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Characterization of ATP-dependent proteolysis in embryos of the brine shrimp, *Artemia franciscana*

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Abstract Under anoxia, embryos of *Artemia franciscana* enter a state of quiescence. During this time protein synthesis is depressed, and continued degradation of proteins could jeopardize the ability to recover from quiescence upon return to favorable conditions. In this study, we developed an assay for monitoring ATP/ubiquitin-dependent proteolysis in order to establish the presence of this degradation mechanism in *A. franciscana* embryos, and to describe some characteristics that may regulate its function during anoxia-induced quiescence. For lysates experimentally depleted of adenylates, supplementation with ATP and ubiquitin stimulated protein degradation rates by 92 ± 17% (mean ± SE) compared to control rates. The stimulation by ATP was maximal at concentrations ≥11 $\mu mol \cdot l^{-1}$. In the presence of ATP and ubiquitin, ubiquitin-conjugated proteins were produced by lysates during the course of the 4-h assays, as detected by Western blotting. Acute acidification of lysates to values approximating the intracellular pH observed under anoxia completely inhibited ATP/ubiquitin-dependent proteolysis. Depressed degradation was also observed under conditions where net ATP hydrolysis occurred. These results suggest that ATP/ubiquitin-dependent proteolysis is markedly inhibited under cellular conditions promoted by anoxia. Inhibition of proteolysis during quiescence may be one critical factor that increases macromolecular stability, which may ultimately govern the duration of embryo survival under anoxia.

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Key words Anoxia · Metabolic depression · pH · Protein degradation · Ubiquitin

Abbreviations *AP* alkaline phosphatase · *HPLC* high-performance liquid chromatography · *PCA* perchloric acid · *PKA* cAMP-dependent protein kinase · *PKC* Ca^{2+}-dependent protein kinase

Introduction

When exposed to unfavorable environmental conditions such as anoxia, embryos of the brine shrimp, *Artemia franciscana*, enter a reversible state of quiescence that can last at least 4 years (Clegg 1997). Both anabolic and catabolic processes are arrested, which results in metabolic rates that are extremely low compared to normoxic values (Hand and Gnaiger 1988; Hand 1990, 1995; Hontoria et al. 1993). After 50–60 h of anoxic exposure, heat dissipation is approximately 0.2% of aerobic values and is still declining (Hand 1995). Two hallmarks of anoxia-induced quiescence in these embryos are (1) a decline in pH_i from pH 7.7–7.9 in aerobic cysts to as low as pH 6.3 (Busa et al. 1982; Kwast et al. 1995; Clegg et al. 1995), and (2) a dramatic decrease in intracellular ATP coupled with a proportional increase in AMP (Stocco et al. 1972; Carpenter and Hand 1986; Rees et al. 1989; Anchordoguy and Hand 1994). Because protein synthesis is severely depressed under anoxia (Clegg and Jackson 1989; Hofmann and Hand 1990, 1994; Kwast and Hand 1993, 1996), a continued degradation of proteins could conceivably deplete cellular stores of key regulatory enzymes. This depletion could jeopardize the embryo's ability to recover from its quiescent state upon return to oxygenated conditions. Thus, one might predict that protein degradation would be inhibited in the absence of oxygen.

While there are multiple proteolytic mechanisms available to most cells, it has been suggested that ubiquitin-mediated proteolysis (for review see Ciechanover and Schwartz 1994; Goldberg 1995; Mykles 1998) is

responsible for degrading virtually all regulatory proteins (Ciechanover et al. 1984; Rock et al. 1994). A basic feature of the ubiquitin-dependent proteolytic system is that proteins are tagged for degradation by covalently coupling them to ubiquitin. A large 26S proteasome then recognizes these ubiquitin-conjugated proteins and degrades them (reviewed in Goldberg et al. 1997). Since ubiquitin-dependent proteolysis is ATP dependent and is involved in the turnover of a vast array of proteins, it follows that inhibition of this pathway could protect against protein depletion during quiescence. Indeed, measurement of ubiquitin-conjugated proteins demonstrated a sharp decline in level during entrance into quiescence (Anchordoguy and Hand 1994, 1995). Conjugate concentrations were only 7% of normoxic values following 24 h of anoxia. This result suggests that the conditions imposed by anoxia inhibit ubiquitin conjugation and likely proteolysis. However, the presence or absence of a decline in ubiquitin-dependent protein degradation per se has not been demonstrated. The goal of the following study was to establish the presence of an active ATP- and ubiquitin-dependent proteolytic system in lysates of *A. franciscana* embryos and to determine what factors might regulate its function during quiescence.

