Erratum

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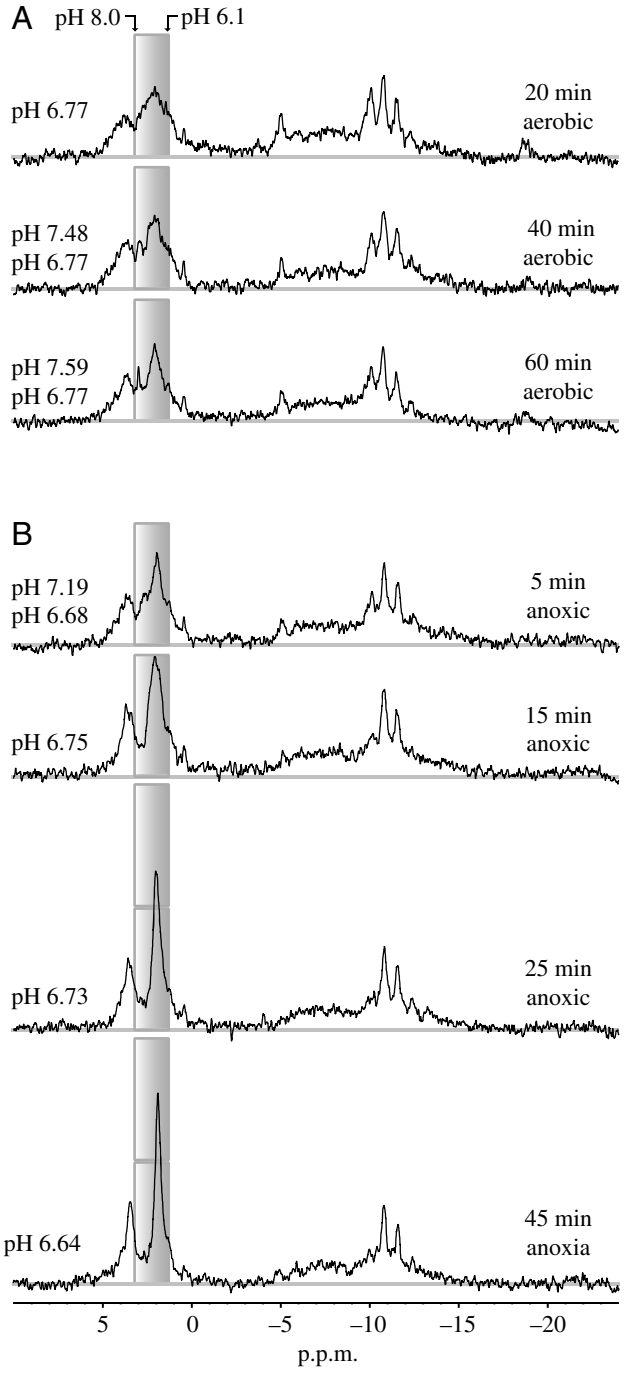
In the final print version and the pdf version available on-line, an incomplete version of Fig. 6 was published, with the lower *x* axis and labels obscured by the figure legend. The full-text version on-line is correct. The complete figure is reproduced below.



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V-ATPase inhibition prevents recovery from anoxia in *Artemia franciscana* embryos: quiescence signaling through dissipation of proton gradients

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Summary

The metabolic downregulation critical for long-term survival of *Artemia franciscana* embryos under anoxia is mediated, in part, by a progressive intracellular acidification. However, very little is known about the mechanisms responsible for the pH transitions associated with exposure to, and recovery from, oxygen deprivation. In the present study, we demonstrate with ^{31}P-NMR that incubation of intact embryos with the V-ATPase inhibitor bafilomycin A_1 severely limits intracellular alkalinization during recovery from anoxia without affecting the restoration of cellular nucleotide triphosphate levels. Based on these data, it appears that oxidative phosphorylation and ATP resynthesis can only account for the first 0.3 pH unit alkalinization observed during aerobic recovery from the 1 pH unit acidification produced during 1 h of anoxia. The additional 0.7 pH unit increase requires proton pumping by the V-ATPase. Aerobic incubation with bafilomycin also suggests that V-ATPase inhibition alone is not enough to induce an acute dissipation of proton gradients under anoxia. In intact embryos, the dissipation of proton gradients and uncoupling of oxidative phosphorylation with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) leads to an intracellular acidification similar to that seen after 1 h of anoxia. Subsequent exposure to anoxia, in the continued presence of CCCP, yields little additional acidification, suggesting that proton gradients are normally dissipated under anoxia. When combined with protons generated from net ATP hydrolysis, these data show that the dissipation of proton chemical gradients is sufficient to account for the reversible acidification associated with quiescence in these embryos.

Key words: vacuolar-type ATPase, CCCP, bafilomycin A_1, quiescence, gradient dissipation, anoxia, acidification, intracellular pH, ^{31}P-NMR.

Introduction

For more than 20 years, the source of the impressive intracellular acidification induced by anoxia in encysted embryos of the brine shrimp, *Artemia franciscana*, has eluded a complete explanation. In the preceding companion paper (Covi and Hand, 2005), we proposed a novel mechanism for regulated acidification of the intracellular space, in which exposure to anoxia would trigger a net flux of protons from acidic compartments to the surrounding cytoplasm. Given the rapid drop in cellular ATP:ADP ratio occurring under anoxia in these embryos (Anchordoguy and Hand, 1994; Stocco et al., 1972), this process would probably involve inactivation of ATP-dependent proton pumping into these compartments (cf. Huss and Wieczorek, 2003; Kettner et al., 2003). Here we demonstrate that *in vivo* inhibition of V-ATPase activity in encysted embryos both induces a reversible intracellular acidification under aerobic conditions and precludes recovery from the intracellular acidification induced by anoxia. In addition, we show that global dissipation of proton gradients with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) yields an acidic intracellular pH (pH_i) similar to that observed after 1 h of anoxia. We argue that, together, these data offer strong support for the hypothesis that proton gradient dissipation is a key contributor to anoxia-induced acidification of the intracellular space in *A. franciscana* embryos. Indeed, when combined with the protons produced by net hydrolysis of ATP (Busa and Nuccitelli, 1984; Kwast et al., 1995), the collapse of proton chemical gradients can fully explain the origin of acid equivalents required for this acidification.

