Oxidative phosphorylation and the realkalinization of intracellular pH during recovery from anoxia in Artemia franciscana embryos

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Oxidative phosphorylation and the realkalinization of intracellular pH during recovery from anoxia in *Artemia franciscana* embryos

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Abstract

The contribution of mitochondrial oxidative phosphorylation to the realkalinization of intracellular pH (pH i) and resynthesis of purine nucleotides during recovery from anoxia was investigated in embryos of *Artemia franciscana* by assessing the sensitivity of mitochondrial respiration to pH, calculating proton consumption by oxidative phosphorylation, and measuring changes in pH i using 31P nuclear magnetic resonance. Following short-term anoxia, pH i increased from 6.7 to 7.7 during 20 min of aerobic recovery and was temporally correlated with a large increase in ATP. State 3 respiration rates of isolated mitochondria were not substantially compromised at the acidic pH corresponding to the pH i during anoxia (pH 6.3-6.8) compared to values obtained at pH 7.7. Both state 3 respiration rates and respiratory control ratios exhibited broad, substrate-specific pH optima, whereas state 4 respiration rates increased gradually with increasing pH. P:O flux ratios were near their mechanistic limits and did not vary appreciably with pH below 7.5. Estimates of intracellular buffering capacity indicate that between 18 and 37 mmol H⁺ (1 cytosol)^-1 must be consumed to elevate pH i from 6.7 to 7.7.

Phosphorylation of mono- and diphosphate purine-nucleotides during the first 20 min of recovery may account for the consumption of up to 4.79 mmol H⁺ (1 cytosol)^-1. An additional 4.77 to 8.18 mmol H⁺ (1 cytosol)^-1 may be consumed through the oxidation of mono- or dicarboxylic acids, respectively, in the Krebs cycle. Taken together, these data are consistent with a role for oxidative phosphorylation in the realkalinization of pH i and resynthesis of purine nucleotides in *A. franciscana* embryos during recovery from anoxia.

Keywords: Mitochondrion; Oxidative phosphorylation; pH, intracellular; Respiration; Anoxia; *(Artemia)*

1. Introduction

In response to anoxia, encysted gastrulae of the brine shrimp *Artemia franciscana* enter a quiescent state in which energy flow is suppressed to exceedingly low values [1-3]. An anoxia-induced acidification of intracellular pH (pH i) from ≥ 7.9 to ≤ 6.8 [4] is associated with the arrest of both catabolic [5-7] and anabolic metabolism [8-11]. Based on calculations of proton yield from the hydrolysis of purine tri- and diphosphate nucleotides during anoxia [12,13], a major source of this proton load has been hypothesized to come from net ATP hydrolysis [13]. Upon reoxygenation, the mechanism(s) responsible for the restoration of cellular conditions conducive to normal development is not known. It has been suggested that pH i must be realkalinized prior to the return to preanoxic metabolic rates (reviewed in [13,7,14]). The objectives of the present study were to measure the time-course of pH i realkalinization during recovery from anoxia and assess the sensitivity of oxidative phosphorylation to acidic pH. Insensitivity to pH would support the hypothesis that mitochondria contribute to the alkalination of pH i through the oxidation of amino and organic acids and resynthesis of ATP pools.

Artificial acidification of pH i in situ inhibits glycolysis in *A. franciscana* embryos at the same loci that are inhibited during anoxia [6], and key enzymes involved in carbohydrate (trehalose) metabolism are inhibited in vitro by acidic pH [15-17]. Considering that the vast majority of cellular energy provided during preemergent development
is derived from large stores of trehalose [18-21], inhibition of glycolysis effectively inhibits the embryo's primary catabolic pathway [6]. However, the time-course of $\Delta pH$ change during recovery in *A. franciscana* embryos has not been well resolved to date by $^{31}$P-NMR [4] and therefore cannot be compared with data on heat dissipation showing the time-course of metabolic recovery upon reoxygenation [1]. In this study, we used a 300 MHz NMR spectrometer to measure changes in $\Delta pH$ during transitions between aerobic and anoxic conditions.