Materials and methods

Lysate preparation and protein-degradation assay

Brine shrimp (*A. franciscana)* embryos from the Great Salt Lake population were purchased from Sander's Brine Shrimp (Ogden, Utah). Hatching percentage for these embryos is approximately 80% (Anchordoguy et al. 1993). Embryos were hydrated and washed in a solution consisting of 0.25 $mol \cdot l^{-1}$ NaCl and 25 $mmol \cdot l^{-1}$ TRIS-HCl buffer (pH 8.0). Since encysted embryos are impermeable to amino acids, endogenous proteins were radiolabeled heterotrophically with [^{14}C]$NaHCO_3$ (3.86 μCi/ml medium) essentially as described by Hofmann and Hand (1990). After 8 h of normoxic incubation in the presence of tracer, embryos were rinsed in 0.25 $mol \cdot l^{-1}$ NaCl and homogenized in three volumes of an ice-cold solution consisting of 10 $mmol \cdot l^{-1}$ TRIS-HCl, 5 $mmol \cdot l^{-1}$ $MgCl_2$, and 10 $mmol \cdot l^{-1}$ KCl (pH 8.0) and centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was depleted of ATP by incubation for 30 min at 25 °C with 0.2 $mmol \cdot l^{-1}$ dinitrophenol and 20 $mmol \cdot l^{-1}$ 2-deoxyglucose, and passage across two sequential Sephadex G-25 desalting columns (Helmerhost and Stokes 1980). The pH was adjusted by adding 1 $mol \cdot l^{-1}$ TRIS-maleate buffer containing 5 $mmol \cdot l^{-1}$ $MgCl_2$, and 10 $mmol \cdot l^{-1}$ KCl (typically pH 7.8, except for the pH-profile experiments) to a final assay concentration of 100 $mmol \cdot l^{-1}$ TRIS-maleate; pH was verified in the reaction mixtures at the time of the proteolysis assay. Samples were kept on ice until assays were performed.

Immediately prior to the proteolysis assay, control reaction mixtures were supplemented with 1 $mmol \cdot l^{-1}$ hemin (proteasome inhibitor, Etlinger and Goldberg 1980) and 100 $\mu mol \cdot l^{-1}$ cyclohexamide (protein synthesis inhibitor). The ATP/ubiquitin-supplemented reaction mixtures contained 50 $\mu mol \cdot l^{-1}$ lanthanum nitrate, 45 $mmol \cdot l^{-1}$ creatine phosphate, 50 $\mu g \cdot ml^{-1}$ creatine phosphate kinase (i.e., ATP regenerating system, as per Tamura et al. 1991; Müller et al. 1980), 100 $\mu mol \cdot l^{-1}$ cyclohexamide, 100 $\mu mol \cdot l^{-1}$ pyrophosphate, 100 $\mu g \cdot ml^{-1}$ ubiquitin from bovine erythrocytes, and various concentrations of ATP (1 $mmol \cdot l^{-1}$ unless otherwise stated). The assay was initiated by raising the temperature to 25 °C. Proteolysis was estimated by following the release of radiolabeled amino acids into perchloric acid (PCA) supernatants. The 26S proteasome, which is responsible for degrading ubiquitin-conjugated proteins, produces small polypeptides (3–20 amino acid residues). Oligopeptidases and exopeptidases may hydrolyze the oligopeptides further to free amino acids (reviewed by Goldberg et al. 1997). Acid precipitation of proteins followed by analysis of the supernatant fraction is well established for measuring ubiquitin-dependent proteolysis (e.g., Breslow et al. 1986; Jahngen et al. 1986; Mathews et al. 1989; Müller et al. 1980). At the appropriate time points, triplicate samples were added to ice-cold PCA to stop the reaction (final concentration of 7% PCA). Samples were centrifuged at 10,000 *g* for 10 min (4 °C). Supernatants were neutralized with 5 $mol \cdot l^{-1}$ K_2CO_3 and centrifuged again at 10,000 *g* for 10 min to remove perchlorate salts. The supernatant was collected, dried overnight at 85 °C to remove any residual [^{14}C]HCO_3^-, and reconstituted with water. Radioactivity was measured by liquid scintillation counting in ScintiVerse II cocktail (Fisher Scientific, Denver, Colo.).

Measurements of adenylates

To verify that the ATP-regenerating system adequately stabilized adenylate concentrations throughout the course of the protein degradation assays, adenylates were quantified in neutralized PCA supernatants by high-performance liquid chromatography (HPLC) or by enzyme-coupled assays. HPLC analyses were performed essentially as described by Rees and Hand (1991) with a 5-μm Spherex-NH_2 aminopropyl column, except that potassium phosphate buffer was usually used instead of ammonium bicarbonate in the mobile phase. When ATP concentration was measured enzymatically, 30 μl of neutralized PCA supernatants were added to a reaction mixture (1.0 ml final volume) containing: 5.6 units of hexokinase, 2.4 units of glucose-6-phosphate dehydrogenase, 1 $mmol \cdot l^{-1}$ $MgCl_2$, 0.2 $mmol \cdot l^{-1}$ ditheithreitol, 0.05 $mmol \cdot l^{-1}$ $NADP^+$, 50 $mmol \cdot l^{-1}$ TRIS-HCl (pH 8.1), and 0.1 $mmol \cdot l^{-1}$ glucose (as per Carpenter and Hand 1986). Fluorometric analyses were performed with excitation/emission wavelengths of 340 nm and 460 nm, respectively.