Descriptive observations of *Artemia* development can be traced to the early 1900s (for review, see Clegg and Conte, 1980), and extensive investigation of the mechanisms involved in the extreme anoxia tolerance displayed by the encysted embryonic stage continues on many fronts (e.g. Clegg et al., 2000; Eads and Hand, 2003; Menze et al., 2005; Warner et al., 2002; Willsie and Clegg, 2002). However, until now, functional examination of these mechanisms *in vivo* has been hampered by the presence of a cyst shell that encapsulates these embryos. This shell can be divided into two main

sections: tertiary envelope (chorion) and embryonic cuticle (Drinkwater and Clegg, 1991), which together present a physical barrier conferring impermeability to all but low-molecular-mass gasses and water (Trotman, 1991). It even appears to prevent the passage of protons (Busa et al., 1982) and volatile solvents such as DMSO (Price, 1967). In our companion paper, however, we demonstrated that removal of the chorion confers permeability to macrolide antibiotics such as bafilomycin A_1, a highly specific and lipid-soluble inhibitor of the V-ATPase (Covi and Hand, 2005). This method of permeabilization is used advantageously in the present study and promises to expand the experimental repertoire available for other *in vivo* experiments in the future with these encysted embryos.

The V-ATPase is a proton pump that is well known for its nearly ubiquitous role in the acidification of intracellular compartments (Forgac, 2000; Futai et al., 2000). These compartments, diagrammed in Fig. 1, include early and late endosomes, Golgi complex, exocytotic and protein trafficking vesicles, lysosomes and yolk platelets (Fagotto, 1995; Nishi and Forgac, 2002). Not surprisingly, a large number of physiological processes depend on V-ATPase activity, including endocytosis, exocytosis, protein targeting, receptor recycling and lysosomal function (Nishi and Forgac, 2002). Of particular relevance when considering *A. franciscana* embryos is the finding that the acidification of yolk platelets for degradation requires an ATP-dependent proton pump in insects (Abreu et al., 2004) and is inhibited by bafilomycin in *Xenopus* oocytes (Fagotto and Maxfield, 1994) and *Rhodnius prolixus* eggs (Motta et al., 2004). The only form of regulation of V-ATPase activity known to have physiological relevance is the dissociation of the peripheral (V_1) sector from the membrane-spanning (V_0) domain (Wieczorek et al., 2003), and this disassembly may be a mechanism for inactivating the V-ATPase when energy availability is limited (Kane and Smardon, 2003).

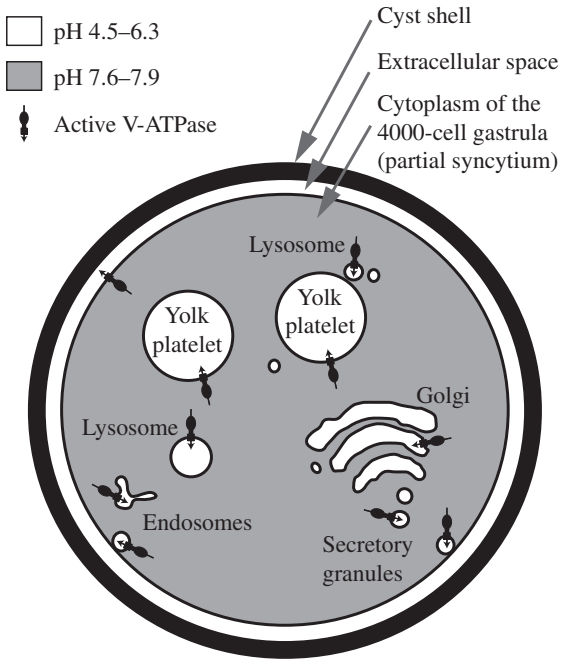

Fig. 1. Diagram describing the potential acidic compartments within a developing encysted gastrula of the brine shrimp, *Artemia franciscana*. Degree of acidification is indicated by grey scale. Maintenance of steady-state pH within acidic compartments is assumed to be regulated largely by ATP-dependent proton pumping, ion leak rates and buffering capacity.

The ability to survive prolonged bouts of anoxia is directly linked to the depression of cellular energy turnover (Hand, 1998). This correlate is exemplified in *A. franciscana* embryos, for which it has reasonably been argued that long-term survivorship under anoxia is accomplished in part through the establishment of a nearly ametabolic state (Clegg, 1997; Warner and Clegg, 2001). We previously proposed that the drop in cellular ATP:ADP ratio and increase in free inorganic phosphate (P_i) are likely to inactivate the V-ATPase within minutes of exposing these embryos to anoxia and that subsequent dissipation of proton chemical gradients could result in a substantial cytoplasmic acidification (Covi and Hand, 2005). Here, we use the technique of phosphorous nuclear magnetic resonance (^{31}P-NMR) to directly test this hypothesis by observing the effects of V-ATPase inhibition and proton gradient dissipation on the pH_i status of intact brine shrimp embryos. The protonophore CCCP is also used to examine the effects of complete dissipation of cellular proton gradients. Specifically, this lipid-soluble weak acid facilitates a comparison of intracellular acidification under anoxia with the acidification induced by combining the uncoupling of oxidative phosphorylation with the dissipation of proton gradients.

Materials and methods

Materials

Instant Ocean was purchased from Aquarium Systems (Eastlake, OH, USA). Gentamycin sulfate and CCCP were obtained from Sigma (St Louis, MO, USA). Bafilomycin A_1 (also referred to as bafilomycin) was either obtained from AG Scientific (San Diego, CA, USA) or LC Laboratories (Woburn, MA, USA). Both bafilomycin and CCCP were dissolved in 100% ethanol, and stock solutions were stored at –20°C. Low endotoxin sucrose for ^{31}P-NMR and respirometry experiments was obtained from Pfanstiehl (Waukegan, IL, USA). All other chemicals used were of the highest grade available.

Animals

Encysted embryos of the brine shrimp *Artemia franciscana* Kellog (Great Salt Lake population) were obtained in the dehydrated state (post-diapause) from Sanders Brine Shrimp Co. (Ogden, UT, USA) in 2001. These post-diapause embryos were stored dry at –20°C and hydrated overnight at 0°C in 0.25 mol l^{-1} NaCl prior to dechorionation or aseptic washing. Dechorionation was performed as previously described (Kwast and Hand, 1993). Aseptic washing was performed using the

same protocol, but the antiformin (NaOH/hypochlorite solution) wash was kept at 0°C to prevent complete removal of the chorion. Aseptically treated embryos were used in the 16 h anoxia study only. Dechorionated embryos were used in all other experiments. Embryos were stored for no more than 16 h in ice cold 0.125 mol l^{-1} NaCl prior to experimentation. All solutions were prepared less than 24 h prior to use, and the length of incubation time for embryos in antiformin was strictly maintained at 20±1 min at room temperature. Developmental staging of embryos was conducted using the same criteria described by Conte et al. (1977).

