 1996). A hemoprotein apparently serves as the oxygen sensor

during the induction of erythropoietin at low oxygen tensions in hepatoma cells (Goldberg *et al.* 1988; Semenza *et al.* 1994), and such a sensor has been implicated in the modulation of gene expression in rat and turtle hepatocytes (Semenza *et al.* 1994; Land and Hochachka, 1995). It is plausible that heme or hemoprotein sensors may be involved in oxygen sensing during the entrance of numerous invertebrate facultative anaerobes into anoxia.

Anaerobic quiescence in embryos of *Artemia franciscana*

When exposed to anoxia, gastrula-stage embryos of the brine shrimp *Artemia franciscana* enter an extreme state of metabolic quiescence during which heat flow is depressed to very low levels (Hand and Gnaiger, 1988; Hand, 1990; Hontoria *et al.* 1993); after 48 h, heat dissipation is 0.2 % of the aerobic value and is still declining (Hand, 1995). As others have argued (Clegg, 1997), an ametabolic state may actually be reached in these embryos at some point under anoxia. The embryos can survive anoxia at room temperature for more than 4 years (Clegg, 1997). The largest transition in intracellular pH (pH_i) ever measured for eukaryotic cells occurs within minutes after exposure to anoxia. As shown in Fig. 2A, pH_i decreases by at least 1 unit to 6.7 within 20 min (Busa *et al.* 1982; Clegg *et al.* 1995; Kwast *et al.* 1995) and to as low as 6.3 after several hours (Busa *et al.* 1982). Upon return of oxygenated perfusion, pH_i is quickly restored to control aerobic values (Fig. 2B). A similar state of quiescence can be promoted under fully aerobic conditions by artificially lowering the pH_i to 6.8 with elevated levels of CO_2 (Busa and Crowe, 1983; Carpenter and Hand, 1986). Biochemical evidence shows that this treatment, termed aerobic acidosis, promotes inhibition at the same steps of the glycolytic pathway as under the natural state of anoxia (Carpenter and Hand, 1986). However, in contrast to anoxia (Stocco *et al.* 1972; Carpenter and Hand, 1986), ATP levels do not change from control levels during the first hours of aerobic acidosis; this observation indicates that a low [ATP] is not a requirement for the metabolic arrest. These and other data (for reviews, see Hand, 1993, 1997; Hand and Hardewig, 1996) suggest a role for pH_i in the depression of metabolism during entry of *Artemia franciscana* embryos into anoxia.

A pH_i change of this magnitude (1 pH unit) is not the common situation in invertebrate facultative anaerobes in response to anoxia (e.g. Ellington, 1983, 1993; Reipschläger and Pörtner, 1996). However, a freshwater leech (*Hirudo medicinalis*) has recently been reported to exhibit a pH_i acidification of 0.96 units under anoxia (Schmidt *et al.* 1996). Similarly, hypoxic potato tubers experience a 0.6 pH unit depression of tissue pH within the first 30 min of hypoxia (Vayda *et al.* 1995). One feature common to all three of these organisms (brine shrimp embryos, leeches, potato tubers) relates to the lack of any substantial quantities of cellular phosphagens. *A. franciscana* embryos have only small quantities of arginine phosphate (S. C. Hand, unpublished observations), and leeches apparently have no phosphagen system whatsoever (Robin and Roche, 1965; Schmidt and Kamp, 1996). While arginine phosphate has been reported in protozoans (*Tetrahymena pyriformis*; Robin and Viala, 1966), I am aware of no report of phosphagens in plants. Thus, large transients in pH_i during anoxia and any subsequent participation of this factor in metabolic depression could perhaps be associated with organisms possessing low levels of phosphagen. Further, all of these organisms experience a sizable drop in ATP levels during anoxia which, at least in the case of *Artemia* embryos, makes a significant contribution to the cellular acidosis (Busa, 1985; Kwast *et al.* 1995).