Western blot analysis of ubiquitin-conjugated proteins

To qualitatively assay for ubiquitin conjugates, samples were subjected to Western blot analysis as described by Anchordoguy and Hand (1994), except polyvinylidene-diflouride (PVDF) membranes were used in place of nitrocellulose. Samples were diluted to a final protein concentration of 1 $mg \cdot ml^{-1}$ in the appropriate volume of sample buffer containing 50 $mmol \cdot l^{-1}$ TRIS-HCl, 20% glycerol, 2% sodium dodecylsulfate (SDS), and 0.4 $mol \cdot l^{-1}$ 2-mercaptoethanol (pH 8.3). Samples (40 μg total lysate protein) were loaded onto a 9% SDS polyacrylamide gel for electrophoresis. Electrophoresis of proteins was conducted with an electrode buffer consisting of 25 $mmol \cdot l^{-1}$ TRIS-HCl, 200 mM glycine, and 0.1% SDS (pH 8.3). Proteins were electro-transferred to the PVDF membrane at 200 mA for 3 h (15 °C) in a transfer solution containing 20 $mmol \cdot l^{-1}$ TRIS-HCl, 144 $mmol \cdot l^{-1}$ glycine, and 25% methanol (pH 8.6). Following electro-transfer, the membrane was heat-fixed at 70 °C for 30 min and blocked in a solution of powdered milk (50 $mmol \cdot l^{-1}$ TRIS-HCl, 3% (wt/v) nonfat dried milk, 0.15 $mol \cdot l^{-1}$ NaCl, and 0.5% (v/v) Triton X-100, pH 7.6) for 1 h. The membrane was incubated for 1 h with a polyclonal antibody specific for conjugated ubiquitin (diluted in the milk solution). The blot was given one 5-min wash in 25 $mmol \cdot l^{-1}$ TRIS-HCl, 100 $mmol \cdot l^{-1}$ NaCl, pH 7.8 (TBS), two washes in TBS with 0.05% (v/v) Triton X-100 (TTBS), and a final wash in TBS. The membrane was then incubated for 1 h with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Bio-Rad), which was diluted in TTBS with 1% BSA. The washing procedure was repeated. The membrane was equilibrated in 50 $mmol \cdot l^{-1}$ glycine and 4 $mmol \cdot l^{-1}$ $MgCl_2$ (pH 9.6) for 15 min. Conjugated ubiquitin was visualized by incubating the blot with 1.5 mg of 5-bromo-

4-chloro-3-inodyl phosphate and 3 mg of nitro blue tetrazolium dissolved in 15 ml of the glycine-$MgCl_2$ solution.

Influence of exogenously added kinases on proteolysis

ATP/ubiquitin-supplemented reaction mixtures were supplemented with 0.1 $\mu g \cdot ml^{-1}$ Ca^{2+}-dependent protein kinase (PKC), 20 $\mu g \cdot ml^{-1}$ phosphatidyl serine, and 0.5 $mmol \cdot l^{-1}$ $CaCl_2$ (PKC reaction mixture), or 1 $mg \cdot ml^{-1}$ cAMP-dependent protein kinase holoenzyme (PKA) and 50 $\mu mol \cdot l^{-1}$ cAMP (PKA reaction mixture). PKA was desalted prior to use by passage across a Sephadex G-25 desalting column. To control for the possibility of non-specific protein effects, bovine serum albumin (BSA) was added to some of the ATP/ubiquitin-supplemented reactions. Proteolysis was determined as above.

Influence of net ATP hydrolysis on proteolysis

In order to address the influence of net ATP hydrolysis on proteolysis, ATP/ubiquitin-supplemented lysates were incubated with 5–100 units/ml alkaline phosphatase (AP) during the proteolysis experiments. To ensure that AP was not influencing proteolysis via protein dephosphorylation, AP was sequestered (60 units/ml final assay volume) in dialysis tubing (mw cutoff = 12–14 kDa; Spectra/Por, Spectrum Medical Industries, Los Angeles, Calif.) to prevent access to lysate proteins. The influence of potential products of ATP hydrolysis (adenosine, adenosine monophosphate, adenosine diphosphate, hypoxanthine, inosine, and inosine monophosphate) were evaluated by supplementing reaction mixtures with 1 $mmol \cdot l^{-1}$ of each compound.

Results

Presence of ubiquitin-dependent proteolysis and validation of assay

The addition of ATP to embryo lysates stimulated protein degradation approximately twofold compared to the control reactions without adenylates, as measured by the release of radioactivity into the PCA-soluble supernatant fraction across the 4-h incubation period (Fig. 1). The degradation was further enhanced by adding ATP and exogenous ubiquitin together. The latter observation indicates that levels of endogenous ubiquitin typically present in lysates (approximately 5.5–6 nmol of free ubiquitin per gram wet mass; cf. Anchordoguy and Hand 1994, 1995) were apparently not sufficient to support maximal rates of ubiquitin-dependent protein degradation in vitro. The concentration dependency of the process on ATP is shown in Table 1, where it can be seen that 11 $\mu mol \cdot l^{-1}$ ATP is sufficient to stimulate maximal degradation.

Western blots of reaction mixtures sampled as a function of incubation time indicate that these preparations are competent to catalyze the conjugation of ubiquitin to protein substrates. By using an antibody specific for ubiquitin conjugates, an increase in conjugates can be documented when ATP and exogenous ubiquitin are present in reaction mixtures (Fig. 2). In contrast, the control reactions without ATP and exogenous ubiquitin have low and constant concentrations of ubiquitin conjugates.

