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# REACTIVATION OF UBIQUITINATION IN *ARTEMIA FRANCISCANA* EMBRYOS DURING RECOVERY FROM ANOXIA-INDUCED QUIESCENCE

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## Summary

**Encysted gastrulae of *Artemia franciscana* are known to enter a reversible state of quiescence promoted by anoxia, during which the half-life of cytochrome oxidase is prolonged up to 77-fold. This observation suggests that proteolytic pathways within mitochondria are inhibited, and indeed the suppression of the initial step in ubiquitin-mediated proteolysis under anoxia has been reported. Given that active embryos require efficient degradation of macromolecules, we investigated the reactivation of ubiquitination during recovery from anoxia and aerobic acidosis (elevated $CO_2$ levels under aerobic conditions). During 6 h of recovery from anoxia, the levels of ubiquitin-conjugated proteins rose 6.5-fold, reaching 78 % of the pre-anoxia (control) values. Concomitant with the elevation in ubiquitin conjugates was a sharp decline in AMP level, a rise in ATP level and an alkalization of intracellular pH. Our results suggest that the reinitiation of ubiquitin conjugation is partially dependent on decreasing AMP and/or increasing ATP levels. However, when anoxic embryos were transferred to aerobic acidosis, which promotes a return to control (aerobic) levels of adenylates yet maintains the acidic intracellular pH, 71 % of the total suppression of ubiquitination still remained. This observation reveals a predominant role of intracellular alkalization in the reactivation of ubiquitination during recovery. We suggest that the rapid reversibility of the factors regulating ubiquitin conjugation allows *Artemia* embryos readily to reinitiate the degradation of proteins *via* the ubiquitin-mediated pathway during recovery.**

Key words: *Artemia franciscana*, ubiquitin, recovery, anoxia, brine shrimp, intracellular pH, ATP, AMP.

## Introduction

A wide variety of organisms are known to enter a reversible state of quiescence in response to harsh environmental conditions (Hochachka and Guppy, 1987; Hand, 1991). The quiescent state is characterized by a reduced metabolism that includes a suppression of protein turnover, i.e. downregulation of both synthesis and degradation (Clegg and Jackson, 1989; Hofmann and Hand, 1990; Anchordoguy *et al.* 1993). Although proteolysis must be curtailed in order to preserve the cellular machinery necessary for recovery, reactivated cells require efficient turnover of macromolecules. Both of these criteria can be met if arrested degradative pathways are maintained in a condition poised for the return to normal activity. We focused our investigation on the energy-dependent, ubiquitin-mediated proteolytic pathway. This pathway has been studied for almost 20 years and is present in a wide variety of organisms (Goldberg *et al.* 1976; Hershko *et al.* 1978; Rechsteiner, 1987; Hershko and Ciechanover, 1992). In the present report, we investigate factors controlling the reinitiation of ubiquitination, the rate-limiting step in ubiquitin-mediated proteolysis.

Embryos of the brine shrimp *Artemia franciscana* are classic examples of organisms that enter a reversible, quiescent state in response to a variety of environmental stimuli (Drinkwater and Clegg, 1991). In response to anoxia, this organism suppresses glycolysis (Dutrieu and Crestia-Blanchine, 1966; Ewing and Clegg, 1969; Carpenter and Hand, 1986) and protein turnover (Anchordoguy *et al.* 1993) and dramatically reduces dissipative heat flow (Hand and Gnaiger, 1988; Hand, 1990; Hontoria *et al.* 1993). The degree to which metabolism is suppressed in *A. franciscana* embryos under anoxia is considerably more severe than that described for most facultative anaerobes (Gnaiger, 1983*a*,*b*; Hand, 1993). ATP levels in these embryos, unlike those in most vertebrate species, fall precipitously upon exposure to anoxia (Carpenter and Hand, 1986; Rees *et al.* 1989; Anchordoguy and Hand, 1994). This net hydrolysis of ATP results in the production of protons, and intracellular pH is lowered from at least 7.9 to 6.8 within 90 min (Busa *et al.* 1982). The role of acidification in coordinating metabolic arrest has been supported by experiments in which embryos are acidified under fully aerobic

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conditions by exposure to high levels of $CO_2$ (60 % $CO_2$:40 % $O_2$). Embryos subjected to this treatment, termed aerobic acidosis, possess an intracellular pH of 6.8 and exhibit metabolic suppressions similar to those seen under anoxia (Busa and Crowe, 1983; Carpenter and Hand, 1986; Hand and Gnaiger, 1988; Hofmann and Hand, 1990). One critical difference between anoxia and aerobic acidosis is that ATP levels initially remain high under the latter condition whereas they plummet under anoxia (Carpenter and Hand, 1986; Anchordoguy and Hand, 1994).