NMR spectroscopy

Proton-decoupled ^{31}P-NMR spectra were collected at a frequency of 202.458 MHz at a temperature of 298±0.1 K using a Brucker (Bellerica, MA, USA) AMX-500 spectrometer. 2048 data points were collected over a spectral width of 82 p.p.m., and datasets were zero-filled to 32 K. Except during the first hour of anoxia and recovery from anoxia, 3072 transients were recorded over a period of 10 min in each experiment. During these transitions between aerobic and anoxic conditions, 1536 transients were recorded over 5 min in order to provide better time resolution of pH shifts. Data were exponentially multiplied by 10 Hz line-broadening prior to Fourier transformation. Zero reference was established using a 85% H_3PO_4 standard. Field drift was assessed by the acquisition of 85% H_3PO_4 spectra before and after each experiment. A deuterium lock was not required, as field drift in our system (0.057 Hz h^{-1}) was much lower than resolution (0.4 Hz data $point^{-1}$).

Embryos were packed by gravity into a 10 mm glass NMR tube, and movement during perfusion was prevented by placing a foam plug at the top of the embryo column. For bafilomycin treatments and controls, embryos were superfused from the bottom of the tube with 0.2 mol l^{-1} sucrose. For CCCP treatment and 16 h anoxia study, embryos were superfused with 0.25 mol l^{-1} NaCl. It should be noted that control spectra obtained using sucrose superfusion were indiscernible from those obtained with NaCl superfusion (data not shown). Both sucrose and NaCl solutions were sterile filtered before use, and the final concentration of ethanol during treatments was 0.2%. Maintenance of aerobic conditions and induction of anoxia during *in vivo* spectroscopy were conducted as described by Kwast et al. (1995). In brief, embryos were superfused with oxygen saturated medium at a rate of 2 ml min^{-1}, and anoxia was induced by stopping perfusion. As discussed by Kwast et al. (1995), the use of oxygen-saturated medium has no noticeable affect on embryonic development, and stopping superfusion provides the most rapid production of anoxic conditions. Peak identification was accomplished by comparison with published spectra (Busa et al., 1982; Kwast et al., 1995). Intracellular pH was estimated from the chemical shift (resonance frequency) of P_i using a titration curve generated in our facility (Fig. 2C). This standard curve was produced using a solution of 10 mmol l^{-1} P_i, 110 mmol l^{-1} KCl and 20 mmol l^{-1} NaCl, and pH was set by the combination of mono- and dibasic phosphate.

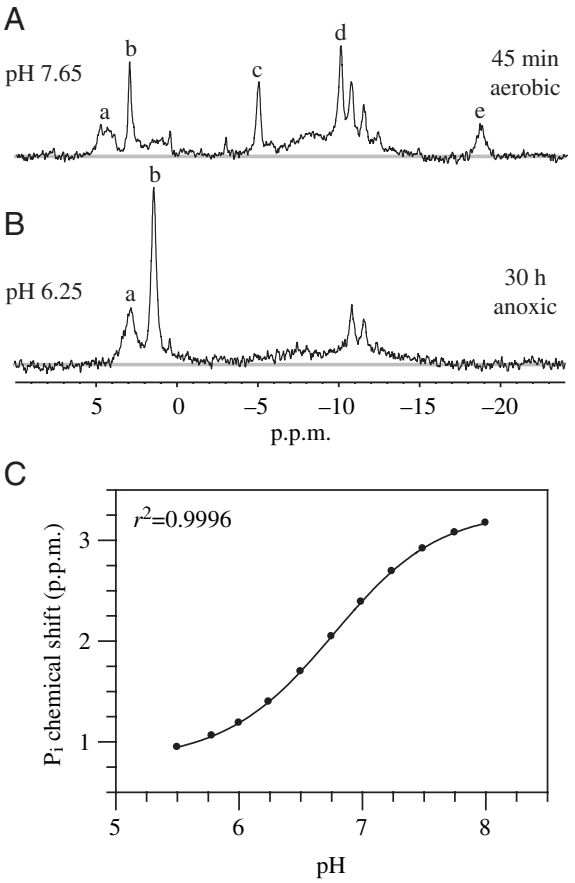

Fig. 2. Representative ^{31}P-NMR spectra of intact *A. franciscana* embryos collected after (A) 45 min of aerobic development or (B) 30 h of anoxia following 1 h of aerobic development. Duration and nature of treatments are listed to the right of each spectrum. Average embryonic (intracellular) pH is listed to the left. Predominant chemical shifts for phosphate nuclei are identified by letters: a, phosphomonoesters; b, inorganic phosphate (P_i); c, γ-NTP and β-NDP; d, α-NTP and NDP; e, β-NTP. (C) A standard curve relating the chemical shift of P_i (δ) to solution pH was used to determine the average intracellular pH during treatments. This curve is described by the equation pH=6.76+log[(δ–0.82)/(3.31–δ)].

Hatching assays

Hatching success was observed for embryos used in ^{31}P-NMR experiments. To remove inhibitors and ethanol from the incubation medium after NMR measurements were made, embryos were gently resuspended in the NMR tube using 35‰ artificial seawater at room temperature, and care was taken to use only the embryos that were contained within the spectrometer detection window. These embryos were transferred to a Durawipe (Johnson and Johnson Advanced Materials Co., New Brunswick, NJ, USA) filter funnel, rinsed with artificial seawater and resuspended in fresh 35‰ artificial seawater for 15–30 min prior to transferring to cell culture plates for hatching tests. Embryos were allowed to develop in the dark for 66–69 h before counting. This incubation period was sufficient for completion of hatching, as no further

development was observed in individuals that did not reach the naupliar stage after 66 h. It is relevant to note that recovery of pHi after removal of bafilomycin was not tested, as it is extremely difficult to remove bafilomycin from *A. franciscana* embryos. For example, the depressed hatching rates caused by exposure to 4 μmol l^{-1} bafilomycin for 24 h at 0°C (10% hatch) are only slightly reversed by 5.5 days of washing in inhibitor-free medium at 0°C (21% hatch). Given the low K_D (10^{-8} mol l^{-1}) and lipophilic nature of bafilomycin (Drose and Altendorf, 1997), this could be the result of strong binding between inhibitor and enzyme, or a product of slow diffusion out of the lipid-rich cell mass of these embryos.

Respirometry

Respiration measurements were conducted in a solution of 0.2 mol l^{-1} sucrose. CCCP was added 2.75 h after embryos were placed in respiration medium at 25°C, and the final ethanol concentration after addition was 0.5%. Respiration medium was sterile filtered prior to use, and oxygen consumption due to any bacterial contamination was limited by the inclusion of 50 μg ml^{-1} gentamycin sulfate. Gentamycin sulfate has no noticeable effect on survivorship of *A. franciscana* embryos (data not shown).