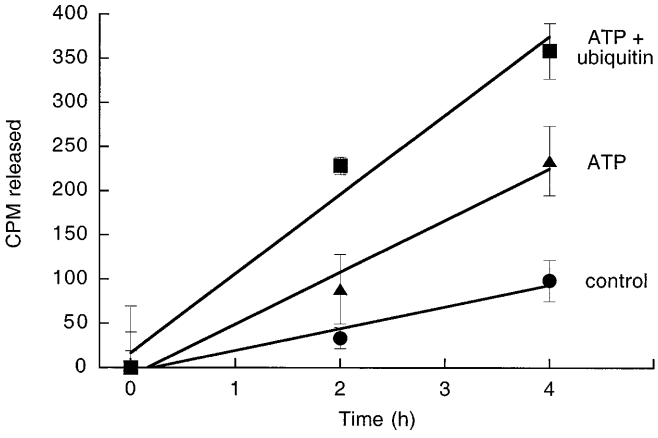

Fig. 1 Stimulation of protein degradation in lysates of *Artemia franciscana* embryos by ATP and ubiquitin. *Circles* represent reaction mixtures in the absence of ATP and without exogenous ubiquitin. *Triangles* represent reaction mixtures containing 1 $mmol \cdot l^{-1}$ ATP, which was held constant by an ATP-regenerating system. *Squares* represent reactions with 1 $mmol \cdot l^{-1}$ ATP and 24 $\mu mol \cdot l^{-1}$ exogenous ubiquitin. All reactions contained about 5.8 $mg \cdot ml^{-1}$ protein and were at conducted at pH 7.9. Values represent means ± SE, $n = 3$

The creatine phosphate/creatine phosphokinase ATP-regenerating system effectively controlled ATP concentrations throughout the 4-h degradation assays. ATP values (mean ± SE) at time zero were 1.01 ± 0.02 $mmol \cdot l^{-1}$ and at hour 4 were 1.05 ± 0.07 $mmol \cdot l^{-1}$. AMP was low throughout the assay, but a small increase in AMP occurred (time 0 = 0.065 ± 0.006 mM; hour 4 = 0.175 ± 0.015 $mmol \cdot l^{-1}$). ADP varied from 0 $\mu mol \cdot l^{-1}$ to 40 $\mu mol \cdot l^{-1}$ during the assay (data not shown).

Taken together, these data support the presence of a functional ATP/ubiquitin-dependent pathway for protein degradation in *A. franciscana* embryos. The result extends the previous observations that ubiquitin-conjugated proteins decrease and free ubiquitin increases in these embryos during anoxic exposure (Anchordoguy and Hand 1994, 1995). The actual degradation process stimulated by ATP and ubiquitin can be measured in lysates from these embryos.

Table 1 Dependence of protein degradation on ATP concentration. Values are mean ± SE, $n = 3$

Total ATP in assay mixture	Cpm released per 4 h
None detectable (control[a])	325 ± 48*
11 $\mu mol \cdot l^{-1}$	685 ± 14**
20 $\mu mol \cdot l^{-1}$	695 ± 23**
60 $\mu mol \cdot l^{-1}$	666 ± 28**
110 $\mu mol \cdot l^{-1}$	685 ± 16**
510 $\mu mol \cdot l^{-1}$	621 ± 14**
1 $mmol \cdot l^{-1}$	651 ± 52**

[a] Control assays do not contain exogenous ubiquitin or the ATP-regenerating system. Addition of the regenerating system to lysates results in the accumulation of 10 $\mu mol \cdot l^{-1}$ ATP by the end of the assay

*,** Values that differ in the numbers of asterisks assigned are significantly different from each other (ANCOVA, $P < 0.05$)

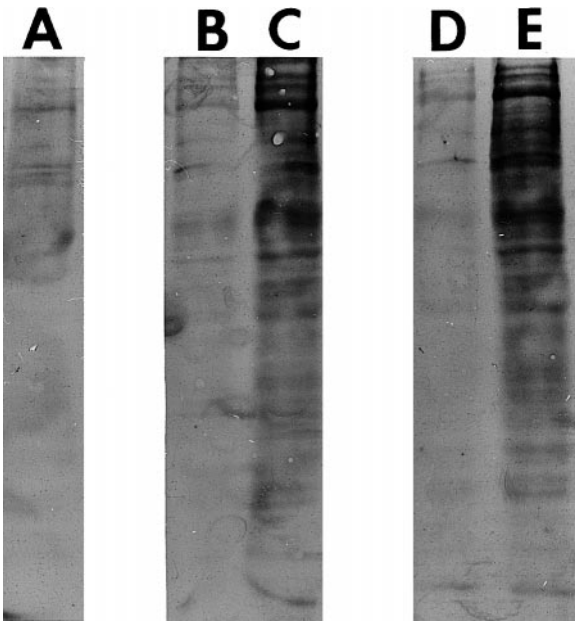

Fig. 2 Western blot of ubiquitin conjugates during the 4-h protein degradation assay. *Lane A* represents levels of ubiquitin conjugates in lysate immediately prior to initiation of the proteolysis assay (0 h). Control reactions were not supplemented with ATP or ubiquitin and were sampled at 2 h (*lane B*) and 4 h (*lane D*) during the assay. ATP/ubiquitin-stimulated reactions were also sampled at 2 h (*lane C*) and 4 h (*lane E*)

pH dependency of protein degradation

Both ATP/ubiquitin-dependent protein degradation and the unstimulated control degradation are diminished when pH is lowered from 7.9 to 6.7 (Fig. 3a). However, the stimulated rate is more sensitive to pH than is the unstimulated rate, as supported by ANCOVA ($P < 0.05$). When the unstimulated rate is subtracted, the pH sensitivity of ATP/ubiquitin-stimulated degradation can be evaluated directly (Fig. 3b). The subtraction increases variability in the resulting pH profile. Even so, it is apparent that as pH is lowered from 7.7 to 6.4, a range which approximates the pH_i change experienced by these embryos during anoxic exposure (Busa et al. 1982), the rate of ATP/ubiquitin-stimulated degradation is strongly inhibited to values approaching zero (r^2 for regression line = 0.59; slope significantly different from zero, $P < 0.05$). The rate of degradation in the absence of ATP and exogenous ubiquitin is inhibited 28% across this same pH range.