That protons are consumed during mitochondrial oxidative phosphorylation is supported by both theoretical considerations (e.g., [22,23]) and experimental data [24]. However, for oxidative phosphorylation to contribute to the realalkalinization of $\Delta pH$, in *A. franciscana* embryos, it must be assumed that mitochondrial oxidative phosphorylation is not severely compromised by acidic pH. In addition, an oxidizable carbon source must be available. Even though trehalose-based glycolytic flux is restricted at low pH, [6], sufficient quantities of free amino acids [25] and other organic acids [8,2] are presumably available for oxidation. Thus, mitochondrial oxidative phosphorylation could potentially provide a mechanism to solve the two major problems preventing the resumption of normal development following bouts of anoxia in *A. franciscana* embryos: the resynthesis of triphosphate purine-nucleotides and realalkalinization of $\Delta pH$.

Direct evidence for a role of mitochondria in recovery could be obtained by exposing the embryos to an uncoupler of mitochondrial oxidative phosphorylation during anoxia and measuring $\Delta pH$ and cellular purine nucleotides upon reoxygenation. However, because the outer shell of these embryos is impermeable to virtually anything but water and low-molecular-weight gases [26], alternative approaches are required. We chose to measure the effect of extramitochondrial pH on state 3 and state 4 respiration, P:O flux ratios, and respiratory control ratios (RCRs) of isolated mitochondria using a variety of oxidizable substrates. These data indicate only a minor influence of acidic pH on mitochondrial respiration and, when coupled with direct measurements of $\Delta pH$ during recovery, support a role for oxidative phosphorylation in recovery from anoxia prior to the re-establishment of trehalose-based glycolysis.

2. Materials and experimental procedures

2.1. Source and preparation of embryos

Encysted embryos of *A. franciscana* were from the Great Salt Lake, UT, and obtained through Sanders Brine Shrimp Co. (Ogden, UT). Embryos were hydrated and washed as previously described [9]. Hatching percentage was 78.1 ± 3.7% (mean ± S.E., n = 21), as determined by Anchordoguy et al. [27].

$$\text{2.2. }^{31}\text{P-NMR measurement of } \Delta pH$$

$^{31}$P-NMR was used to estimate $\Delta pH$ in intact *A. franciscana* embryos during transitions between aerobic and anoxic conditions. NMR studies were performed with a 7.05 Tesla 10 cm vertical bore cryomagnet and an AM-300 spectrometer employing a 10 mm broadband digital probe (Bruker Instruments, Billerica, MA). Data files were collected using a tip angle of 30°, a sweep width of 10 KHz, and 2K data arrays. Relaxation delays of 0.1 s were employed. Free induction decays composed of 3000 transients (5 min acquisition time) were exponentially multiplied by 10 Hz line-broadening prior to Fourier transformation. As there was no discernible creatine phosphate present in *A. franciscana* embryos and arginine phosphate was very low, the gamma phosphate resonance of ATP using fully oxygenated embryos was set at $-2.5$ ppm [29]. The difference in chemical shift between $\gamma$ ATP and creatine phosphate is a constant. Field drift in our system was minimal and was excluded by acquisition of proton spectra prior to and following serial phosphorus acquisitions [30]. Intracellular pH was estimated from the chemical shift of inorganic phosphate according to the formula

$$\text{pH} = 6.8 + \log_{10} \left( (\sigma - 3.4)/(5.7 - \sigma) \right),$$

where $\sigma$ is the observed chemical shift of inorganic phosphate relative to creatine phosphate, 6.8 is the $pK$ value for inorganic phosphate, and 5.7 and 3.4 are the chemical shift values for fully dibasic and monobasic phosphate, respectively. These data were derived from titration curves generated in our NMR laboratory and published previously [28]. The limit of resolution of this technique was about 0.05 ppm with our acquisition parameters, corresponding to approx. 0.08 pH units.