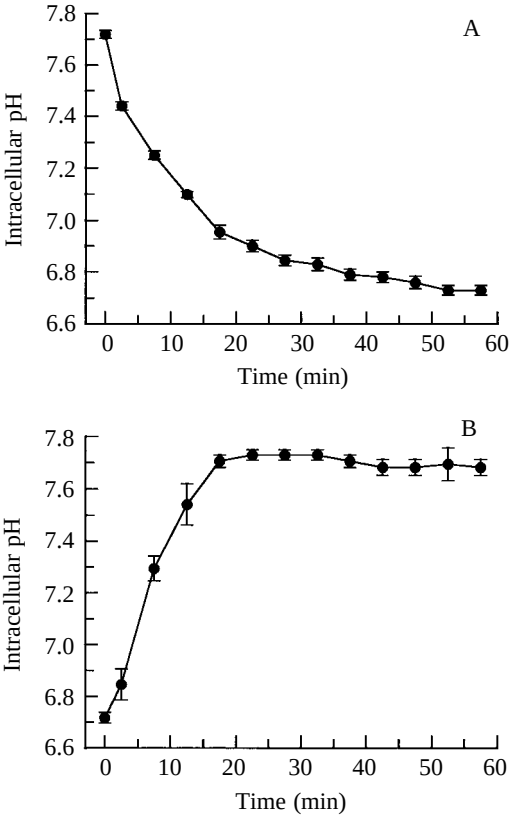

Fig. 2. ^{31}P nuclear magnetic resonance measurements of intracellular pH during transitions from oxygen saturation to anoxia (A) and from anoxia to oxygen saturation (B) in encysted embryos of *Artemia franciscana*. Data are presented as means ± S.E.M. for nine (A) and six (B) independent experiments (redrawn from Kwast *et al.* 1995).

Arrest of translation during anaerobic quiescence

A global arrest of cytoplasmic protein synthesis occurs during entry into anoxia-induced quiescence in *Artemia* embryos (Clegg and Jackson, 1989; Hofmann and Hand, 1990, 1994). The phenomenon can be demonstrated *in situ* by following the incorporation of radiolabeled amino acids into specific proteins (e.g. subunits of cytochrome *c* oxidase), as well as *in vitro* by estimating the total translational capacity of cell-free lysates prepared from aerobic, anoxic and aerobic acidotic embryos. As illustrated in Table 1, lysates of anoxic and aerobic acidotic embryos displayed rates of incorporation

Table 1. *Incorporation of radiolabeled leucine into protein by cell-free lysates of* Artemia franciscana *embryos*

Experiment	Replicate	Aerobic control	Treatment
Anoxic quiescence	1	251 730±4508*	49 067±1004
	2	400 120±4642	24 402±462
Aerobic acidosis	1	317 573±11 547	9582±2310
	2	364 563±5245	4448±1413

*Values are expressed as cts min^{-1} h^{-1} (mean ± S.E.M.) of triplicate assays performed on a single lysate. Lysates from aerobic embryos were assayed at pH 7.9 and those from quiescent embryos were assayed at pH 6.8.

After Hofmann and Hand (1994).

of radiolabeled leucine that were only a few per cent of those measured for lysates from aerobic embryos, when each lysate was assayed at the respective pH value measured for each treatment *in vivo*. Because artificial acidification of embryos under fully aerobic conditions promoted a depression of translation comparable to that seen under anoxia, the results indicate a signalling role for pH_i in the metabolic arrest. The levels of endogenous template were quantitatively the same in all lysates (Hofmann and Hand, 1992), and addition of exogenous mRNA did not alter the measured translation rates. Thus, the acute blockage of amino acid incorporation was apparently not due to message limitation. When lysates from each treatment were assayed over a range of physiologically relevant pH values (pH 6.4–8.0), amino acid incorporation in lysates from quiescent embryos was consistently lower than that in aerobic controls. Thus, a stable alteration in translational capacity of quiescent lysates was indicated (for example, phosphorylation of initiation factors), and a direct effect of protons on the translational machinery was not apparently the sole explanation for the translational arrest in lysates from quiescent embryos. Tissue acidosis in potato tuber under anoxia has been causally linked to inhibition of protein synthesis and the aberrant association of elongation factor EF-1α with polysomes (Vayda *et al.* 1995).