Influence of protein kinases on proteolysis

Addition of Ca^{2+}-dependent PKC had no effect on the rate of ATP and ubiquitin-stimulated protein degradation (data not shown). However, PKA significantly enhanced ATP/ubiquitin-stimulated degradation (Fig. 4; ANCOVA, $P < 0.05$). cAMP added alone had no effect. The value in the presence of PKA was 128.8 ± 4.3%

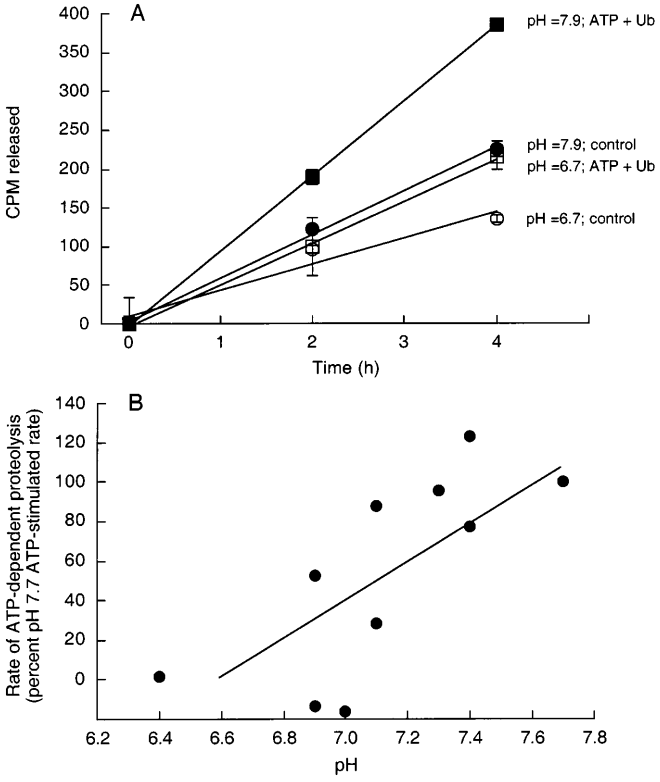

Fig. 3A, B Effects of pH on proteolysis. **A** *Circles* represent reaction mixtures in the absence of ATP and without exogenous ubiquitin. *Squares* represent reaction containing 1 mmol·l^{-1} ATP and 100 μg·ml^{-1} ubiquitin. *Closed symbols* represent reactions conducted at pH 7.9, and *open symbols* were at pH 6.7. Values represent means ± SE, $n = 3$. **B** Effects of pH on ATP/ubiquitin-stimulated protein degradation. Data were pooled from three preparations and are expressed as a percentage of the rate observed at pH 7.7

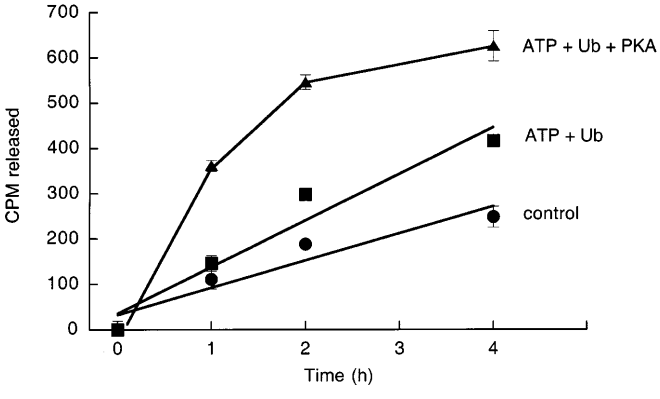

Fig. 4 Effect of exogenous cAMP-dependent protein kinase (PKA) on protein degradation in lysates from *A. franciscana* embryos. Controls (*circles*) represent samples not supplemented with ATP or ubiquitin. Reactions supplemented with 1 mmol·l^{-1} ATP and 100 μg·ml^{-1} ubiquitin are indicated by *squares*. The PKA treatment (*triangles*) was identical to the ATP/ubiquitin reaction, but supplemented with 50 μmol·l^{-1} cAMP and 1 mg·ml^{-1} PKA. Values represent means ± SE, $n = 3$

(mean ± SE, $n = 8$) of the ATP/ubiquitin-stimulated degradation value. PKA also increased the level of ubiquitin-conjugated proteins in the lysates compared to

the levels seen with ATP and ubiquitin alone, as shown by Western blot analysis (Fig. 5).

However, further experimentation revealed that this stimulation of degradation was apparently independent of any event involving phosphorylation. Omission of cAMP from the reaction mixture did not reduce the stimulatory effect of PKA on proteolysis (data not shown). When lysates were incubated in the presence of potent inhibitors of PKA [the isoquinolone derivative, A1 (Lu et al. 1996), or protein kinase inhibitor peptide 635.20 (Walsh et al. 1990)], the effect was not diminished. Heat-denaturation of PKA (boiling or 56 °C for 20 min) prior to addition did not prevent the stimulatory effect (data not shown). Finally, the substitution of PKA with bovine serum albumin did not result in enhanced proteolysis (data not shown). Thus, the stimulation by PKA does not appear to be due to a nonspecific protein effect.