Dechorionated embryos were blotted dry, and 2–3 mg (approximately 230–350 embryos) were weighed directly in a 1 ml Eppendorf tube. Embryos were resuspended in respiration medium within 4–6 min of blotting dry and immediately transferred to a water-jacketed respiration chamber (model RC350; Strathkelvin Instruments, Glasgow, Scotland, UK) that was maintained at 25°C. Oxygen tension was measured in 1.5 ml of medium using a polarographic oxygen electrode (Strathkelvin model 1302) under room lighting, and data were collected with DataCan V acquisition software from Sable Systems (Las Vegas, NV, USA). Raw data were analyzed with DatGraf software from Cyclobios (Innsbruck, Austria). Oxygen consumption by the electrodes, back diffusion into the system, and electrode time constants were corrected for as previously described (Kwast et al., 1995). Oxygen tension was recorded continuously for 6.5 h with gentle stirring, and the electrode was raised at regular predetermined intervals to introduce air for gas exchange. In this way, CO_2 accumulation was limited, and oxygen tension was prevented from dropping below 65% air saturation. It is important to note that, while we observed no lysis or homogenization of embryos during respirometry experiments, continuous stirring does appear to increase permeability of the cuticle and decrease subsequent hatching success in controls. Thus, respiration effects observed with stirring cannot be directly compared with ^{31}P-NMR, which has no detrimental effect on hatching rates for control treatments.

Results

Spectra obtained from aerobic (Fig. 2A) and anoxic (Fig. 2B) *A. franciscana* embryos were very similar in appearance to those previously published by our lab and others (Busa et al., 1982; Kwast et al., 1995). Average embryonic (intracellular) pH was estimated using the chemical shift of P_i and a standard curve relating the P_i chemical shift to solution pH (Fig. 2C). For encysted embryos, intracellular pH after 1 h of superfusion was estimated by ^{31}P-NMR to be 7.70±0.04 (mean ± S.E.M.; N=7). This is slightly lower than the pH of ⩾7.9 reported by Busa et al. (1982) but identical to the 7.70±0.02 reported by Kwast et al. (1995). During 1 h of anoxia, intracellular pH decreased to 6.66±0.03 (N=3), and restoration of aerobic superfusion after 1 h of anoxia produced a rapid alkalinization to pH 7.64±0.05 (N=3) over a period of 22.5 min. Both of these measurements are also very similar to those reported by Kwast et al. (1995) for 1 h of anoxia (6.73±0.02) and 20 min of aerobic recovery (7.73±0.02) following 2 h of anoxia. The lowest pH we observed was 6.25, which was recorded after 30 h of continuous anoxia (Fig. 2B). It is relevant to note that the ^{31}P-NMR observations made by Kwast et al. (1995) employed aseptically treated embryos (chorion intact) superfused with 0.25 mol l^{-1} NaCl, while those discussed above were made using dechorionated embryos superfused with 0.2 mol l^{-1} sucrose. This demonstrates that both steady-state pH_i under aerobic conditions and the transitions in pH_i associated with exposure to anoxia are insensitive to the surrounding medium even if the chorion is removed.

Aerobic superfusion of encysted embryos produced a single P_i resonance during the first 1.5 h of development, after which a second resonance appeared slightly upfield (to the right) of the first (Fig. 3A,B). The pH indicated by both of these chemical shifts was relatively stable in control embryos until exposure to anoxia (Fig. 3A), upon which they merged and moved progressively upfield in accordance with the predicted intracellular acidification. Relative to the control, exposure to 4 μmol l^{-1} bafilomycin beginning at 1.5 h of development had no noticeable effect on steady-state pHi, acidification induced by anoxia or alkalinization during aerobic recovery (Fig. 3A). By contrast, when the embryos were incubated with bafilomycin for 24 h at 0°C (metabolism and development blocked by cold) prior to ^{31}P-NMR, steady-state aerobic pHi was noticeably acidified, and realkalinization of pHi after anoxia was severely blunted (Fig. 4A). After 20 min of aerobic incubation, a single broad P_i resonance indicated a slight acidification in embryos pretreated for 24 h with bafilomycin (Fig. 4A,B). Recovery from this acidification appears to be asynchronous, given that the breadth of the P_i peak lessens during aerobic incubation (Fig. 4B). Importantly, the three phosphate resonances produced by di- and trinucleotide phosphates (NDP and NTP; primarily ATP, ADP, GTP and GDP) appear to be unaffected by bafilomycin treatment (compare Fig. 4B to Fig. 2A), which indicates that the embryos are indeed viable and metabolically active after the 24 h treatment with bafilomycin.

Following 1 h of aerobic superfusion, exposure to 1 h of anoxia (26 h exposure to bafilomycin) produced a 0.87 unit acidification, which was accompanied by a large reduction in the size of the NTP and NDP chemical shifts (Fig. 4C). This response is very similar to the anoxic response originally

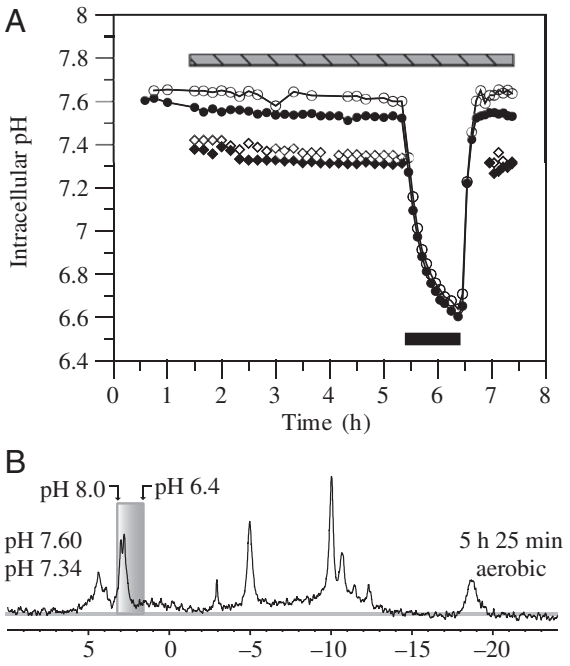

Fig. 3. Intracellular pH and NTP/NDP status of dechorionated *A. franciscana* embryos during acute treatment with bafilomycin as observed with ^{31}P-NMR. (A) Intracellular pH is plotted as a function of time for two P_i chemical shifts identified during early development. Circles represent the downfield (left) P_i resonance, while diamonds represent the upfield (right) P_i resonance. Open symbols indicate 4 μmol l^{-1} bafilomycin treatment while filled symbols indicate 0.2% ethanol control treatment. Period of bafilomycin or ethanol treatment is indicated by a hashed bar, and anoxic period is indicated by a solid bar. (B) Representative spectrum for aerobic embryos, demonstrating the presence of two distinct P_i peaks, which appear as a part of normal aerobic development. Two pH values are given to indicate that a range of pH is evident from the observed P_i peaks.