Experiments with isolated mitochondria from *Artemia* embryos indicate that arrest of protein synthesis also occurs in this cellular compartment. The rate of protein synthesis in isolated mitochondria from these embryos is acutely sensitive to pH (Kwast and Hand, 1993, 1996*a*), with an 80 % reduction in incorporation of radiolabeled amino acids as pH is lowered from the optimum (7.7–7.5) to pH 6.7, the measured pH_i under anoxia (Fig. 3). The depression of protein synthesis appears to be global in that no qualitative differences are detectable in the array of translation products synthesized (Kwast and Hand, 1996*a*). Intramitochondrial levels of adenine and guanine nucleotides did not differ as a function of extramitochondrial pH under aerobic conditions (Kwast and Hand, 1996*a*) and, therefore, alterations in the mitochondrial energy status cannot account for the observed depression of protein synthesis at low pH. Another way by which pH could potentially affect organellar protein synthesis is by altering the rate of transport

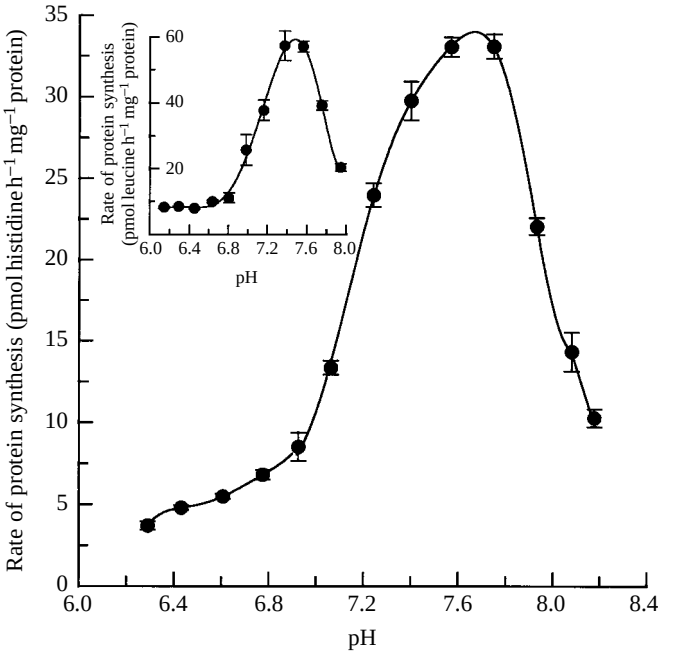

Fig. 3. pH profile of the rate of protein synthesis by isolated mitochondria from *Artemia franciscana* embryos. Data are means ± S.E.M. for three incubations (redrawn from Kwast and Hand, 1996*a*).

of amino acids into the matrix. However, similar pH profiles for protein synthesis were obtained with either [^{3}H]leucine or [^{3}H]histidine (Fig. 3) (which are known to use different transporters; Halling *et al.* 1973; Nichols and Ferguson, 1992), as well as with [^{3}H]methionine (see Kwast and Hand, 1996*a*). Consequently, if pH influences amino acid transport, the effect spans multiple transporters to a similar degree.