Effect of net ATP hydrolysis on proteolysis

When net ATP hydrolysis is promoted in the lysate by addition of AP, protein degradation was decreased to 23 ± 5% (mean ± SE, $n = 6$; ANCOVA, $P < 0.05$) of the ATP and ubiquitin-stimulated rate (Fig. 6a). AP addition resulted in a decreased accumulation of ubiqutin-conjugated proteins compared to ATP/ubiquitin-supplemented reaction mixtures (Fig. 7). Proteolysis was inhibited only when the concentration of AP was sufficient to severely reduce the ATP concentration in the reaction mixture (Fig. 6b). However, ATP did not decline to values that limit ATP/ubiquitin-dependent proteolysis (cf. Table 1). When lower concentrations of AP were utilized (e.g. 5–15 units ml^{-1}),

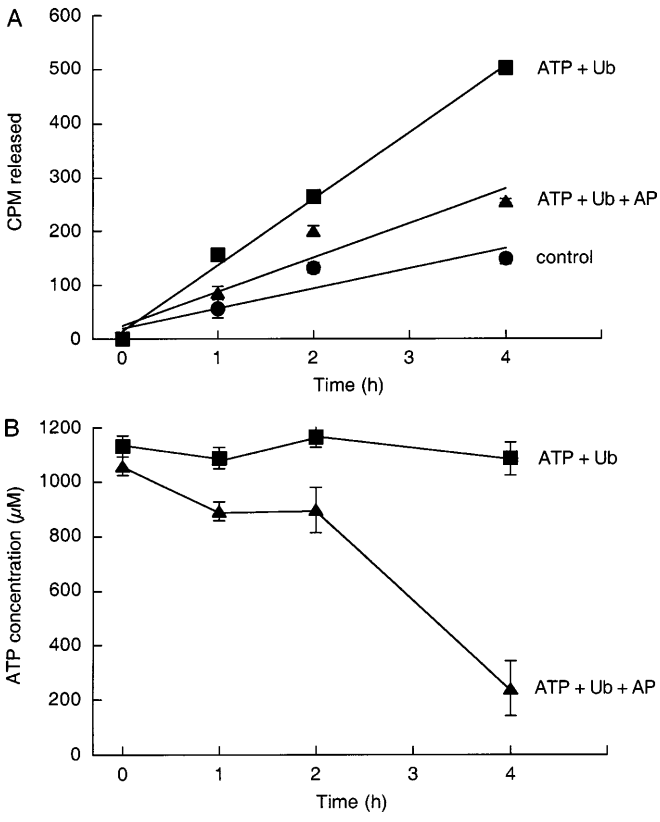

Fig. 6A, B Effect of alkaline phosphatase supplementation on proteolysis and ATP levels. **A** *Circles* represent reaction mixtures in the absence of ATP and without exogenous ubiquitin (control). *Squares* represent reaction mixtures containing 1 $mmol \cdot l^{-1}$ ATP and 100 $\mu g \cdot ml^{-1}$ ubiquitin (*ATP + Ub*). *Triangles* represent reaction mixtures identical to the ATP reaction mixtures but supplemented with 60 U ml^{-1} alkaline phosphatase (*ATP + Ub + AP*). **B** ATP levels during the reaction in the presence of AP. Values represent means ± SE, $n = 3$

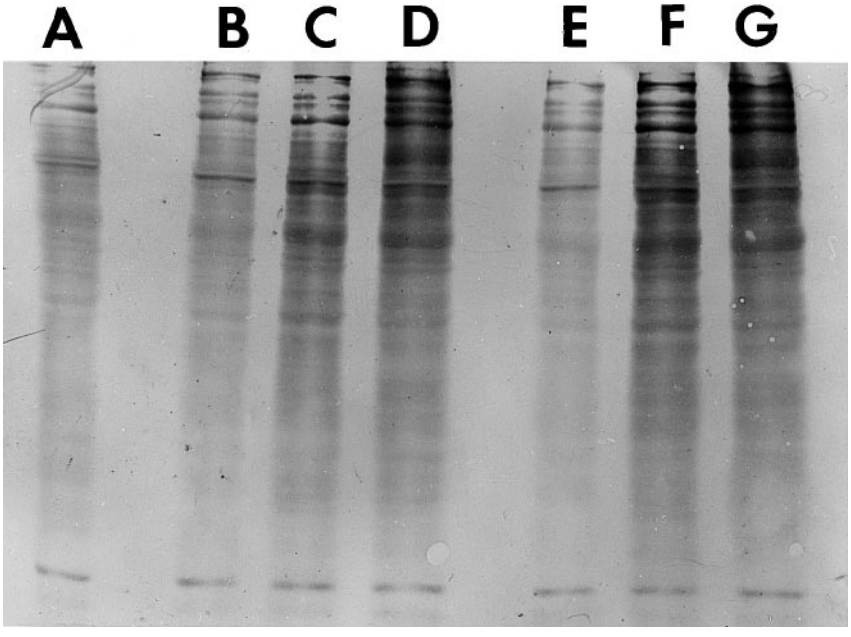

Fig. 5 Effect of PKA supplementation on ubiquitin conjugate levels. *Lane A* represents levels of ubiquitin conjugates in lysate immediately prior to initiation of the proteolysis assay (0 h). Control reactions were not supplemented with ATP or ubiquitin and were sampled at 2 h (*lane B*) and 4 h (*lane E*) during the assay. ATP/ubiquitin-stimulated reactions were also sampled at 2 h (*lane C*) and 4 h (*lane F*). Finally, ATP/ubiquitin-stimulated reactions which were supplemented with PKA were sampled at 2 h (*lane D*) and 4 h (*lane G*)