described by Busa et al. (1982), suggesting that acidification mechanisms are still functional after prolonged bafilomycin exposure. By contrast, intracellular alkalinization during aerobic recovery was severely inhibited in embryos exposed to prolonged bafilomycin treatment (Fig. 4A). While an initial rapid alkalinization of 0.3 pH units was observed during the first 17.5 min of aerobic recovery, no further shift in the predominant P_i resonance was observed. The broad nature of the phosphate peak indicated that heterogeneity of pH was still present during recovery from anoxia (Fig. 4D). Indeed, 2 h after the restoration of aerobic superfusion, the P_i resonance indicated that the average intracellular pH ranged from 7.19 to 6.72. It should be noted that we cannot be certain whether this pH range is entirely the product of heterogeneity among individuals or whether it is also the result of variation in pH among multiple compartments within individual embryos. It is also important to note that NTP and NDP resonance signatures returned within 17.5 min of reestablishing aerobic conditions (Fig. 4D), which indicates that oxidative phosphorylation was restored.

The disparity in response observed between acute and prolonged treatments with bafilomycin suggests a time dependency for loading effective quantities of the inhibitor. This possibility is supported by the observation that pre-incubation of dechorionated embryos with 1 μmol l^{-1} bafilomycin for 24 h on ice did not impair recovery from anoxia (data not shown), while 4 μmol l^{-1} bafilomycin clearly did. Movement of the inhibitor across the embryonic cuticle may be too slow to effect a change in pH status during acute treatment.

In addition to macrolide antibiotics, dechorionated embryos of *A. franciscana* are also permeable to the protonophore CCCP. Incubation with 50 μmol l^{-1} CCCP at 25°C produced a rapid rise in oxygen consumption by whole embryos (Fig. 5), as would be predicted if the protonophore reached the mitochondrial inner membrane and uncoupled oxidative phosphorylation. This initial increase was followed by prolonged decrease in oxygen consumption consistent with increasing mortality, which is expected to accompany complete uncoupling oxidative phosphorylation (Fig. 5). Pre-incubation with 20 μmol l^{-1} CCCP for 30 h at 0°C (metabolism and development blocked by cold) has a large effect on the metabolic status and pH_i of dechorionated embryos when re-warmed to 25°C. In these CCCP-treated embryos, the predominant P_i resonance, even though quite broad, indicates an acidified pH_i of 6.77 after a brief 20 min of aerobic incubation (Fig. 6A). Also, as expected from exposure to an uncoupler of oxidative phosphorylation, NTP and NDP resonance peaks are greatly reduced relative to control embryos (Fig. 6A *versus* Fig. 2A). The broadness of the main P_i resonance, and presence of a small additional alkaline peak, may indicate differential permeability to CCCP among embryos. Upon exposure to anoxia, very little shift was observed for the main (acidic) P_i resonance (Fig. 6B), although the broadness of the peak decreased. Concurrently, the remaining NTP and NDP signals disappeared. Based on the nature of this main P_i resonance, a key point to be made is that exposure to anoxia induced little additional acidification beyond that already caused by CCCP.

Recovery from the intracellular acidification induced by anoxia is just as rapid after 16.5 h of exposure as it is after 2 h. Previous observations demonstrated that after 2 h of anoxia, pH_i alkalinizes from 6.7 to 7.7 within the first 20 min of reoxygenation (Kwast et al., 1995). The present study shows that after 16.5 h of anoxia the restoration of pH_i to aerobic values also occurs within the first 20 min of reoxygenation, even though pH dropped to a low of 6.35 under anoxia (Fig. 7). This rapid alkalinization of intracellular pH stands in contrast with the relatively lengthy time required for full acidification to pH 6.35.

Discussion

In the companion paper (Covi and Hand, 2005), we demonstrated that dechorionated embryos of the brine shrimp, *Artemia franciscana*, are permeable to macrolide antibiotics such as bafilomycin and that exposure to sub-micromolar