Hofmann and Hand (1990) showed that the synthesis of cytochrome *c* oxidase (COX) is inhibited in *A. franciscana* embryos subjected to either anoxia or aerobic acidosis. The half-life of this critical mitochondrial enzyme is extended 77-fold under anoxia and sevenfold during aerobic acidosis (Anchordoguy *et al.* 1993). The complete mechanism by which enzyme half-life is extended has not been fully elucidated. Clegg *et al.* (1994) have recently reported the presence of a 26 kDa protein that may serve as a protective molecular chaperone in *A. franciscana* embryos during anoxia. The inhibition of proteolytic pathways also deserves study. Considering that much of protein degradation is energy-dependent (Rivett, 1989; Goldberg, 1990), reduced rates of proteolysis under conditions of low cellular ATP levels (e.g. anoxia) are not entirely unexpected and have been observed in other systems (Simpson, 1953; Schweiger *et al.* 1956; Goldberg *et al.* 1976). However, the extension of COX half-life in *A. franciscana* embryos exposed to aerobic acidosis (i.e. high ATP, low intracellular pH) indicates that acidification alone can depress rates of protein degradation.

In the light of the observations mentioned above, we began an investigation of the ubiquitin-mediated proteolytic system in *A. franciscana* embryos (Anchordoguy and Hand, 1994). This pathway utilizes the covalent attachment of ubiquitin to target proteins for proteolysis (Hershko and Ciechanover, 1992). In addition to being energy-dependent (Hershko *et al.* 1978; Ciechanover, 1987; Hershko and Ciechanover, 1992) and pH-sensitive (Muller *et al.* 1980), this degradative pathway is apparently active within mitochondria (Magnani *et al.* 1991) and is responsible for the turnover of short-lived metabolic proteins (Rechsteiner, 1987; Rivett, 1990). Evidence from several systems suggests that the ATP-dependent conjugation of ubiquitin to proteins determines the rate of proteolysis *via* this pathway (Hershko *et al.* 1978; Munro and Pelham, 1985). Under anoxia, ubiquitin conjugates are actually suppressed in *A. franciscana* embryos, and regulation of ubiquitin-mediated proteolysis seems to occur at or before this conjugation step (Anchordoguy and Hand, 1994).

In the present study, we investigate the reactivation of ubiquitination during recovery of *A. franciscana* embryos from exposure to both anoxia and aerobic acidosis. In particular, we focus on how the depletion of AMP, the regeneration of ATP and the corresponding alkalization during recovery from anoxia correlate with increases in ubiquitin-conjugated proteins. Our results suggest that these changes allow *A. franciscana* embryos readily to resume the targeting of proteins for ubiquitin-mediated degradation.

## Materials and methods

### *Preparation of samples*

Embryos of the brine shrimp *Artemia franciscana* (maxima grade) were obtained from Sanders Brine Shrimp Company (Ogden, UT, USA) and stored at −20 °C. Embryos were hydrated in tap water (0 °C) overnight and washed as previously described (Carpenter and Hand, 1986). 5 g of hydrated embryos was filtered and blotted before being transferred to flasks containing 100 ml of 0.25 mol $l^{-1}$ NaCl. For a given experiment, flasks were connected in series by gas-impermeable tubing (Viton) and bubbled vigorously with air for 4 h at 20 °C. After this aerobic incubation, embryos were subjected to either anoxia (100 % $N_2$) or aerobic acidosis (60 % $CO_2$:40 % $O_2$) for 24 h as previously described (Carpenter and Hand, 1986). In experiments where anoxic embryos were exposed sequentially to aerobic acidosis, flasks were bubbled with 100 % $N_2$ for 24 h, then switched to 60 % $CO_2$:40 % $N_2$ for 30 min to allow equilibration of $CO_2$, before finally being subjected to 60 % $CO_2$:40 % $O_2$. At each time point, a flask containing embryos was placed in an isopropanol/ice bath (−15 °C) to cool the sample rapidly (Anchordoguy and Hand, 1994). Embryos from one flask were divided and homogenized at 0 °C in either buffer A (for conjugated ubiquitin) or buffer B (for free ubiquitin) as described by Pickart *et al.* (1991). Buffer A consisted of 50 mmol $l^{-1}$ Tris–HCl, pH 8.3, 20 % glycerol, 2 % SDS and 0.4 mol $l^{-1}$ $\beta$-mercaptoethanol; buffer B contained 50 mmol $l^{-1}$ sodium MOPS, pH 8.3, 20 mmol $l^{-1}$ EDTA, 0.3 mmol $l^{-1}$ phenylmethylsulphonyl fluoride, 0.3 mmol $l^{-1}$ sodium *p*-tosyl-L-lysine chloromethyl ketone, 4 $\mu$g $ml^{-1}$ leupeptin, 2 % Triton X-100 and 5 mmol $l^{-1}$ *N*-ethylmaleimide. The homogenate was centrifuged at 10 000 ***g*** for 15 min and the supernatant was stored at −20 °C until quantification as described below.

### *Quantification of ubiquitin conjugates*

A modification of the solid phase assay of Haas and Bright (1985) was employed as previously described (Anchordoguy and Hand, 1994). This procedure (confetti blot) eliminates autoradiography and allows for the direct quantification of ubiquitin conjugates with a gamma counter. Briefly, a confetti blot utilized individual nitrocellulose disks (7 mm diameter) to immobilize protein from samples of embryo extracts. After being heat-treated (70 °C for 30 min), disks were transferred to blocking solution for 1 h (Anchordoguy and Hand, 1994). The disks were then incubated in a solution containing an antibody that specifically recognizes conjugated ubiquitin (Haas and Bright, 1985). After 1 h, the disks were extensively washed and incubated in $^{125}$I-labelled Protein A (Anchordoguy and Hand, 1994). Each disk was then thoroughly washed and counted individually in a gamma counter. Ubiquitin conjugate standards were synthesized

using reticulocyte fraction II as described by Haas and Bright (1985). A standard curve ranging from 0.01 to 0.1 pmol of conjugated ubiquitin was generated with each assay. Typically, standard errors for triplicate samples were less than 3 % of the mean.