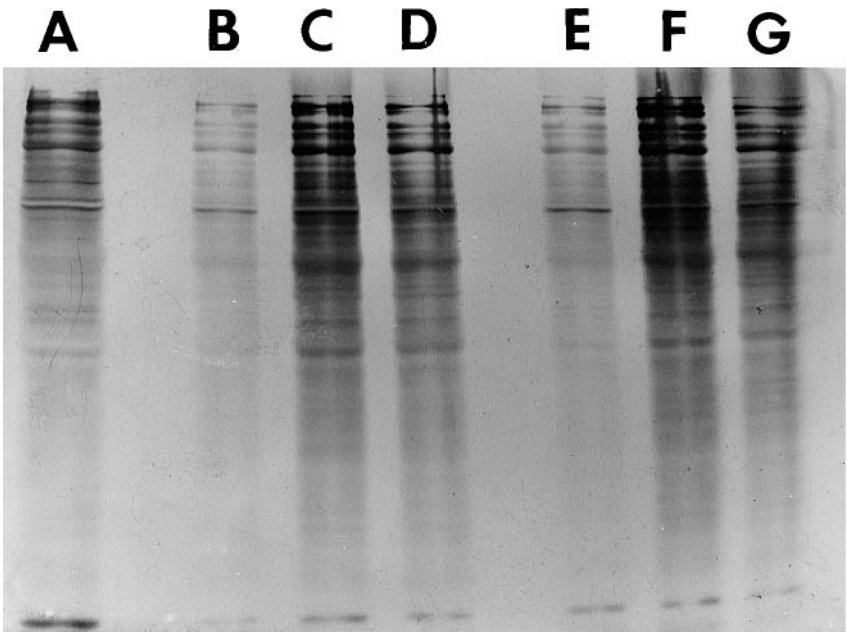

Fig. 7 Effect of AP on levels of ubiquitin conjugates. *Lane A* represents levels of ubiquitin conjugates in lysate immediately prior to initiation of the proteolysis assay (0 h). Control reactions were not supplemented with ATP or ubiquitin and were sampled at 2 h (*lane B*) and 4 h (*lane E*) during the assay. ATP/ubiquitin-stimulated reactions were also sampled at 2 h (*lane C*) and 4 h (*lane F*). Finally, ATP/ubiquitin-stimulated reactions which were supplemented with 60 U ml^{-1} AP were sampled at 2 h (*lane D*) and 4 h (*lane G*)

proteolysis was unchanged from that observed in ATP/ubiqutin-supplemented reaction mixtures (ANCOVA, $P > 0.05$), and ATP remained constant throughout the 4-h experiments (data not shown). Thus, even though ATP levels in these experiments (Fig. 6) were still fully saturating for proteolysis, it appears that a process or product linked to the net hydrolysis of ATP may result in inhibition of protein degradation. The inhibitory action of AP was not the result of protein dephosphorylation, because identical results were obtained when AP was sequestered in dialysis tubing during the assay. This treatment decreased protein degradation to 29.3% (ANCOVA, $P < 0.05$) of the ATP/ubiqutin-stimulated rate and also severely lowered ATP concentration in the reaction mixture.

A variety of potential products of ATP hydrolysis (e.g., adenosine, adenosine monophosphate, adenosine diphosphate, hypoxanthine, inosine, inosine monophosphate, and phosphate) were assessed for their ability to inhibit degradation. However, none were found to be inhibitory (data not shown).

Discussion

Results of this study establish the presence of an active ubiquitin-dependent proteolytic mechanism in embryos of *A. franciscana* and describe some potential mechanisms for its regulation during anoxia-induced quiescence. Because quiescence is characterized by dramatic decreases in both pH and ATP concentration, these factors are likely candidates for regulation of proteolysis. Previous studies with rabbit reticulocyte lysates indicated that proteolysis was both pH- and ATP-dependent (Müller et al. 1980). The present study supports a role for alterations in both pH and ATP in the regulation of ubiquitin-mediated proteolysis during anoxia-induced quiescence. In lysates of *A. franciscana,* ATP/ubiquitin-dependent proteolysis is completely inhibited by lowering the pH from 7.7 to 6.4, which represents the physiological range for the embryos under normoxic versus anoxic conditions (Fig. 3b). ATP concentrations below 11 $\mu mol \cdot l^{-1}$ may also restrict proteolysis (Table 1), and the net hydrolysis of ATP per se appears to inhibit ATP/ubiquitin-dependent proteolysis.

The ubiquitin-dependent pathway for proteolysis utilizes ATP at perhaps two steps. The first step of the ubiquitin conjugation process is ATP dependent (Haas and Rose 1982). The ubiquitin-activating enzyme (E_1) utilizes ATP in forming a thiolester with ubiquitin. In rabbit reticulocytes, the $k_{1/2}$ for ATP for this reaction was calculated to be 36 $\mu mol \cdot l^{-1}$ (Haas and Rose 1982). The role of ATP hydrolysis in promoting degradation at the level of proteasome function is not fully resolved. The apparent K_m of the proteasome for ATP is 12–15 $\mu mol \cdot l^{-1}$ (Hoffman and Rechsteiner 1997). However, this ATP requirement may be related to chaperoning activity that facilitates movement of protein substrates into the proteasome, and not proteolysis per se (reviewed in Goldberg et al. 1997; Tanaka 1998). Nevertheless, ubiquitin-mediated proteolysis is dependent on ATP hydrolysis. In the present study, ATP concentration had to be lowered below 11 $\mu mol \cdot l^{-1}$ before any change in degradation rate could be detected (Table 1). This low concentration range for ATP sensitivity is generally consistent with the mammalian studies above. While such low concentrations of ATP are not usually seen in mammalian cells (cf. Atkinson 1977), it is possible that ATP concentrations may approach these values during anoxia-induced quiescence in *A. franciscana* embryos and thereby limit proteolysis. Several studies indicate

that ATP is very low but detectable by chemical measurement in these embryos during the initial hours of quiescence (Stocco et al. 1972; Carpenter and Hand 1986; Rees et al. 1989; Anchordoguy and Hand 1994).