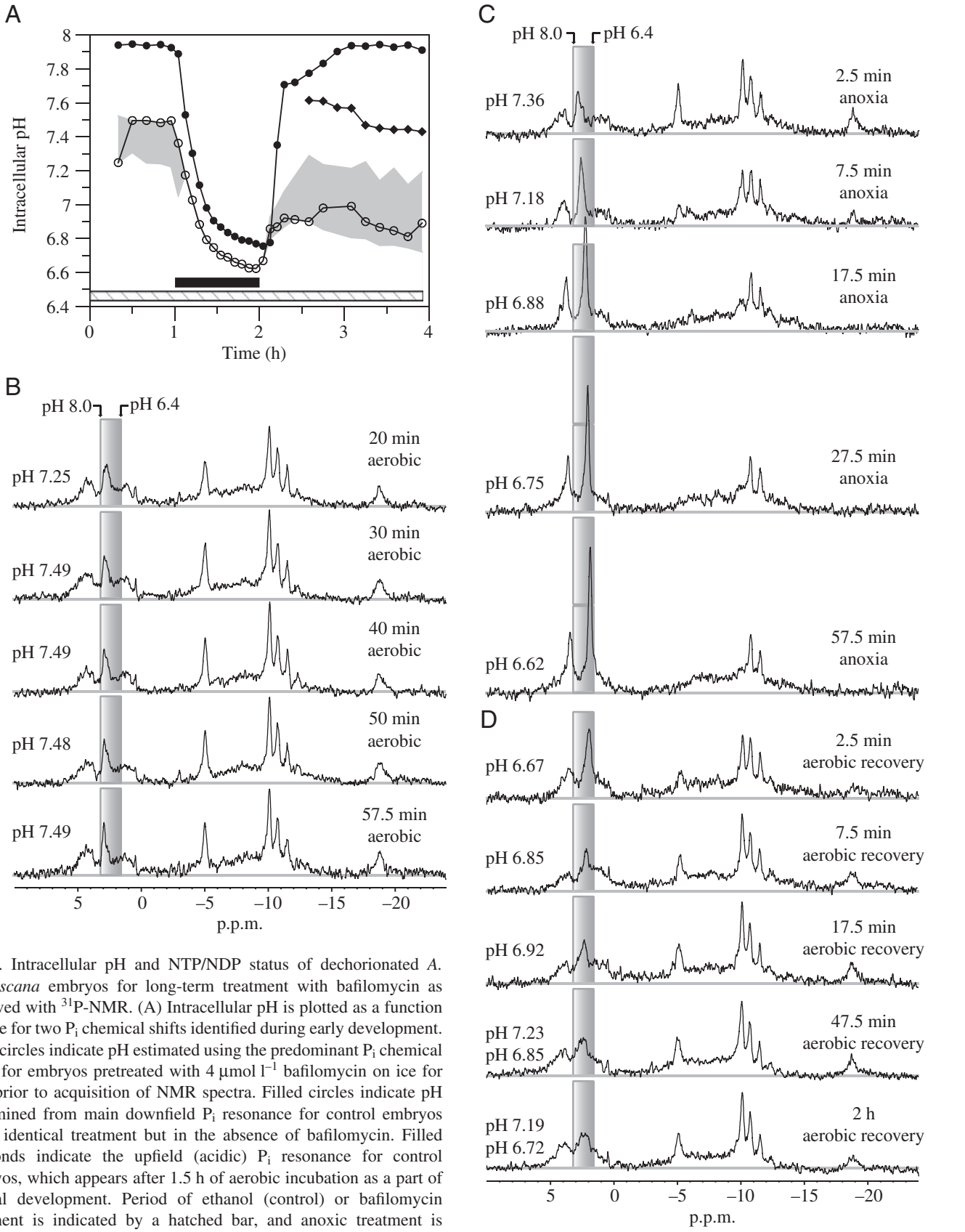

Fig. 4. Intracellular pH and NTP/NDP status of dechorionated *A. franciscana* embryos for long-term treatment with bafilomycin as observed with ^{31}P-NMR. (A) Intracellular pH is plotted as a function of time for two P_i chemical shifts identified during early development. Open circles indicate pH estimated using the predominant P_i chemical shifts for embryos pretreated with 4 μmol l^{-1} bafilomycin on ice for 24 h prior to acquisition of NMR spectra. Filled circles indicate pH determined from main downfield P_i resonance for control embryos given identical treatment but in the absence of bafilomycin. Filled diamonds indicate the upfield (acidic) P_i resonance for control embryos, which appears after 1.5 h of aerobic incubation as a part of normal development. Period of ethanol (control) or bafilomycin treatment is indicated by a hatched bar, and anoxic treatment is indicated by a solid bar. Spectra used for plotting bafilomycin treatment are selectively displayed for (B) 1 h of aerobic development, followed by (C) 1 h of anoxia-induced quiescence and (D) 2 h of subsequent aerobic recovery. Control spectra are not shown. All shaded boxes are identical, and serve to emphasize changes in chemical shift and shape of the P_i peaks.

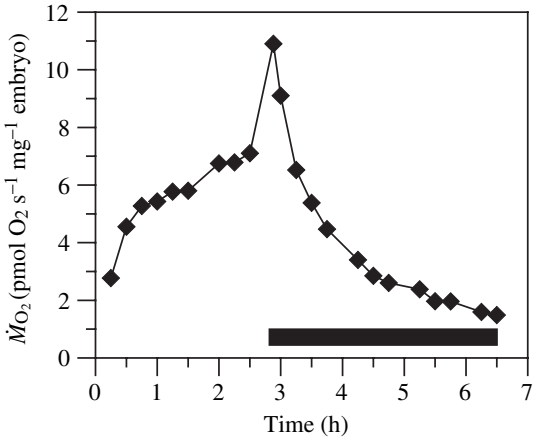

Fig. 5. Effect of 50 µmol l^{-1} CCCP on oxygen consumption ($\dot{M}_{O_2}$) by dechorionated *A. franciscana* embryos. Embryos were allowed to develop for 2.75 h before the addition of the protonophore CCCP. Period of incubation with CCCP is indicated by a solid bar.

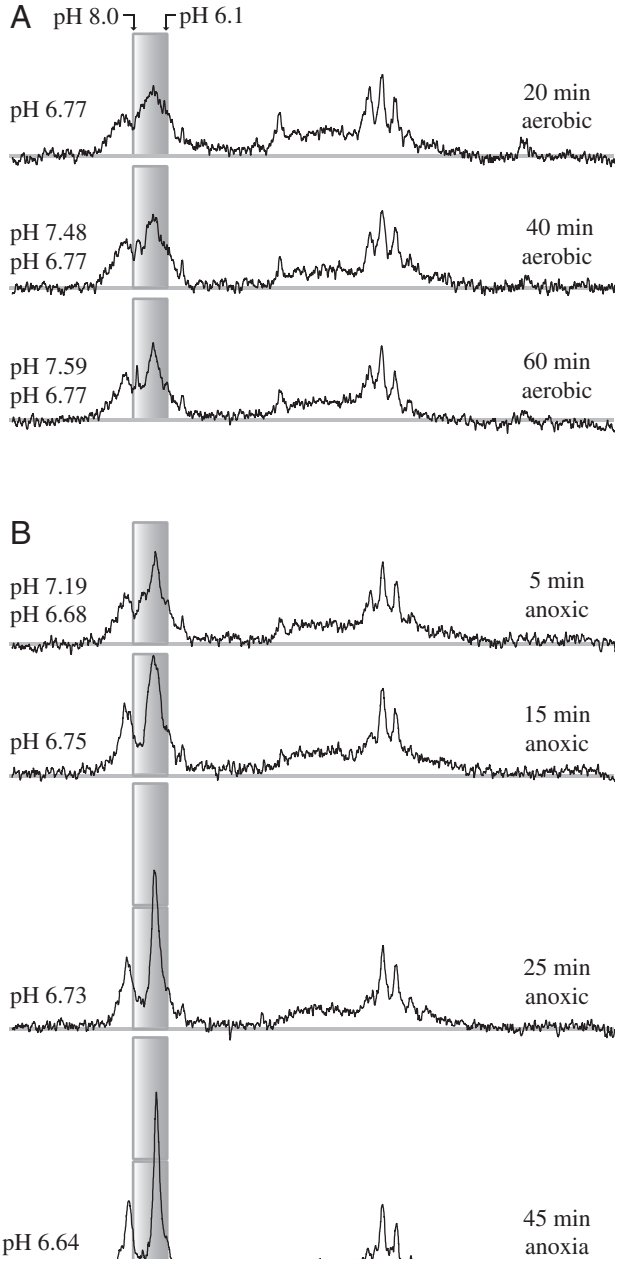

Fig. 6. Representative ^{31}P-NMR spectra for long-term treatment of dechorionated embryos with CCCP. Embryos were incubated for 30 h on ice in a sterile-filtered solution of 0.25 mol l^{-1} NaCl and 20 µmol l^{-1} CCCP. ^{31}P-NMR spectra were subsequently acquired during 1 h of superfusion with CCCP under (A) aerobic conditions, (B) followed by anoxia.

concentrations of this V-ATPase inhibitor arrests development of the anoxia-tolerant gastrula. The primary goals of the current study were to determine whether the effects of bafilomycin are linked to intracellular acidification *via* the dissipation of proton gradients normally maintained by the V-ATPase during aerobic development and, if so, to determine whether such dissipation is involved in the acidification observed under anoxia. For intact dechorionated embryos, we succeeded in demonstrating that (1) prolonged incubation with bafilomycin produces a moderate but reversible intracellular acidification, (2) exposure to bafilomycin does not affect the capacity for acidification under anoxia, (3) prolonged exposure to bafilomycin precludes recovery from acidification induced by anoxia and (4) the lipid-soluble protonophore CCCP also penetrates the embryonic cuticle and produces an acidification similar to that occurring after 1 h of anoxia. Together, these data confirm the presence of functional V-ATPase during the anoxia-tolerant stage of development, provide direct evidence of the importance of this proton pump during recovery from anoxia and suggest that global dissipation of proton gradients occurs during the first hour of exposure to anoxia.