The remaining possibility was that extramitochondrial pH inhibits protein synthesis through changes in matrix pH, i.e. that there were proton-sensitive translational components localized within the mitochondrion. Measurement of matrix pH as a function of extramitochondrial pH across the relevant physiological range (pH 7.9–6.3) showed that matrix pH was very responsive to external proton concentration (Kwast and Hand, 1996*b*). In fact, because ΔpH across the inner membrane of *Artemia* mitochondria decreases somewhat at lower extramitochondrial pH values, matrix pH undergoes a more severe acidification than external pH across the above range (Fig. 4). Second, experiments in the presence and absence of nigericin (a H^+/K^+ exchanger that abolishes mitochondrial ΔpH; Nicholls and Ferguson, 1992) were performed to establish whether protein synthesis was responding directly to matrix pH. The pH optimum for protein synthesis showed an alkaline shift from approximately 7.5 in the absence of nigericin to 8.2 in the presence of nigericin (Fig. 5). Considering that steady-state matrix pH was 8.2 at the optimal extramitochondrial pH of 7.5 in control mitochondria, the 0.7 unit shift in extramitochondrial pH optimum seen upon abolishing the ΔpH clearly indicated that protein synthesis was responding to matrix pH.

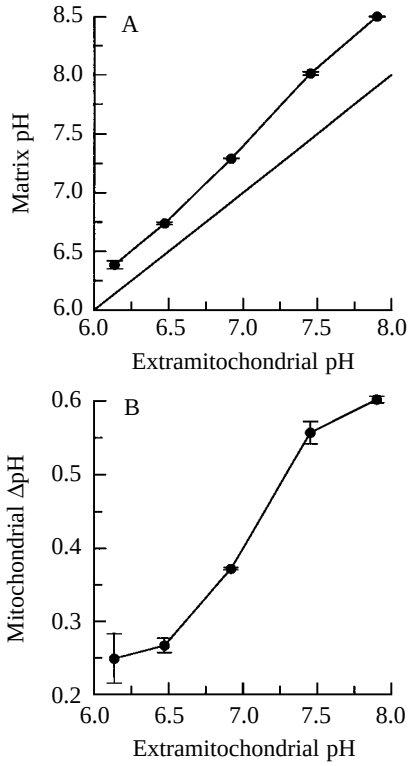

Fig. 4. Matrix pH and ΔpH as a function of extramitochondrial pH in isolated mitochondria from *Artemia franciscana* embryos. The line of unity is indicated in A. Data are the means ± S.E.M. for three independent determinations at each pH (redrawn from Kwast and Hand, 1996*b*).

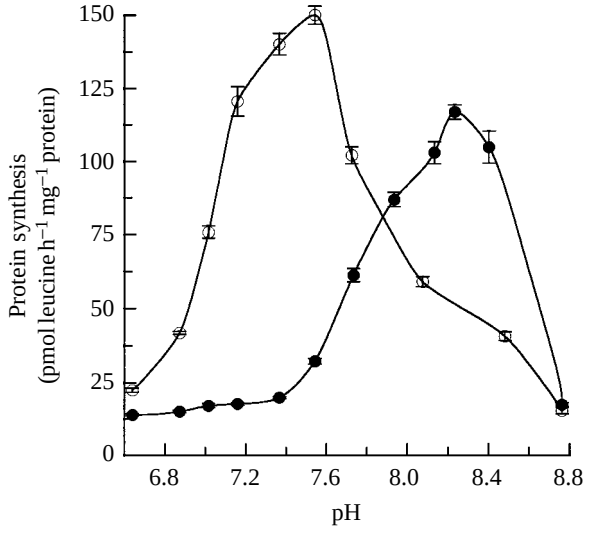

Fig. 5. The shift in the pH optimum for protein synthesis in the presence and absence of nigericin in isolated mitochondria from *Artemia franciscana* embryos. Open symbols are assays performed in the absence of nigericin and filled symbols are assays performed with 100 µmol l^{-1} nigericin. Data are means ± S.E.M. for three independent determinations at each pH (redrawn from Kwast and Hand, 1996*b*).