A novel, but currently unexplained, observation from the present study is that net ATP hydrolysis promotes inhibition of ATP/ubiquitin-dependent proteolysis. It is interesting to note that the inhibition of protein degradation as a result of net ATP hydrolysis occurs despite the presence of sufficient ATP to fully support degradation. Specifically, ATP/ubiquitin-stimulated degradation was inhibited under conditions where ATP concentration was still above 200 $\mu mol \cdot l^{-1}$ (Fig. 6). These data suggest that some product of ATP hydrolysis may be responsible for the observed inhibition. Net ATP hydrolysis in mammalian cells during hypoxic insult can lead to the large accumulation of several products of ATP hydrolysis, including adenosine, adenosine monophosphate, adenosine diphosphate, hypoxanthine, inosine, and xanthine (e.g., Pillwein et al. 1987; van Bilsen et al. 1989). Similarly, in the sipunculid worm, *Sipunculus nudus,* anoxia and hypercapnia resulted in an approximately fourfold greater adenosine concentration as compared to aerobic controls. However, none of these metabolites, added individually, inhibited ATP/ubiquitin-dependent proteolysis in our assays. Perhaps a complex interaction of ATP metabolites may be required to explain the observation.

Recent work performed with mammalian erythrocytes has indicated that the ubiquitin-activating enzyme (E_1) can be covalently modified by phosphorylation, which increases ATP/PP_i exchange (indicator of activity) by approximately twofold (Kong and Chock 1992; Cook and Chock 1995). Exogenous PKA significantly enhanced degradation in *A. franciscana* lysates compared to ATP/ubiquitin-stimulation alone (Fig. 4) and increased the level of ubiquitin conjugates as well (Fig. 5). However, our data show that this enhanced degradation is independent of protein phophorylation per se. Since both conjugation and proteolysis were increased, it is possible that the kinase may interact specifically with a component of the conjugating system (most likely E_1).

Western blot analyses revealed that changes in levels of ubiquitin-conjugates were always paralleled by changes in proteolysis rate in the same direction (Figs. 2, 7). These data suggest that the primary site for regulation of proteolysis in *A. franciscana* embryos is at the level of ubiquitin-conjugation rather than at the proteasome. Similarly, the degradation of abnormal proteins during heat shock appears to be controlled by the rate of ubiquitination (Munro and Pelham 1985). Interestingly, ubiquitin conjugates diminished more under anoxia than they did under the artificial state of aerobic acidosis (Anchordoguy and Hand 1994). Aerobic acidosis is characterized by low pH, but in contrast to what is observed under anoxia, ATP availability remains high for the first few hours (Busa and Crowe 1983). Data on the half-life of cytochrome-*c*-oxidase (COX) show a similar trend in that the half-life of COX was extended approximately 77-fold under anoxia, but only 7-fold under aerobic-acidosis (Anchordoguy et al. 1993). Taken together, these data further support our observation that the net hydrolysis of ATP promotes inhibition of ubiquitin-mediated proteolysis.

Several studies have examined proteolytic mechanisms in *A. franciscana* that are not ATP/ubiquitin dependent. Osuna et al. (1977) concluded that there was virtually undetectable proteolytic activity in the encysted embryo, but it should be noted that their methodology would not have measured ATP-dependent processes. Other studies have revealed the presence of proteases in the embryo, but they appear to be either compartmentalized (i.e., in lysosomes) or masked by endogenous protease inhibitors (see Nagainis and Warner 1979; Warner and Shridhar 1980; Perona and Vallejo 1982, Warner 1989a, b). Proteases increase in activity during the embryonic-larval transition, and in at least one case, this change has been correlated with a shift in the ratio of an endogenous protease inhibitor to its target protease (Warner et al. 1997). Several studies have demonstrated the presence of multiple proteases in nauplius larvae of *A. franciscana* (Garesse et al. 1980; Pan et al. 1991; Warner et al. 1995). However, except for ubiquitin-mediated proteolysis, there appears to be little extra-lysosomal protein degradation available to the encysted embryo.

The data presented here support a downregulation of ubiquitin-mediated proteolysis in the quiescent embryo. The decline in pH associated with entrance to quiescence appears to be one mechanism promoting proteolytic arrest. Decline in ATP concentration in vivo to low levels may also be a contributor, as well as net hydrolysis of ATP per se. These data extend, and are fully consistent with, the data of Warner et al. (1997) who found no change in total protein in encysted embryos after 1 month of anoxic exposure. This latter observation is supported by the study from Clegg (1997), which showed no decrease in radiolabeled proteins after a full year of anoxia. The present study indicates that arrest of ubiquitin-dependent proteolysis likely explains a substantial share of the increased protein stability observed under anoxia. Ultimately, the stability of macromolecules may dictate survivorship during quiescence.

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