Embryos were packed by gravity into a 10 mm glass NMR tube. Approx. 4 to 5 g of hydrated embryos were used for each experiment. Embryos were superfused using the spatial arrangement described by Busa et al. [4] with minor modifications. Oxygen-saturated superfusion solution (0.25 M NaCl) was pumped through the NMR tube with a peristaltic pump at a rate of 2 ml/min. It is appropriate to note that exposure of these embryos to 100% oxygen has no effect on the kinetics of hatching or total hatchability [4]. Anoxia was imposed by halting the superfusion. This method gives essentially the same pH profile as does switching the superfusion to nitrogen-saturated (nominally O$_2$-free) medium; an additional advantage of initiating anoxia by halting the superfusion is the decrease in the time lag prior to onset of the pH decline (see [4]). To initiate recovery from anoxia, superfusion with oxygen-saturated medium was resumed.

2.3. Measurements of mitochondrial respiration

Mitochondria were isolated from *A. franciscana* embryos as previously described [11]. 30 μl of a mitochon-
drial suspension (100–150 μg of mitochondrial protein) was added to 1.47 ml of respiration medium consisting of 500 mM sucrose, 150 mM KCl, 50 mM 3-(N-morpholino)propanesulfonic acid (Mops), 10 mM KH₂PO₄, 2 mM MgCl₂, 1 mM EGTA and 0.5% (w/v) fatty-acid-free bovine serum albumin (BSA; fraction V, Sigma). The pH of the respiration medium was adjusted with either HCl or NaOH and encompassed the range of measured pHᵢ in A. franciscana embryos under both steady-state anoxic and aerobic conditions. No detectable change in pH occurred during the time-course of any given respiratory measurement. Protein concentration was determined according to the methods of Peterson [31].

Oxygen uptake was measured with Strathkelvin model 1302 oxygen electrodes coupled to model 781 oxygen meters. The glass respiration chambers (Strathkelvin RC 350) were held at 25°C and stirred with glass-covered magnetic stir bars. The oxygen electrodes were calibrated before each experiment with air- and nitrogen-saturated solutions. The partial pressure of O₂ (pO₂) in the chambers was digitally recorded with the Datacan V data-acquisition program (Sable Systems, Salt Lake City, UT).

Following temperature equilibration of the mitochondria in respiration medium, 15 μl of substrate (final concentrations: 10 mM glutamate with 0.5 mM malate, 0.2 mM palmitoyl-L-carnitine with 0.5 mM malate, 15 mM pyruvate with 0.5 mM malate, or 5 mM succinate with 10 μM rotenone) was added to the chamber and pO₂ during state 2 respiration recorded for several minutes. Then 45 μl of ADP was added (150 μM final concn.) and pO₂ during state 3 respiration recorded. Measurements of pO₂ continued for at least 5 min after the depletion of ADP during state 4 respiration.

All substrates were added at concentrations determined to be saturating. The small quantity of ‘sparking’ substrate (0.5 mM malate) included with glutamate, palmitoyl-L-carnitine, and pyruvate gave no discernible state 3 respiration when used alone (data not shown). To account for variation among mitochondrial preparations, respiration rates with succinate were measured for each preparation and compared to the mean for all experiments. A percentage adjustment was then applied to the other substrates to allow direct comparison of state 3, state 4, and RCR values among all substrates. Total nucleotide concentration in the cytosol-t (pH unit)-¹ by using the tissue water value of 0.57% for dechorionated A. franciscana embryos (cf. [34]).