Depression of macromolecular degradation under anoxia

Because the energetically expensive process of protein synthesis is restricted during prolonged periods of anoxia-induced quiescence in *Artemia* embryos, the question arises as to how these organisms are able to maintain the functional integrity of their cellular machinery, a process that normally requires replacement of macromolecules through biosynthesis. Consequently, we investigated the rates of protein turnover in *Artemia* embryos subjected to anoxia and aerobic acidosis (Anchordoguy *et al.* 1993). By measuring the turnover of cytochrome *c* oxidase (COX), we calculated that, compared with aerobic conditions, the half-life of this protein is extended 77-fold under anoxia, but only sevenfold during aerobic acidosis. These results suggested that the intracellular acidification occurring under both conditions may contribute to the extension of the half-life of COX. In addition, the much longer half-life of COX under anoxia indicated that some factor related to the reduced availability of ATP might also be important in regulating COX degradation (Anchordoguy *et al.* 1993).

To examine the regulation of protein degradation during quiescence, we chose to focus on ATP-dependent, ubiquitin-mediated proteolysis – a pathway prominently involved in the turnover of short-lived metabolic proteins (Rechsteiner, 1987). Briefly, the ubiquitin-mediated degradation pathway utilizes the covalent attachment of ubiquitin to target proteins for proteolysis. Proteins conjugated to ubiquitin are then rapidly degraded by a specific 26S proteosome that cleaves off ubiquitin before hydrolyzing the targeted protein (Rechsteiner, 1987; Ciechanover, 1994; Goldberg, 1995). Further, this ATP-dependent degradation is markedly suppressed at pH values of 7.0 and below (Müller *et al.* 1980).

The initial step in the ubiquitin-mediated degradation pathway is inhibited during entry into quiescence, as indicated by levels of conjugated ubiquitin (Fig. 6). The levels of ubiquitin-conjugated proteins in *Artemia* embryos drop to 37 % of control (aerobic) values during the first hour of anoxia and reach 7 % in 24 h (Anchordoguy and Hand, 1994). Associated with the rapid fall in [ATP] (Fig. 6) is a reciprocal rise in [AMP] (Carpenter and Hand, 1986). The very low $K_{1/2}$ value for ATP (0.04 mmol l^{-1}) (Haas and Rose, 1982) makes it unlikely that low cellular [ATP] limits ubiquitination except under the most severe conditions. Conversely, Hershko *et al.* (1978) demonstrated a progressive inhibition of proteolysis in the presence of increasing AMP concentrations, from which an apparent K_i for AMP of approximately 0.15 mmol l^{-1} was derived. Consequently, inhibition of ubiquitination by AMP may explain why proteolysis in intact cells is suppressed before ATP levels drop sufficiently to limit this process. Conjugated ubiquitin levels recover rapidly upon returning embryos to aerobic conditions (Fig. 7).

It also appears that a major contributor to the drop in levels of ubiquitin conjugates during quiescence in *Artemia* embryos is the acidification of pH_i (Anchordoguy and Hand, 1994, 1995). Despite constant [ATP], levels of ubiquitin conjugates decreased rapidly to 58 % of aerobic values within 1 h of