### *Quantification of free ubiquitin*

The levels of free (unconjugated) ubiquitin in *A. franciscana* extracts were determined by the radioimmunoassay procedure described by Haas *et al.* (1985). Briefly, ubiquitin (Sigma Chemical Co., St Louis, MO, USA) was radiolabelled with $^{125}$I using Iodogen (Pierce, Rockford, IL, USA). Iodinated ubiquitin and antibodies specific for free ubiquitin were diluted in a 45 % saturated ammonium sulphate fraction of human (TJA) plasma containing 2.5 mmol $l^{-1}$ dithiothreitol for use in the radioimmunoassay. A standard curve was prepared by diluting free ubiquitin in buffer B containing 1 mg $ml^{-1}$ bovine serum albumin to achieve concentrations of 1–50 pmol of ubiquitin per 50 $\mu$l. After an overnight incubation (4 ˚C), antibody-bound $^{125}$I-labelled ubiquitin was precipitated with polyethylene glycol (20 000 $M_r$), centrifuged at 7000 ***g*** for 20 min, and quantified with a gamma counter (Anchordoguy and Hand, 1994).

### *Quantification of adenylates*

Experiments identical to those described above were conducted in order to determine adenylate levels. After being cooled below 0 ˚C in the isopropanol/ice bath, each flask was kept on ice until being further processed in a controlled-temperature room (5 ˚C). Embryos were blotted, and triplicate samples were immediately frozen and pulverized in liquid nitrogen with a mortar and pestle (Rees and Hand, 1991). Samples of powdered embryos were then homogenized in 6 % perchloric acid and centrifuged at 10 000 ***g*** for 10 min. The supernatant was neutralized with 5 mol $l^{-1}$ $K_2CO_3$ and centrifuged to remove perchlorate salts. The samples were stored at −70 ˚C until quantification by HPLC as previously described (Rees and Hand, 1991). The pellet from the initial centrifugation was resuspended in 0.5 mol $l^{-1}$ NaOH and analyzed for protein using a modified Lowry protein assay (Peterson, 1977).

## Results

### *Recovery from anoxia*

After 24 h of anoxia, the levels of conjugated ubiquitin decreased to 12 % of control (aerobic) values (Fig. 1), consistent with our previous report (Anchordoguy and Hand, 1994). Upon the reintroduction of aerobic conditions, the levels of ubiquitin conjugates increased rapidly to 53 % of control values within 30 min, and reached 78 % after 6 h (Fig. 1). These changes in ubiquitin conjugation were strongly correlated with alterations in the adenylate pool. ATP rose from 15 % after 24 h of anoxia to 97 % of control values within 30 min (Fig. 1), consistent with the earlier report by Stocco *et al.* (1972). Conversely, a decrease in AMP levels was coincident with the initial high rate of ATP regeneration (Table 1).

In response to the increase in conjugated ubiquitin, free ubiquitin levels decreased dramatically during recovery from anoxia (Fig. 2). The amount of free ubiquitin declined by 28 % within 30 min, before dropping below control values by 3 h. These changes are sufficient to account for the incorporation of free ubiquitin into conjugates. As noted previously (Anchordoguy and Hand, 1994), direct comparison of free and conjugated ubiquitin levels is complicated by the markedly

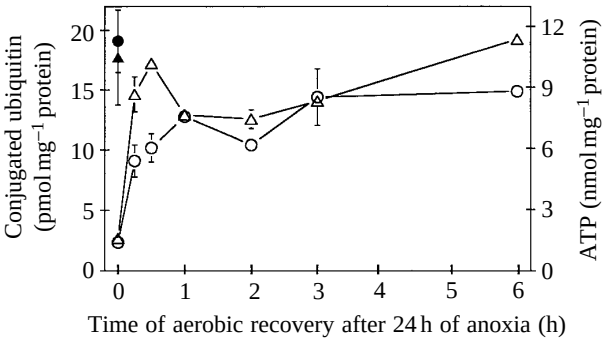


Fig. 1. Changes in levels of conjugated ubiquitin (○) and ATP (△) during aerobic recovery after 24 h of anoxia. Control (aerobic) levels of ubiquitin conjugates (●) and ATP (▲) are shown. Each point represents a mean ± S.E.M.; *N*=3. Where error bars are absent, they are smaller than the size of the symbol.