The ^{31}P-NMR data presented here suggest that the sequestration of protons in compartment(s) with low P_i content is the major contributor to intracellular alkalinization during aerobic recovery from anoxia. Following 1 h of anoxia in the presence of 4 µmol l^{-1} bafilomycin, NTP chemical shifts reappear less than 30 min after the restoration of oxygenated conditions. This is consistent with the restoration of oxidative phosphorylation. During the same period, however, only a 0.3 unit alkalinization of pHi (6.6–6.9) is observed in embryos sufficiently loaded with bafilomycin (Fig. 4). Using these data, and assuming that buffering capacity is relatively constant between pH 6.7 and 7.7, one can estimate that oxidative phosphorylation and the resynthesis of ATP combined could only account for ~30% of the protons required for the full recovery of pHi after 1 h of anoxia. This value is at the lower end of the range estimated by Kwast et al. (1995). Their calculations suggest that oxidative phosphorylation, under acidic conditions known to inhibit carbohydrate metabolism in *A. franciscana* embryos, could account for 13–45% of the proton consumption required to shift pHi from the anoxic value of 6.7 to the aerobic value of 7.7, while the resynthesis of NTP could contribute another 13–27% over the same pH range. Our data contribute to this work by demonstrating that the V-ATPase is active at a pH of ⩽6.9 and that proton pumping by

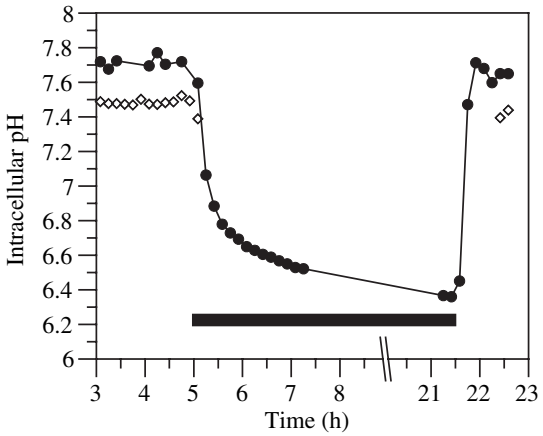

Fig. 7. Intracellular pH status for dechorionated *A. franciscana* embryos during exposure to, and recovery from, 16.5 h of anoxia. Intracellular pH is plotted as a function of time for two P_i chemical shifts identified during early development for aseptically treated embryos superfused with 0.25 mol l^{-1} NaCl. Solid circles indicate pH determined from main downfield (alkaline) P_i resonance, and open diamonds indicate the upfield (acidic) P_i resonance. Period of anoxic treatment is indicated by a solid bar. Treatment conditions used mimic those employed in the anoxia studies of Kwast et al. (1995).

this enzyme is responsible for a large fraction of the alkalinization occurring during aerobic recovery following 1 h of anoxia.

Recovery from intracellular acidification in *A. franciscana* embryos is more likely to involve sequestration of protons in intracellular compartments than secretion of protons across the plasma membrane. While the V-ATPase has been observed to function in proton secretion in the gas gland cells of eel swimbladder when pH_i is low (Sotz et al., 2002), this recovery mechanism would be less effective in *A. franciscana* embryos because the extracellular compartment is spatially constrained by a cuticle and likely to have a buffering capacity equal to or less than the cytosol. Thus, although it is possible that protons are extruded to the extracellular space during recovery from the intracellular acidification induced by anoxia, it seems more probable that alkalinization of pH_i would involve the sequestration of protons within intracellular compartments. These compartments would require a P_i content low enough relative to the cytosol to make their visualization with ^{31}P-NMR difficult. In this way, the protons used to acidify the main intracellular compartment under anoxia would effectively be masked during recovery and aerobic development. As mentioned earlier, compartments involved in this event could include lysosomes, transport vesicles, Golgi apparatus and yolk platelets. It seems unlikely that such organelles would 'hyper acidify' at a time when metabolic processes are being restarted, because their functional capacity could be adversely affected. It is more probable that they serve as proton reservoirs that are emptied upon exposure to anoxia and refilled during aerobic recovery. This would suggest that dissipation of proton gradients under anoxia contributes to the observed intracellular acidification.

While incubation with bafilomycin did produce a slight acidification of the intracellular space under aerobic conditions, the transient nature of this acidification suggests that V-ATPase inhibition alone is not enough to induce a rapid dissipation of proton gradients. One might speculate that inactivation of proton pumping would be coordinated with the opening/activation of dissipative paths if proton gradients collapse under anoxia. Opening of dissipative channels might be triggered directly by oxygen removal; mechanisms of oxygen sensing is an exciting area of current research in many organisms including *A. franciscana* embryos (Kwast and Hand, 1996).

Even if the aerobic embryos are able to maintain pH_i in the absence of V-ATPase activity, inhibition of this enzyme is still likely to affect development given its broad functional repertoire. Such is the case for *Xenopus*, in which bafilomycin treatment appears to block both yolk platelet degradation and cellular differentiation (Fagotto and Maxfield, 1994). These results are consistent with our observations, which demonstrate that the development of brine shrimp embryos is arrested by incubation in low concentrations of bafilomycin (Covi and Hand, 2005), despite an ability to maintain pH_i and NTP status for at least 5 h during exposure to this inhibitor. One potential explanation would be an inability to initiate the first round of lysosome-mediated yolk degradation, which peaks at the start of emergence (Perona et al., 1988; Perona and Vallejo, 1989). This possibility is supported by the observation that both yolk degradation and development can be reversibly inhibited in *Artemia* embryos by incubation with lysosomotrophic agents (Perona et al., 1987).