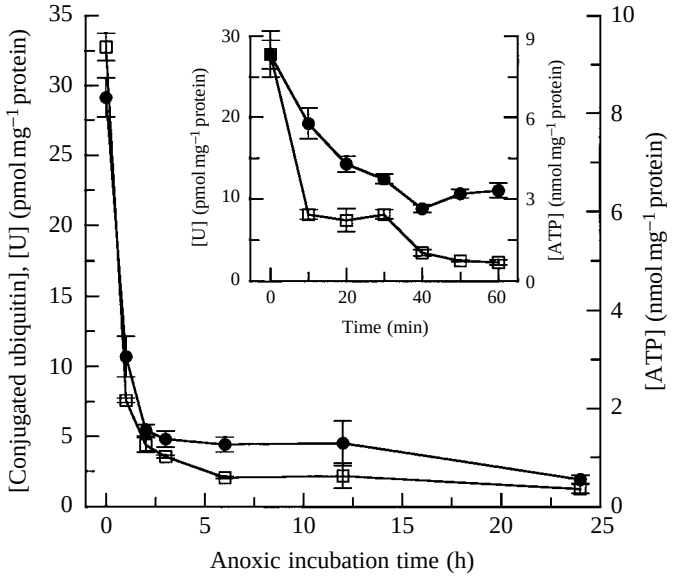

Fig. 6. Changes in the levels of conjugated ubiquitin (filled circles) and ATP (open squares) during entry into anoxia for *Artemia franciscana* embryos. Each point represents the mean ± 1 S.E.M., *N*=3. Where error bars are absent, the error is less than the size of the symbol (redrawn from Anchordoguy and Hand, 1994).

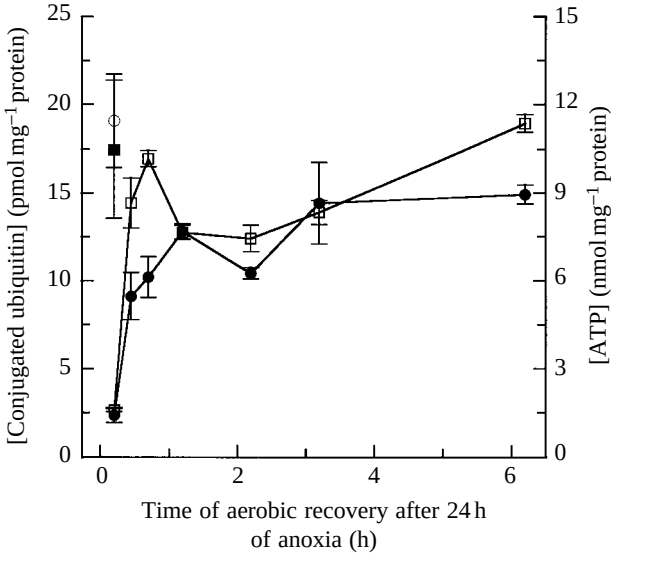

Fig. 7. Changes in the levels of conjugated ubiquitin (filled circles) and ATP (open squares) during aerobic recovery after 24 h of anoxia for *Artemia franciscana* embryos. Control (aerobic) levels of ubiquitin conjugates (open circles) and ATP (filled squares) are shown. Each point represents the mean ± 1 S.E.M., *N*=3. Where error bars are absent, the error is less than the size of the symbol (redrawn from Anchordoguy and Hand, 1995).

aerobic acidosis (Fig. 8). A slower decline in levels of conjugates after 6 h was associated with gradually decreasing ATP levels (Fig. 8). Thus, while the proximate mechanism for the suppression of ubiquitination has not been proved,

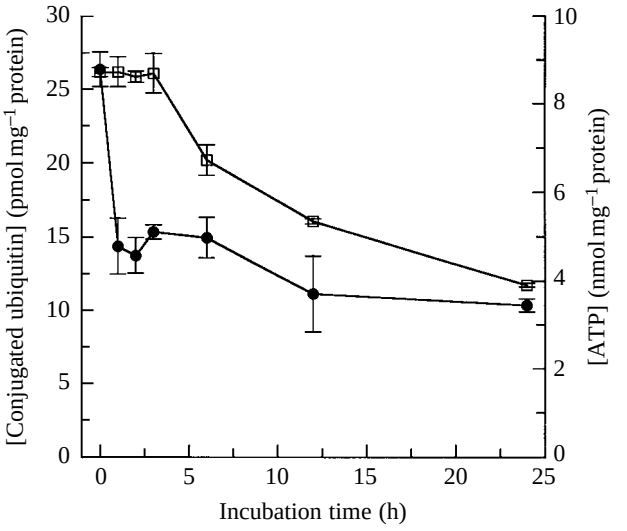

Fig. 8. Changes in the levels of conjugated ubiquitin (filled circles) and ATP (open squares) during 24 h of aerobic acidosis for *Artemia franciscana* embryos. Each point represents the mean ± 1 S.E.M., *N*=3 (redrawn from Anchordoguy and Hand, 1994).

alterations in the adenylate pool and the decrease in pH_i both appear to contribute to the suppression of ubiquitination. In addition, preliminary observations with lysates from *Artemia* embryos suggest that phosphorylation can also alter ubiquitin-dependent degradation rate (F. vanBreukelen and S. C. Hand, unpublished observations).