Table 1. *Response of AMP levels in* Artemia franciscana *embryos to anoxia, aerobic acidosis and various recovery periods*

| | Condition of embryos | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Recovery time (h) | | | | | |
| Treatment/recovery | Aerobic (4 h) | Anoxic (24 h) | Aerobic acidosis (24 h) | 0.25 | 0.5 | 1 | 2 | 3 | 6 |
| Anoxia/aerobic recovery | 0.26±0.08* | 7.32±0.46 | – | 1.32±0.13 | 2.66±0.07 | 3.71±0.17 | 2.90±0.14 | | |
| Anoxia/aerobic acidosis recovery | 0.38±0.01 | 7.50±0.19 | – | 0.92±0.04 | 0.37±0.06 | 0.26±0.05 | 0.25±0.06 | 0.23±0.06 | 0.29±0.02 |
| Aerobic acidosis/aerobic recovery | 0.20±0.06 | – | 2.60±0.41 | 0.23±0.09 | 0.24±0.05 | 1.19±0.02 | 1.13±0.07 | 0.52±0.10 | 0.26±0.01 |

*Values are expressed in nmol $mg^{-1}$ protein and represent means ± S.E.M. (*N*=3).

Table 2. *Response of conjugated ubiquitin levels in* Artemia franciscana *embryos to anoxia, aerobic acidosis and various recovery periods*

| | Condition of embryos | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Time of aerobic recovery (h) | | | | | |
| Treatment/recovery | Aerobic (4 h) | Anoxic (24 h) | Aerobic acidosis (24 h) | 0.25 | 0.5 | 1 | 2 | 3 | 6 |
| Anoxia/aerobic recovery | 33.8* | 0.8 | – | 7.7 | 11.5 | 12.4 | 12.3 | 26.5 | 29.2 |
| Aerobic acidosis/ aerobic recovery | 33.8 | – | 4.9 | 9.1 | 11.4 | 14.1 | 28.5 | 31.1 | 32.7 |
| | | | | Aerobic acidosis recovery time (h) | | | Aerobic recovery time (h) | | |
| | | | | 1 | 3 | 6 | 1 | 3 | 6 |
| Anoxia/aerobic acidosis recovery/aerobic recovery | 24.5 | 3.6 | – | 4.6 | 7.5 | 10.1 | 18.8 | 24.5 | 35.1 |

*Values are expressed as percentage of conjugated ubiquitin, calculated per gram embryo.

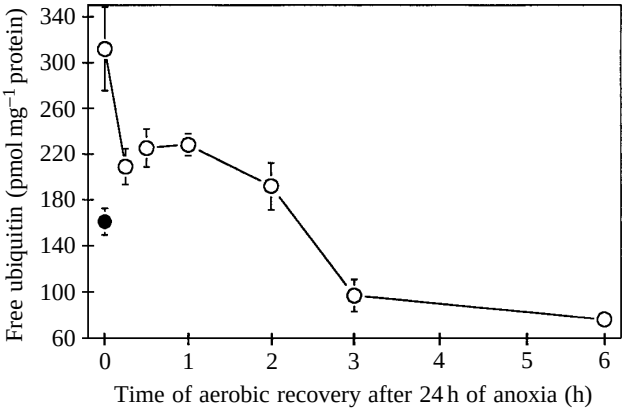


Fig. 2. Changes in levels of free ubiquitin (○) during aerobic recovery after 24 h of anoxia. Control (aerobic) levels of free ubiquitin (●) are shown. Each point represents mean ± S.E.M.; *N*=3. Where error bars are absent, the error is smaller than the size of the symbol.

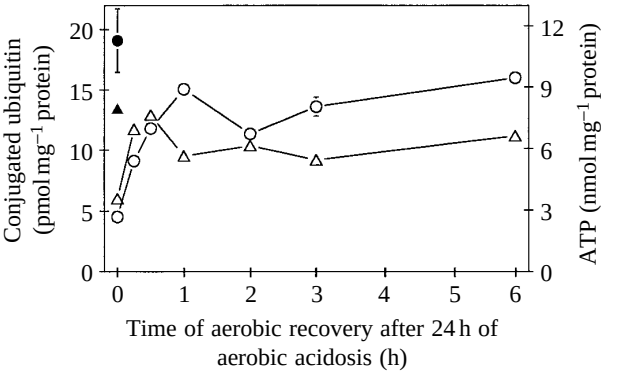


Fig. 3. Changes in levels of conjugated ubiquitin (○) and ATP (△) during aerobic recovery after 24 h of aerobic acidosis. Control (aerobic) levels of ubiquitin conjugates (●) and ATP (▲) are shown. Each point represents mean ± S.E.M.; *N*=3. Where error bars are absent, the error is smaller than the size of the symbol.

different buffers required to prepare samples for quantification. Assuming equal extraction efficiency of the two buffers, comparison of free and conjugated ubiquitin levels can be approximated per gram embryo (Pickart *et al.* 1991; Anchordoguy and Hand, 1994). As seen in Table 2, the proportion of total ubiquitin in the conjugated form declined from 33.8 % in aerobic embryos to 0.8 % after 24 h of anoxia. The proportion of conjugated ubiquitin increased rapidly during the initial 15 min of recovery and gradually approached control levels over 6 h (Table 2).