2.4. Intracellular buffering capacity

Intracellular buffering capacity in A. franciscana embryos was estimated according to previously described methods [33]. Hydrated embryos were incubated in 0.25 M NaCl for 6 h at room temperature (22–23°C) on an orbital shaker. After dechorionation [11], embryos were blotted of excess water and 0.5 g homogenized in 10 ml of 0.9% (w/v) NaCl in a Thomas Teflon-glass homogenizer. The homogenates were equilibrated to room temperature and constantly stirred during titrations. The volume of NaOH (0.1 N) required to titrate the homogenates from pH 6.7 to 7.7 was recorded. A Radiometer model PHM84 pH meter with a GK 2401C electrode was used to measure pH. Buffering capacity was also measured in the supernatant fraction after the homogenate was centrifuged at 3000 × g for 10 min. Buffering capacity in mmol H⁺ (g wet tissue weight)⁻¹ (pH unit)⁻¹ was converted to mmol H⁺ (1 cytosal)-¹ (pH unit)⁻¹ by using the tissue water value of 0.57% for dechorionated A. franciscana embryos (cf. [34]).

2.5. Calculations of proton consumption during oxidative phosphorylation and statistical analyses

Calculations of proton consumption during oxidative phosphorylation were made using the proton stoichiometry for ATP hydrolysis obtained from the contour diagrams of Alberthy [35]. These pH-dependent values were used to calculate a weighted average across the pH range of 6.7 to 7.7. Intracellular free Mg²⁺ concentration was assumed to be 2 mM for all calculations. All statistical analyses were performed using SPSS (Chicago, IL).

3. Results

3.1. NMR measurements of pHᵢ during transitions between aerobic and anoxic conditions

Fig. 1A and 1B show typical NMR spectra of oxygenated and anoxic embryos, respectively. Dramatic
changes in the magnitude of the purine nucleotide triphosphate (NTP) resonances are apparent. Upon stopping the aerobic superfusion to initiate the transition into anoxia, \( \text{pH}_i \) in *A. franciscana* embryos rapidly declined from 7.70 ± 0.02 to 6.90 ± 0.02 (mean ± S.E., \( n = 9 \)) in the first 20 min and gradually declined to 6.73 ± 0.02 after 60 min (Fig. 2A). These results are similar to those obtained by Busa et al. [4], who observed a decrease in \( \text{pH}_i \) of ≥ 1 pH unit after 60 min of anoxia. Following 2 h of anoxia, \( \text{pH}_i \) rapidly increased upon reoxygenation from 6.72 ± 0.02 to 7.73 ± 0.02 (mean ± S.E., \( n = 6 \)) during the first 20 min and thereafter remained relatively constant (Fig. 2B). Busa et al. [4] did not report changes in \( \text{pH}_i \) during aerobic recovery from anoxia due to the apparent masking of the alkaline \( \text{Pi} \) resonance by a large phosphomonoester peak. Our use of a higher-field instrument allowed for better time resolution and magnetic field homogeneity, enabling us to assign \( \text{pH}_i \) values at each stage during transitions between anoxic and aerobic conditions.

Fig. 1. Representative \( ^{31}\text{P-NMR} \) spectra of *A. franciscana* embryos obtained during aerobic (A) and anoxic (B) conditions. Each spectrum was obtained from 3000 transients during a 5 min acquisition period. Free induction decays were exponentially multiplied using 10 Hz linebroadening prior to Fourier transformation. The abscissa is chemical shift in ppm (1 ppm = 121.5 Hz). Chemical assignments (based on chemical shift) were: 1, phosphomonoesters; 2, inorganic phosphate; 3, unknown (likely an acidic, compartmentalized pool of inorganic phosphate (see Ref. [4])); 4, \( \gamma\text{-NTP} \) (mostly ATP) and \( \beta\text{-NDP} \); 5, \( \alpha\text{-NTP} \) and NDP; 6 and 7, uridine diphosphoglucose; and 8, \( \beta\text{-NTP} \).