It has been reported that an abundant 26 kDa protein undergoes translocation to the nucleus during anoxia in *Artemia* embryos and that the reverse translocation occurs when anoxic embryos are returned to aerobic conditions (Clegg *et al.* 1994, 1995). Furthermore, the 26 kDa protein appears to associate with an increased variety of soluble proteins under anoxia. Thus, it is possible that the protein may serve as a protective molecular chaperone during prolonged anoxia and other forms of stress (Liang *et al*. 1997).

A stabilization of mRNA parallel to that seen for protein may also occur during quiescence in *Artemia* embryos. The amounts of mRNA for a mitochondrial-encoded subunit of cytochrome *c* oxidase (COX I) and for nuclear-encoded actin were recently quantified during aerobic development, anoxia and aerobic acidosis (Hardewig *et al.* 1996). The levels of both COX I and actin transcripts increased significantly during aerobic development. The ontogenetic increase in levels of these mRNAs was blocked by anoxia and aerobic acidosis. The levels of COX I and actin mRNA did not decline appreciably during 6 h bouts of quiescence. Mitochondrial mRNA is typically unstable, with a half-lives ranging between 15 min and 3.5 h in mammalian cells (Gelfand and Attardi, 1981) and spanning a similar range in yeast (Min and Zassenhaus, 1993). These constant levels of COX I mRNA and actin mRNA in quiescent compared with developing embryos could be explained by reduced transcription rates combined with an enhanced longevity of existing mRNA. One advantage of

stabilizing mRNA under anoxia could be the quick resumption of translation as soon as oxygen is returned to the embryos.

Support for a mitochondrially located oxygen sensor

As documented above, mitochondrial protein synthesis can be acutely depressed by acidifying extramitochondrial pH. However, this process is also inhibited in *Artemia* mitochondria by the removal of oxygen in the absence of any pH change (Kwast and Hand, 1996*a*,*b*). Compared with aerobic conditions, an inhibition of protein synthesis by 77 % was achieved by placing isolated mitochondria under anoxia (pH 7.5). When pH was lowered to 6.8 under anoxia, the inhibition reached approximately 90 %. To investigate the mechanism by which oxygen removal *per se* inhibited protein synthesis, a number of factor were evaluated.

Quantifying the energy status of mitochondria showed that matrix [ATP]:[ADP] and [GTP]:[GDP] ratios declined measurably with increasing time under anoxia at constant pH (Kwast and Hand, 1996*a*). Addition of ATP to the mitochondrial suspension at the onset of anoxia, however, stabilized the [ATP]:[ADP] ratio at aerobic values but did not rescue protein synthesis. Thus, changes in the levels of matrix adenylates under anoxia are not responsible for the arrest of protein synthesis. While we could not stabilize the modest decrease in the [GTP]:[GDP] ratio under anoxia, this reduction occurred across a range that should not affect protein synthesis on the basis of data for the cytoplasmic compartment, and thus it is likely that other factors are responsible for the oxygen-dependent arrest (see Kwast and Hand, 1996*a*).