### *Recovery from aerobic acidosis*

The levels of conjugated ubiquitin in embryos subjected to 24 h of aerobic acidosis decreased to 24 % of control (aerobic) values (Fig. 3). During recovery, conjugate levels rose rapidly to 62 % of control levels within 30 min (Fig. 3). After 6 h, conjugated ubiquitin levels were 84 % of control values, similar to that seen during recovery from anoxia (Fig. 1). ATP levels during recovery from aerobic acidosis correlated strongly with changes in levels of ubiquitin conjugates, rising to control values within 30 min (Fig. 3). As seen during recovery from anoxia, AMP levels reflected changes in ATP (Table 1). However, the accumulation of AMP during aerobic acidosis was much less than under anoxia. Free ubiquitin levels decreased with increases in conjugated ubiquitin over the course of recovery (data not shown). This trend is reflected in the proportions of ubiquitin conjugated to protein, which decreased to 4.9 % after 24 h of aerobic acidosis and increased steadily over 6 h of recovery (Table 2).

### *Exposure of anoxic embryos to aerobic acidosis*

To separate the effects of changes in adenylates from those of alkalization during recovery, embryos exposed to 24 h of anoxia were allowed to ‘recover’ under conditions where intracellular acidification was maintained. As seen in Fig. 4, ATP levels were very low after 24 h of anoxia. After exposure to aerobic acidosis, ATP levels increased substantially and reached control values within 30 min, despite maintenance of intracellular acidification. ATP levels remained constant and comparable to control values throughout the 6 h of aerobic

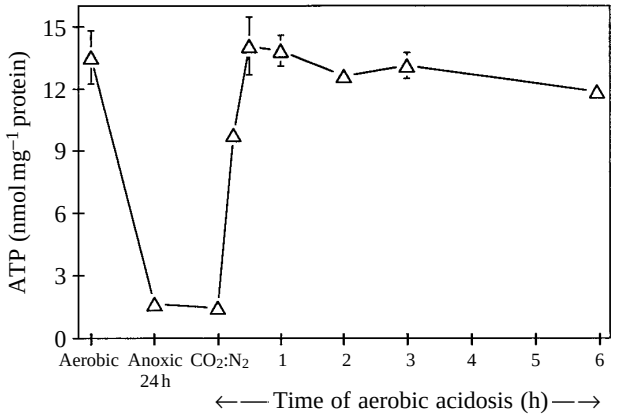


Fig. 4. Changes in ATP levels during sequential exposure of aerobic embryos to 24 h anoxia and 6 h aerobic acidosis. Each point represents mean ± S.E.M.; *N*=3. Where error bars are absent, they are smaller than the size of the symbol.

acidosis (Fig. 4). It is appropriate to note, however, that the ATP level slowly declines if this treatment is continued for a period of days (57.8±1.1 % and 26.7±0.6 % of control after 1 and 3 days, respectively; mean ± S.E.M., *N*=3). Levels of AMP dropped in response to ATP regeneration, and were maintained at low levels under aerobic acidosis (Table 1). Interestingly, levels of ubiquitin conjugates rose from 19 % after 24 h of anoxia to only 43 % of control values after 6 h of aerobic acidosis (Fig. 5). Therefore, maintenance of intracellular acidosis during reoxygenation precluded full reactivation of ubiquitination.

### *Aerobic recovery from sequential exposure to anoxia and aerobic acidosis*

After exposure to anoxia and aerobic acidosis, ATP levels increased slightly during the subsequent exposure to aerobic conditions (Fig. 5). Conjugated ubiquitin levels increased to 83 % of control values within 1 h and reached control values

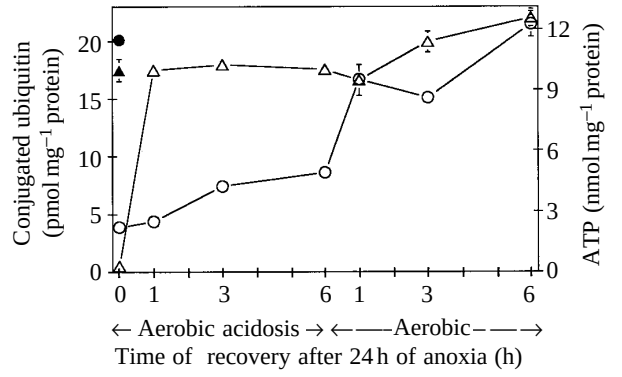


Fig. 5. Changes in levels of conjugated ubiquitin (○) and ATP (△) during sequential exposure of aerobic embryos to 24 h of anoxia, 6 h of aerobic acidosis and 6 h of aerobic recovery. Control (aerobic) levels of ubiquitin conjugates (●) and ATP (▲) are shown. Each point represents mean ± S.E.M.; *N*=3. Where error bars are absent, they are smaller than the size of the symbol.