Fig. 2. NMR measurements of intracellular pH during transitions from aerobic to anoxic (A) and anoxic to aerobic (B) conditions in encysted embryos of *A. franciscana*. Data are presented as means ± S.E. for nine (A) and six (B) independent experiments.

Fig. 3. Effect of extramitochondrial pH on state 3 and state 4 respiration rates in isolated mitochondria from *A. franciscana* embryos using glutamate (A), palmitoyl-L-carnitine (B), pyruvate (C), and succinate (D) as oxidizable substrates. State 3 (open symbols) and state 4 (filled symbols) respiration rates are presented as means ± S.E. (\( n = 3 \)).
3.2. Rates of mitochondrial respiration as a function of pH

State 3 respiration rates (Fig. 3, open symbols) and RCRs (Fig. 4) showed broad substrate-dependent pH optima. Maximal state 3 respiration rates and RCRs occurred at pH 6.0–6.5 with glutamate, pH 6.8 with pyruvate, and pH 7.0–7.5 with both succinate and palmitoyl-L-carnitine. The highest state 3 respiration rates were obtained with 0.2 mM palmitoyl-L-carnitine and the lowest with 10 mM glutamate (Fig. 3B and 3A, respectively). Overall, state 3 respiration rates differed little at pH 6.3 compared to pH 7.7 (corresponding to anoxic and aerobic pH values at steady state, respectively). Specifically, state 3 rates were higher at pH 6.3 versus 7.7 for glutamate (+15%) but lower when palmitoyl-L-carnitine (−20%), pyruvate (−5%), or succinate (−30%) were used as carbon substrates. State 3 respiration was not observed with alanine, aspartate, proline, or propionate either with or without ‘sparking’ substrates (data not shown). State 4 respiration rates increased significantly (P < 0.0001) with increasing extramitochondrial pH, independent of the substrate used (Fig. 3, filled symbols). An increase in state 4 respiration with pH has been reported for mitochondria from other species [36–39]. Total P:O ratios (Table 1) were near their mechanistic limit (1.5 for succinate and 2.5 for all other substrates used (cf. [40]). P:O ratios did not vary appreciably with pH, except for a decline in total P:O ratios above pH 7.5. P:O ratios were not compromised at low pH values.

3.3. Intracellular buffering capacity

From titrations of A. franciscana homogenates between pH 6.7 and 7.7, we estimated intracellular buffering capac-

<table>
<thead>
<tr>
<th>pH</th>
<th>6.0</th>
<th>6.5</th>
<th>6.8</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate P:O</td>
<td>2.26 ± 0.17</td>
<td>2.21 ± 0.09</td>
<td>2.32 ± 0.08</td>
<td>1.93 ± 0.11</td>
<td>2.15 ± 0.08</td>
<td>1.49 ± 0.04</td>
<td>n.a.</td>
</tr>
<tr>
<td>Palmitoyl-L-carnitine P:O</td>
<td>2.50 ± 0.02</td>
<td>2.51 ± 0.04</td>
<td>2.52 ± 0.06</td>
<td>2.48 ± 0.04</td>
<td>2.46 ± 0.06</td>
<td>2.07 ± 0.03</td>
<td>1.57 ± 0.05</td>
</tr>
<tr>
<td>Pyruvate P:O</td>
<td>2.39 ± 0.14</td>
<td>2.51 ± 0.06</td>
<td>2.59 ± 0.05</td>
<td>2.47 ± 0.08</td>
<td>2.50 ± 0.01</td>
<td>1.53 ± 0.00</td>
<td>1.41 ± 0.04</td>
</tr>
<tr>
<td>Succinate P:O</td>
<td>1.42 ± 0.04</td>
<td>1.39 ± 0.01</td>
<td>1.40 ± 0.04</td>
<td>1.41 ± 0.04</td>
<td>1.44 ± 0.05</td>
<td>1.29 ± 0.04</td>
<td>0.83 ± 0.06</td>
</tr>
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</table>