If the inhibition of protein synthesis by removal of oxygen was caused simply by blockage of the electron transport chain, then chemical anoxia should elicit a quantitatively identical response. However, chemical anoxia (with fully saturating levels of cyanide and antimycin A under aerobic conditions) did not inhibit protein synthesis nearly to the same degree as anoxia (Fig. 9). The mitochondrial NAD(P)H pools were fully reduced after 2–3 min with all three treatments, so differences in redox state apparently cannot account for the discrepancies among these treatments. One explanation for this cyanide- and antimycin-insensitive, but hypoxia-sensitive, pattern of protein synthesis depression is the presence of a molecular oxygen sensor within the mitochondrion. It has been reported previously that the erythropoietin pathway in oxygen-sensing cells can be modulated by hypoxia but not by cyanide poisoning (Goldberg *et al.* 1988; Semenza *et al.* 1994) or other respiratory chain inhibitors (Tan and Ratcliffe, 1991). The mechanisms for cellular oxygen sensing, as well as the signal transduction pathways that regulate the subsequent gene expression, are receiving considerable attention (for a review, see Bunn and Poyton, 1996).

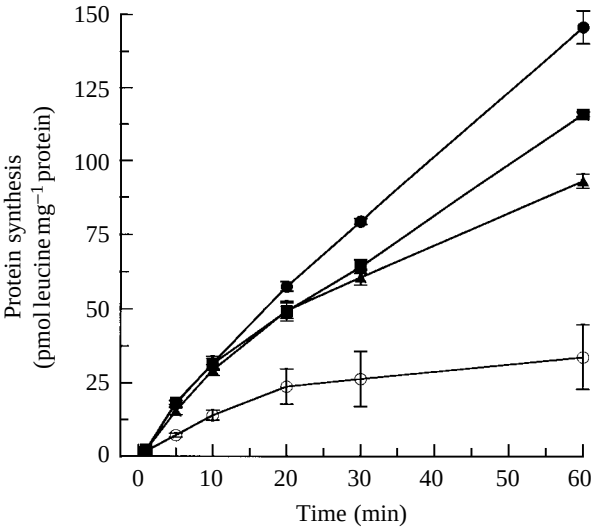

Fig. 9. Comparison of the effects of antimycin A, KCN and anoxia on protein synthesis in isolated mitochondria from *Artemia franciscana* embryos. Filled circles are normoxic controls, squares are assays containing 100 μmol l^{-1} antimycin A, triangles are assays containing 500 μmol l^{-1} KCN and open circles are anoxic assays. Data are means ± S.E.M. for at least three independent assays from multiple mitochondrial preparations at each time point (redrawn from Kwast and Hand, 1996*b*).

Conclusions

Our long-term research goals are to define the fundamental biological criteria that must be met to deploy a global response, such as quiescence, and to identify the underlying physiological mechanisms for satisfying these requirements. Understanding the mechanisms responsible for the coordinated arrest of gene expression and for reductions in macromolecular degradation during anoxia-induced quiescence will have applications for many biological systems where survival depends upon successful recovery from physical and chemical insults. From an ecological perspective, such information may help explain how environmental stimuli that promote entry into quiescence are linked to the requisite physiological responses.

Multiple signals are apparently operative in promoting the suite of biochemical and physiological adjustments that accompanies anaerobic quiescence in *Artemia franciscana* embryos. The recent evidence summarized above for mitochondria suggests that direct sensing of molecular oxygen (or oxygen by-products) may be another mechanism, in addition to acidosis, for down-regulation of biosynthesis in the mitochondrial compartment. Our preliminary results from mitochondrial preincubations with Co^{2+} and Ni^{2+} are consistent with the possible involvement of heme or hemoproteins in the oxygen-sensing mechanism (S. C. Hand and K. Kwast, unpublished observations). We are currently exploring the possibility that oxygen sensing may also have some relationship to biosynthetic depression in the cytosol. In contrast to the apparent role of pH_i in translational control and protein stability discussed above (Anchordoguy *et al.* 1993; Anchordoguy and Hand, 1994, 1995; Warner *et al.* 1997), less is known about the parallel processes of transcription and mRNA stability during quiescence. In terms of recovery from long-term anoxia,

insurmountable difficulties could arise if the entire pool of mRNA species had to be resynthesized *de novo* prior to resuming the energy flows characteristic of developing embryos.

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