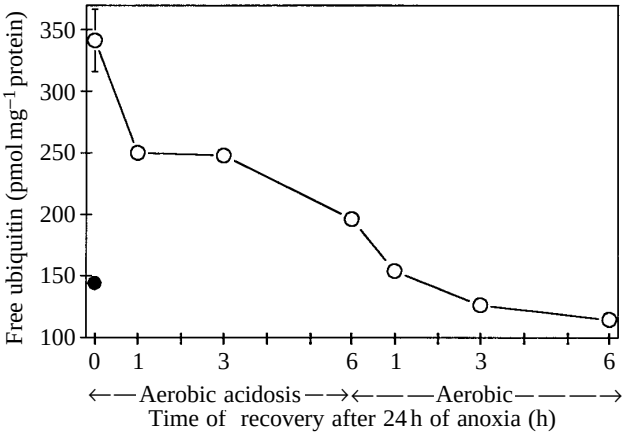


Fig. 6. Changes in levels of free ubiquitin (○) during sequential exposure of aerobic embryos to 24 h of anoxia, 6 h of aerobic acidosis and 6 h of aerobic recovery. Control (aerobic) levels of free ubiquitin (●) are shown. Each point represents mean ± S.E.M.; *N*=3. Where error bars are absent, they are smaller than the size of the symbol.

within 6 h (Fig. 5). The increases in ubiquitin conjugates corresponded to decreases in free ubiquitin levels, which dropped steadily over 6 h of aerobic acidosis and reached control values after aerobic exposure (Fig. 6). These changes in free and conjugated ubiquitin levels are reflected in the proportions of total ubiquitin bound to protein, which increased gradually over a 6 h exposure to aerobic acidosis and then more rapidly under aerobic conditions (Table 2). Therefore, when taken together, these data strongly support a role for realkalization, separate from any additional influence of adenylates, in the return of ubiquitin conjugates to control levels.

## Discussion

Reactivating the cellular pathways for protein degradation would seem necessary after a period of quiescence, yet few studies have focused on this aspect of recovery in eukaryotic organisms. Goldberg *et al.* (1976) employed mutants and various metabolic inhibitors to investigate proteolysis in *Escherichia coli*. By removing glucose from the medium, these authors demonstrated that ATP-depleted bacteria were unable to break down protein. Proteolysis was immediately restored when cells were allowed to regenerate ATP either by glycolysis or by oxidative phosphorylation (Goldberg *et al.* 1976). Furthermore, the inhibition of proteolysis persisted under conditions permitting aerobic respiration in the absence of phosphorylation.

In our previous work with *A. franciscana* embryos, we have observed a strong correlation between ATP levels and the inhibition of the initial step of ubiquitin-mediated proteolysis (Anchordoguy and Hand, 1994). Although the levels of conjugated ubiquitin declined rapidly upon exposure to either anoxia or aerobic acidosis, suppression was greater under the former condition. The present work describes the release of this inhibition upon the return to aerobic conditions. The

reactivation of ubiquitination after 24 h of either anoxia or aerobic acidosis is rapid and correlates with rising ATP levels (Figs 1, 3). However, elevated ATP levels *per se* may not be the proximal factor responsible for reactivation of ubiquitination. It is important to note that AMP levels decrease inversely with increasing ATP (Table 1).

In an *in vitro* study comparing proteolysis in the presence and absence of an ATP-regenerating system in reticulocytes, Hershko *et al.* (1978) observed that reticulocyte extracts incapable of regenerating ATP displayed markedly suppressed rates of proteolysis despite levels of ATP known to stimulate this process. This finding led the authors to propose that a degradation product of ATP might be responsible for this inhibition. While no effect of ADP accumulation was reported, Hershko *et al.* (1978) demonstrated a progressive inhibition of proteolysis in the presence of increasing AMP concentrations. A mechanistic basis for the inhibitory effect of AMP on ubiquitination was later provided by Haas and Rose (1982). In addition, these authors concluded that changes in pyrophosphate and ATP levels may modulate the extent to which AMP inhibition is realized *in vivo* (Haas and Rose, 1982).

Currently, sufficient data are not available to assess the role of pyrophosphate or to separate increasing ATP from decreasing AMP levels in recovering *A. franciscana* embryos. However, a closer analysis of the data from Hershko *et al.* (1978) allows us to calculate an apparent inhibition constant $K_i$ for AMP of approximately 0.15 mmol $l^{-1}$. This value is probably an underestimate considering that the authors also reported ATP hydrolysis during their incubations, which probably generated additional (unmeasured) AMP. As a result, the actual value for $K_i$ could be 2–3 times higher (0.3–0.45 mmol $l^{-1}$). Regardless of the exact value, it is important to note that AMP levels in *A. franciscana* embryos increase from 0.09 to 1.16 mmol $l^{-1}$ after 4 h of anoxia (Rees *et al.* 1989), and therefore decreasing AMP levels during recovery could contribute significantly to the observed reactivation of ubiquitination (Table 1). Furthermore, a regulatory role for AMP levels is consistent with the low concentration required for half-maximal rate (Haas and Rose, 1982), which suggests that declining cellular ATP can only limit ubiquitination under the most severe conditions.

In addition to the dependence of ubiquitin-mediated proteolysis on adenylates, the pathway is also known from *in vitro* work to be acutely pH-sensitive (Muller *et al.* 1980). Since alterations in adenylate levels normally affect intracellular pH in *A. franciscana* embryos, we separated these factors by exposing anoxic embryos to aerobic acidosis (Figs 4, 5). While ATP rapidly rose to control levels, levels of ubiquitin conjugates increased only slightly, revealing the substantial role of intracellular acidification in suppressing ubiquitination. Quantitatively, these data indicate that approximately 71 % of the total suppression of ubiquitination under anoxia can be attributed to pH sensitivity. This percentage is in good agreement with the reduction of ubiquitin conjugate levels during the initial hours of aerobic acidosis, despite unaltered ATP levels (74 %, Anchordoguy and Hand, 1994).