Data are presented as means ± S.E. (n = 3).
ity to be 37.4 ± 0.5 mmol H⁺ (1 cytosol)⁻¹ (pH unit)⁻¹ and, following low-speed centrifugation of the homogenate, 17.9 ± 0.4 (mean ± S.E., n = 3). If the homogenization procedure artificially increased the total number of titratable sites [41], then measurements of buffering capacity using the centrifuged extract may more accurately assess intracellular buffering capacity. In comparison, Busa [13] calculated a value of 32 mmol H⁺ (1 cytosol)⁻¹ (pH unit)⁻¹ from 3¹P-NMR estimates of pH₁ following 30 min incubations of embryos in solutions with varying percentages of CO₂ [5]. Considering that in vivo estimates of buffering capacity typically are on the order of 2-fold lower than in vitro estimates (e.g., [41,42]), and that these embryos have exceedingly low carbonic anhydrase activity (Hand, unpublished data), it is possible that extending the CO₂ equilibration time might lower the NMR estimate.

3.4. Calculations of proton consumption by oxidative phosphorylation

Based on the preceding range of intracellular buffering capacity, the removal of 18 to 37 mmol H⁺ (1 cytosol)⁻¹ would be needed to elevate pH₁ from 6.7 to 7.7 during the first 20 min of aerobic recovery. In order to estimate the consumption of protons by the oxidation of mono- and dicarboxylic acids in the TCA cycle, we used values of 2 mol H⁺ and 3.5 mol O₂ consumed per mol of dicarboxylic acid oxidized and 1 mol H⁺ and 3 mol O₂ consumed per mol of monocarboxylic acid oxidized [43]. Oxygen consumption during the first 20 min of aerobic recovery was estimated from heat dissipation (3625 mJ (g dry embryo)⁻¹ [1]) using a measured oxycaloric value of −226 kJ heat (mol O₂)⁻¹ [2]. We calculated that 14.3 mmol O₂ (1 cellular water)⁻¹ are consumed during this period, assuming 1.12 g H₂O (g dry embryo)⁻¹ (cf. [34]). In comparison, a similar value of 14.8 mmol O₂ (1 cellular water)⁻¹ is obtained from respiration data reported by Clegg [44] during the first 20 min of recovery following 2 h of anoxia. Thus, between 4.79 and 8.18 mmol H⁺ (1 cytosol)⁻¹ could be consumed during the first 20 min of recovery if aerobic metabolism during this period were fuelled by mono- or dicarboxylic acids, respectively.

From the data of Stocco et al. [45], a total of approx. 3.74 mmol NTP equivalents per liter of cytosol are synthesized during the first 20 min of recovery, assuming (1) 55% cellular water (chorionated embryos (cf. Ref. [34])), (2) free interconversion of all purine mono- and diphosphate nucleotides to ATP, and (3) synthesis of all NTP from NDP or NMP. The change in adenine nucleotide concentrations during the first 20 min of aerobic recovery was calculated to be 2.10 mmol ATP equivalents (1 cytosol)⁻¹ (Fig. 1 in [45]). The estimate of the change in guanine nucleotides (1.64 mmol GTP equivalents (1 cytosol)⁻¹) required the use of data collected over a different time-course (Table 5 in Ref. [45]) than that used in the present study. Using a weighted average of 0.64 mmol H⁺ consumed per mmol ADP phosphorylated to ATP between pH 6.7 and pH 7.7 (or 1.28 mmol H⁺ consumed per mmol AMP phosphorylated to ATP), the consumption of 4.79 mmol H⁺ (1 cytosol)⁻¹ can result from the resynthesis of purine di- and triphosphate nucleotides. When combined with the proton consumption from the oxidation of mono- or dicarboxylic acids in the TCA cycle, a substantial fraction of the proton consumption required to elevate pH₁ from 6.7 to 7.7 during the first 20 min of aerobic recovery probably results from mitochondrial metabolism. These results are summarized in Table 2.