Importantly, ^{31}P-NMR experiments using CCCP support previous theoretical calculations, which suggested that the dissipation of proton concentration gradients will have a large impact on pH_i of encysted embryos. We estimated that the dissipation of proton gradients across the membranes of Golgi complex, endosomal and exocytotic vesicles, lysosomes and yolk platelets in *A. franciscana* embryos could release enough protons to account for 50% of the acidification occurring during the first hour of exposure to anoxia (Covi and Hand, 2005). Here, we directly tested the effect of proton gradient dissipation with the protonophore CCCP. If a global dissipation of proton gradients contributes to pH_i acidification under anoxia, then exposure of aerobic embryos to CCCP should induce a pH_i similar to that observed in anoxic embryos. This acidification would be produced by both a net hydrolysis of ATP – due to an uncoupling of oxidative phosphorylation – and a dissipation of proton concentration gradients. In addition, subsequent exposure of CCCP-treated embryos to anoxia should not cause further acidification of the already acidic pH_i produced by CCCP exposure. These responses are in fact exactly what we observed in whole embryos incubated with this protonophore (Fig. 6). Thus, it appears that global proton gradient dissipation occurs under anoxia, and this event contributes to the acidification observed in quiescent embryos.

There is a mismatch between the kinetics of acidification under anoxia and alkalinization upon reoxygenation, which is

consistent with the involvement of proton gradient dissipation in the acidification event. Under anoxia, pH_i declines approximately 0.8 units during the first 22.5 min of exposure (Busa et al., 1982; Kwast et al., 1995). This rapid drop is followed by a slower phase of acidification during which pH_i decreases another 0.2 units over a period of 30 min (57.5 min total anoxia exposure). Busa et al. (1982) observed that this slow phase of acidification continues overnight, eventually reaching a pH of 6.3 ($\geqslant$1.4 unit total acidification). Our results support this finding, as we observed a pH of 6.36 in embryos incubated for 16.5 h under anoxia, and 6.25 after 30 h of exposure. However, unlike the acidification occurring under anoxia, alkalinization of pH_i during recovery is remarkably fast. Kwast et al. (1995) demonstrated that pH_i is completely restored within the first 22.5 min of reoxygenation following 2 h of anoxia. Our work extends this finding by demonstrating a 1.35 unit alkalinization during only 25 min of aerobic recovery following 16.5 h of anoxia (Fig. 7). One potential explanation for the disparity in kinetics between the two transitions could be that proton gradient dissipation under anoxia is slow relative to the resequestration of protons by active transport during recovery.

In conclusion, the data presented here demonstrate that the dissipation of proton gradients under anoxia helps to produce an intracellular acidification that is critical to the induction of quiescence in encysted embryos of the brine shrimp, *Artemia franciscana*. Taking into account changes in the metabolic state of the embryo, we constructed a schematic model of the role played by proton chemical gradients during the induction of, and recovery from, anoxia-induced quiescence (Fig. 8). In this model, exposure to anoxia induces a drop in cellular ATP as oxidative phosphorylation is arrested. Within the first 5 min of exposure to anoxia, this leads to a cessation of proton transport by pumps such as the V-ATPase and an activation of proton dissipative paths. The combined result is a 1 unit drop in intracellular pH over approximately 1 h. The return of oxidative phosphorylation upon reoxygenation of the embryos causes ATP levels to rise, which in turn activates the V-ATPase. Subsequent proton pumping into the intracellular compartment causes further alkalinization of pH_i, which in turn helps to facilitate the resumption of development and metabolic processes inhibited by low pH. Importantly, this novel mechanism for regulated acidification/alkalinization of the intracellular space may represent a critical adaptive trait conferring extreme anoxia tolerance to this species. Such an adaptive trait could answer the question of what sets the anoxia tolerance of *Artemia* embryos apart from that of animals such as goldfish and turtles, which was recently posed by Guppy and Withers (1999).

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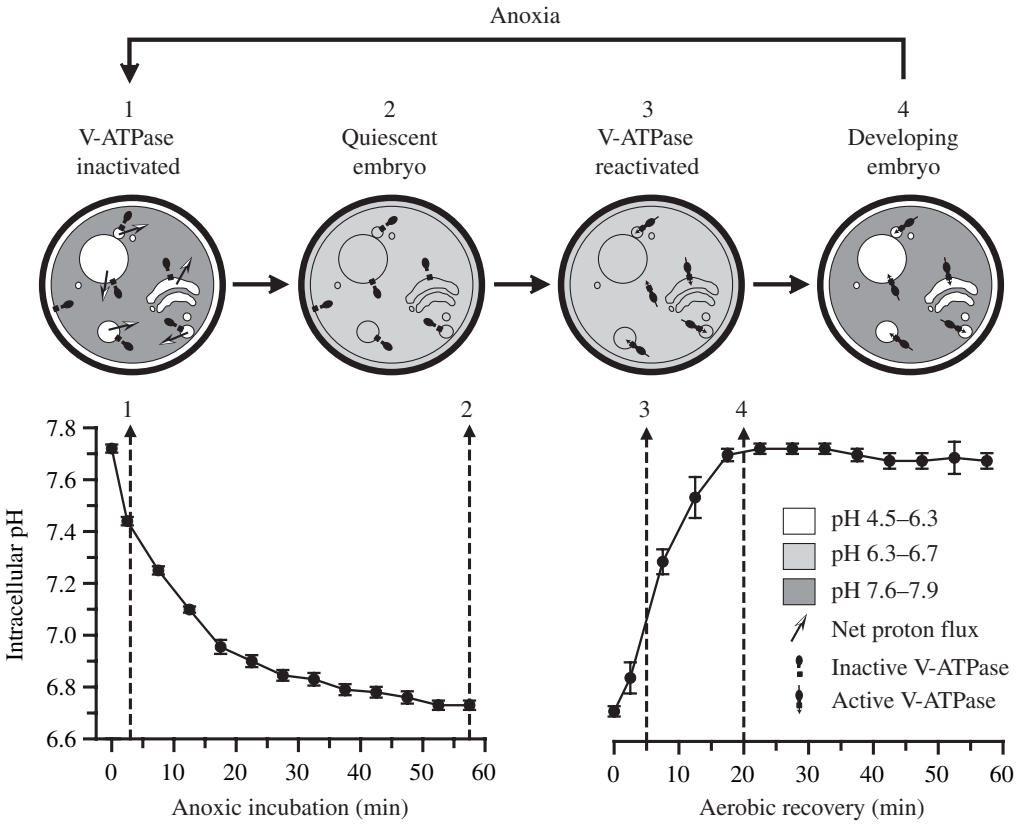

Fig. 8. Schematic representation for the role of proton concentration gradients in intracellular pH shifts induced by transitions between anoxic and aerobic states in encysted embryos of the brine shrimp, *Artemia franciscana*. Plots were modified from Kwast et al. (1995), and the data were derived from ^{31}P-NMR estimates of intracellular pH. Broken lines indicate time points corresponding to the embryo diagrams above and are numbered accordingly.

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