Finally, a brief discussion is warranted concerning the increase in ATP to control levels under aerobic acidosis, where carbohydrate metabolism is known to be severely restricted, i.e. pH 6.8 (Figs 4, 5). This finding suggests that oxidative phosphorylation itself is not severely suppressed by low pHi and that sufficient carbon substrate of some type (e.g. amino acids or other organic acids) is available to mitochondria. Since anabolic, energy-consuming pathways are arrested during aerobic acidosis (Hofmann and Hand, 1990), even limited energy production by the mitochondrion under these conditions could restore the anoxia-depleted ATP levels to aerobic values.

In the present study, we have quantified the regeneration of ATP, the depletion of AMP and the reactivation of ubiquitination during recovery of *A. franciscana* embryos from anoxia and aerobic acidosis. Our results demonstrate that the changes in adenylate levels and levels of ubiquitin conjugates during quiescence are rapidly reversible. While the full reactivation of ubiquitination during recovery is dependent upon the regeneration of ATP and/or AMP depletion, intracellular alkalization (either directly or indirectly) appears to be the predominant factor regulating this process *in vivo*. Considering the key role of ubiquitin-mediated proteolysis in the turnover of metabolic proteins, it seems likely that this pathway is critical for normal development in *A. franciscana* embryos. Although proteolysis was not directly measured in this study, we suggest that rapid reactivation of ubiquitin conjugation allows the embryo to reinitiate degradation *via* the ubiquitin-mediated pathway.

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## References

ANCHORDOGUY, T. J. AND HAND, S. C. (1994). Acute blockage of the ubiquitin-mediated proteolytic pathway during invertebrate quiescence. *Am. J. Physiol.* **267**, R895–R900.

ANCHORDOGUY, T. J., HOFMANN, G. E. AND HAND, S. C. (1993). Extension of enzyme half-life during quiescence in *Artemia* embryos. *Am. J. Physiol.* **264**, R85–R89.

BUSA, W. B. AND CROWE, J. H. (1983). Intracellular pH regulates transitions between dormancy and development of brine shrimp (*Artemia salina*) embryos. *Science* **221**, 366–368.

BUSA, W. B., CROWE, J. H. AND MATSON, G. B. (1982). Intracellular pH and the metabolic status of dormant and developing *Artemia* embryos. *Archs Biochem. Biophys.* **216**, 711–718.

CARPENTER, J. F. AND HAND, S. C. (1986). Arrestment of carbohydrate metabolism during anaerobic dormancy and aerobic acidosis in *Artemia* embryos: determination of pH-sensitive control points. *J. comp. Physiol.* B **156**, B451–B459.

CIECHANOVER, A. (1987). Mechanisms of energy-dependent intracellular protein breakdown: The ubiquitin-mediated proteolytic pathway. In *Lysosomes: Their Role in Protein Breakdown* (ed. H. Glaumann and F. J. Ballard), pp. 561–602. New York: Academic Press.

CLEGG, J. S. AND JACKSON, S. A. (1989). Aspects of anaerobic metabolism of *Artemia* cysts. In *The Cellular and Molecular Biology of* Artemia *Development* (ed. A. H. Warner, J. Bagshaw and T. MacRae), pp. 1–15. New York: Plenum Press.

CLEGG, J. S., JACKSON, S. A. AND WARNER, A. H. (1994). Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*. *Expl Cell Res.* **212**, 77–83.

DRINKWATER, L. E. AND CLEGG, J. S. (1991). Experimental biology of cyst diapause. In Artemia *Biology* (ed. R. A. Browne, P. Sorgeloos and C. N. A. Trotman), pp. 93–117. Boca Raton, FL:CRC Press.

DUTRIEU, J. AND CRESTIA-BLANCHINE, D. (1966). Resistance des oeufs durables hedrates d'*Artemia salina* à l'anoxie. *C. R. hebd. Séanc. Acad. Sci., Paris* D **263**, 998–1000.

EWING, R. D. AND CLEGG, J. S. (1969). Lactate dehydrogenase activity and anaerobic metabolism during embryonic development in *Artemia salina*. *Comp. Biochem. Physiol.* **31**, 297–307.

GNAIGER, E. (1983*a*). Microcalorimetric monitoring of biological activities. Ecological and toxicological studies in aquatic animals. *Science Tools* **30**, 21–26.

GNAIGER, E. (1983*b*). Heat dissipation and energetic efficiency in animal anoxibiosis: Economy contra power. *J. exp. Zool.* **228**, 471–490.

GOLDBERG, A. L. (1990). ATP-dependent proteases in prokaryotic and eukaryotic cells. *Sem. Cell Biol.* **1**, 423–432.

GOLDBERG, A. L., KOWIT, J. D. AND ETLINGER, J. D. (1976). Studies of the selectivity and mechanisms of intracellular protein degradation. In *Proteolysis and Physiological Regulation* (ed. D. W. Ribbons and K. Brew), pp. 313–337. New York, San Francisco, London: Academic Press.