4. Discussion

In anoxia-tolerant vertebrates ([46] and references therein) and some invertebrates ([47,48] and references therein), phosphorylation potential is conserved during anoxia, but in A. franciscana embryos, the majority of cellular ATP is hydrolyzed within the first hours of anoxia [45,6,17]. The hydrolysis of ATP stores coupled with the low to moderate intracellular buffering capacity and a proton-impermeable shell contribute to one of the largest changes in pH₁ ever reported [4]. This pH₁ transient contributes to the induction of a quiescent state (reviewed in [14]) that can last in excess of one year [44]. It has been suggested that, upon reoxygenation, resynthesis of ATP may contribute to the elevation of pH₁ during recovery [12,13,1]. As pointed out earlier, for mitochondrial oxidative phosphorylation to contribute to the realkalinization of pH₁, the following conditions must be met: (1) an oxidizable carbon source must be available and (2) oxidative phosphorylation cannot be severely inhibited at acidic pH₁ (pH 6.3–6.8). Regarding the first condition, Provost [49] calculated that the amount of carbon substrate required to support the observed heat dissipation during the first 20 min of recovery could be met by several intracellular sources (e.g., amino acids, lactate) prior to the reinitiation of glycolysis. The present study indicates that the second condition may also be met. As judged by state 3 respiration rates, P:O ratios, and RCRs, mitochondrial oxidative
phosphorylation is not compromised at the acidic pH characteristic of pH₁ in *A. franciscana* embryos at the beginning of recovery. Thus, it is likely that oxidative phosphorylation contributes to both the realkalinization of pH₁ and resynthesis of purine triphosphate nucleotides during recovery. Calculations indicate that a large fraction (26 to 72%, Table 2) of the proton consumption required to elevate pH₁ from 6.7 to 7.7 during the first 20 min of aerobic recovery may result from the resynthesis of purine triphosphate nucleotides and oxidation of organic acids to CO₂ and H₂O in the TCA cycle.

Of the carboxylic acids examined in this study, both succinate and pyruvate were capable of sustaining high rates of mitochondrial respiration. Because lactate accumulates to a small extent in *A. franciscana* embryos during anoxia [2], pyruvate could be a substrate for mitochondrial oxidation during recovery. Based on the pH profile of state 3 respiration, pyruvate oxidation would be at or near its pH optimum at the beginning of aerobic recovery (pH₁ < 6.7). Of the four amino acids tried (alanine, aspartate, proline, and glutamate), glutamate was the only one that supported state 3 respiration. Glutamate concentration is approx. 8 mM in *A. franciscana* embryos [25]. Of all the substrates examined in this study, palmitoyl-L-carnitine supported the optimum at the beginning of aerobic recovery (pH₁ ~ 6.7). It is feasible that this first phase in metabolic recovery correlates closely with the increase in heat dissipation during recovery. Calculations indicate that a large fraction of heat dissipation indicate recovery is biphasic and may involve separate metabolic events [1]. Considering that glycolysis is inhibited by acidic pH, at the onset of aerobic recovery [6], it is feasible that this first phase in metabolic recovery (first 20 min) is supported by the oxidation of amino or other organic acids. Once pH₁ is partially realkalinized, trehalose-based metabolism can resume and may produce the second phase of heat dissipation. Chemical measurements of cellular ATP show that there is a rapid resynthesis following reoxygenation; within 15 to 20 min, levels are comparable to those of the preanoxic state [45,52]. These observations are consistent with the rapid increase in magnitude of the NTP resonances during recovery observed with NMR (data not shown). The temporal correlations among the increases in heat dissipation, cellular ATP, and pH₁, as well as the relative insensitivity of state 3 respiration to acidic pH, are all consistent with a role for oxidative phosphorylation in the realkalinization of pH₁ and synthesis of purine triphosphate nucleotides during recovery from anoxia in these embryos.

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