HAAS, A. L. AND BRIGHT, P. M. (1985). The immunochemical detection and quantitation of intracellular ubiquitin-protein conjugates. *J. biol. Chem.* **260**, 12464–12473.

HAAS, A. L., MURPHY, K. E. AND BRIGHT, P. M. (1985). The inactivation of ubiquitin accounts for the inability to demonstrate ATP, ubiquitin-dependent proteolysis in liver extracts. *J. biol. Chem.* **260**, 4694–4703.

HAAS, A. L. AND ROSE, I. A. (1982). The mechanism of ubiquitin activating enzyme. *J. biol. Chem.* **257**, 10329–10337.

HAND, S. C. (1990). Heat dissipation during long-term anoxia in *Artemia franciscana* embryos: identification and fate of metabolic fuels. *J. comp. Physiol.* B **160**, 357–363.

HAND, S. C. (1991). Metabolic dormancy in aquatic invertebrates. In *Advances in Comparative and Environmental Physiology*, vol. 8 (ed. R. Gilles), pp. 1–50. Berlin: Springer-Verlag.

HAND, S. C. (1993). $pH_i$ and anabolic arrest during anoxia in *Artemia franciscana* embryos. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, T. Sick, M. Rosenthal and G. van den Thillart), pp. 171–185. Boca Raton, FL: CRC Press.

HAND, S. C. AND GNAIGER, E. (1988). Anaerobic dormancy quantified in *Artemia* embryos: a calorimetric test of the control mechanism. *Science* **239**, 1425–1427.

HERSHKO, A. AND CIECHANOVER, A. (1992). The ubiquitin system for protein degradation. *A. Rev. Biochem.* **61**, 761–807.

HERSHKO, A., HELLER, H., GANOTH, D. AND CIECHANOVER, A. (1978). Mode of degradation of abnormal globin chains in rabbit reticulocytes. In *Protein Turnover and Lysosome Function* (ed. H. L. Segal and D. J. Doyle), pp. 149–169. New York: Academic Press.

HOCHACHKA, P. W. AND GUPPY, M. (1987). *Metabolic Arrest and the Control of Biological Time*, 227pp. Cambridge, MA: Harvard University Press.

HOFMANN, G. E. AND HAND, S. C. (1990). Arrest of cytochrome-c oxidase synthesis coordinated with catabolic arrest in dormant *Artemia* embryos. *Am. J. Physiol.* **258**, R1184–R1191.

HONTORIA, F., CROWE, J. H., CROWE, L. M. AND AMAT, F. (1993). Metabolic heat production by *Artemia* embryos under anoxic conditions. *J. exp. Biol.* **178**, 149–159.

MAGNANI, M., SERAFINI, G., AMTONELLI, A., MALATESTA, M. AND GAZZANELLI, G. (1991). Evidence for a particulate location of ubiquitin conjugates and ubiquitin-conjugating enzymes in rabbit brain. *J. biol. Chem.* **266**, 21018–21024.

MULLER, M., DUBIEL, W., RATHMANN, J. AND RAPOPORT, S. (1980). Determination and characterization of energy-dependent proteolysis in rabbit reticulocytes. *Eur. J. Biochem.* **109**, 405–410.

MUNRO, S. AND PELHAM, H. (1985). What turns on heat shock genes? *Nature* **317**, 477–478.

PETERSON, G. L. (1977). A simplification of the protein assay method of Lowry *et al.* which is generally more applicable. *Analyt. Biochem.* **83**, 346–356.

PICKART, C. M., SUMMERS, R. G., SHIM, H. AND KASPEREK, E. M. (1991). Dynamics of ubiquitin pools in developing sea urchin embryos. *Dev. Growth Differ.* **33**, 587–598.

RECHSTEINER, M. (1987). Ubiquitin-mediated pathways for intracellular proteolysis. *A. Rev. Cell Biol.* **3**, 1–30.

REES, B. B. AND HAND, S. C. (1991). Regulation of glycolysis in the land snail *Oreohelix* during estivation and artificial hypercapnia. *J. comp. Physiol.* B **161**, 237–246.

REES, B. B., ROPSON, I. J. AND HAND, S. C. (1989). Kinetic properties of hexokinase under near-physiological conditions. Relation to metabolic arrest in *Artemia* embryos during anoxia. *J. biol. Chem.* **264**, 15410–15417.

RIVETT, A. J. (1989). High molecular mass intracellular proteases. *Biochem. J.* **263**, 625–633.

RIVETT, A. J. (1990). Eukaryotic protein degradation. *Curr. Opin. Cell Biol.* **2**, 1143–1149.

SCHWEIGER, H. G., RAPOPORT, S. AND SCHOLZEL, E. (1956). Role of non-protein nitrogen in the synthesis of haemoglobin in the reticulocyte *in vitro*. *Nature* **178**, 141–142.

SIMPSON, M. V. (1953). The release of labeled amino acids from the proteins of rat liver slices. *J. biol. Chem.* **201**, 143–154.

STOCCO, D. M., BEERS, P. C. AND WARNER, A. H. (1972). Effect of anoxia on nucleotide metabolism in encysted embryos of the brine shrimp. *Devl Biol.* **27**, 479–493.