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Kurt E. Kwast
University of Colorado Boulder

Steven C. Hand
University of Colorado Boulder

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Oxygen and pH regulation of protein synthesis in mitochondria from *Artemia franciscana* embryos

Kurt E. KWAST* and Steven C. HAND*†‡

*Department of Environmental, Population, and Organismic Biology, and †Graduate Program in Molecular Biophysics, University of Colorado, Boulder, CO 80309-0334, U.S.A.

To identify factors responsible for the down-regulation of mitochondrial biosynthetic processes during anoxia in encysted *Artemia franciscana* embryos, the effects of oxygen limitation and pH on protein synthesis were investigated in isolated mitochondria. At the optimal pH of 7.5, exposure of mitochondria to anoxia decreases the protein synthesis rate by 79 %. Rates were suppressed by a further 10 % at pH 6.8, the intracellular pH (pH_i) measured under anoxia *in vivo*. Matrix pH, measured under identical conditions, was 8.43 ± 0.01 at an extramitochondrial pH of 7.9 (mean $\pm$ S.E.M., $n = 3$), 8.05 ± 0.01 at pH 7.5, and 7.10 ± 0.01 at pH 6.8. The matrix pH did not vary ($P \geqslant 0.20$) as a function of oxygen availability during the 1 h assays. Intramitochondrial purine nucleotides varied little as a function of pH. In contrast, after 1 h of protein synthesis under anoxia, ATP levels decreased by up to 40 %,. whereas AMP, ADP and GDP concentrations increased, and GTP and GMP concentrations remained relatively constant. The addition of 1 mM ATP at the onset of anoxia maintained the ATP/ADP ratio at the aerobic value, but did not stabilize the GTP/GDP ratio or rescue rates of protein synthesis. Thus, at present, we cannot eliminate the possibility that the decrease in the GTP/GDP ratio during anoxia may contribute to the suppression of protein synthesis. The effect of anoxia was reversible; the rate of protein synthesis upon reoxygenation after a 30 min bout of anoxia was comparable ($P = 0.14$) with the pre-anoxic rate (193 ± 17 and 174 ± 6 pmol of leucine per mg of protein respectively; mean $\pm$ S.E.M., $n = 3$). The array of mitochondrial translation products did not differ qualitatively as a function of either oxygen availability or pH. Finally, similar pH profiles for protein synthesis were obtained with either [^{3}H]leucine or [^{3}H]histidine (known to use different transporters). Consequently, it is improbable that the pH-sensitivity of protein synthesis can be explained by a specific protein effect on the import of the radiolabelled amino acid used. In summary, both oxygen limitation and acidic pH suppress rates of mitochondrial protein synthesis and are likely to contribute to the arrest of mitochondrial anabolic processes during anoxia-induced quiescence in *A. franciscana* embryos.

INTRODUCTION

Encysted gastrulae of the brine shrimp *Artemia franciscana* are perhaps the quintessential example of an animal capable of acute metabolic arrest in response to anoxia [1]. These embryos can survive total oxygen deprivation for periods of up to 2 years [2]. In response to anoxia, the embryos enter a quiescent (dormant) state in which energy flow is severely suppressed to values approaching ametabolic [3–6]. An anoxia-induced acidification of intracellular pH (pH_i) by $\geqslant 1$ pH unit [7.8] is thought to promote metabolic arrest of both catabolic ([9,10], reviewed in [4]) and anabolic processes in the cytoplasm [11–13]. For example, a direct role of pH_i acidification has been implicated in the suppression of trehalose metabolism [10,14,15], the primary catabolic pathway supporting pre-emergent development in *A. franciscana* embryos ([16–19], reviewed in [20]). Based on results with a cell-free translation system from these embryos, acidic pH can inhibit components of the cytoplasmic translational machinery; pH-induced covalent modification was also implicated in this down-regulation [12]. In addition, experiments *in vivo* have provided indirect evidence suggesting that pH_i acidification may also affect mitochondrial protein synthesis; cytochrome *c* oxidase (COX) synthesis is suppressed either by anoxia or by the acidification of pH_i with 60 % CO_2 in the presence of 40 % O_2 (aerobic acidosis) [1]. In the present study, we examined the effect of both oxygen limitation and pH on protein synthesis in isolated mitochondria to further our understanding of the regulation of whole-cell metabolism during anoxia-induced quiescence.

In a previous study [21], isolated mitochondria from *A. franciscana* embryos were acutely sensitive to changes in extramitochondrial pH; an 80 % decrease in protein synthesis rates was observed when pH was lowered from the optimum pH of 7.5 to 6.8, the pH_i observed after short-term anoxia *in vivo* [7,8]. The mechanism by which changes in extramitochondrial pH affect rates of mitochondrial protein synthesis is not known. Potential mechanisms include changes in transport (amino acids, adenine nucleotides, or other substrates), the intramitochondrial energy status, or direct influences of pH on the mitochondrial translational machinery. In this study, we examined the effect of pH on intramitochondrial levels of purine nucleotides and determined the change in matrix pH when extramitochondrial pH was varied across the relevant physiological range (pH 6.8–8.0).

On the basis of studies *in vivo* with intact embryos, it can be difficult in some cases to differentiate between the effects of oxygen limitation itself and pH_i acidification on cellular metabolism. For example, a role for oxygen limitation in the suppression of protein synthesis is not directly evident from experiments *in vivo* examining COX synthesis under anoxia and aerobic acidosis [1]. Both anoxia and aerobic acidosis result in the acidification of pH_i, induction of a biochemically similar quiescent state, and the suppression of COX synthesis to a similar extent [1]. This last observation suggests that pH_i

Abbreviations used: BCECF/AM, acetoxymethyl (AM) ester of 2,′7′-bis-(2-carboxyethyl)-5-carboxyfluorescein; COX, cytochrome *c* oxidase; MIM, mitochondrial isolation medium; PSM, protein synthesis medium; pH_i, intracellular pH.

‡ To whom correspondence should be addressed.

acidification is sufficient, in the absence of oxygen limitation, to suppress COX synthesis. Other experiments *in vivo*, in which pH_i was alkalinized during anoxia by the addition of ammonia, show that heat dissipation [3] and COX activity [1] increase in response to elevated pH_i during anoxia. However, in these embryos it is not easy to alter oxygen supply and keep pH_i constant *in vivo*. The use of isolated mitochondria offers the distinct advantage of being able to manipulate both oxygen supply and pH independently during metabolic studies.

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity commercially available and were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. Sucrose was purchased from Pfanstiehl (Waukegan, IL, U.S.A.), and radiolabelled amino acids ([^{3}H]histidine, [^{3}H]leucine and [^{35}S]methionine) were obtained from New England Nuclear (Boston, MA, U.S.A.). The acetoxymethyl (AM) ester of 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Silicone oil (grades 550 and 710) was a gift from Dow Corning (Midland, MI, U.S.A.). Encysted *A. franciscana* embryos (Optima Grade) were obtained from Sanders Brine Shrimp Co. (Ogden, UT, U.S.A.).

Measurements of mitochondrial protein synthesis

Mitochondria were isolated as previously described [21]. For anoxic assays, mitochondria (0.5–1.0 mg of protein/ml) were aerobically preincubated for 10 min at room temperature (22 °C to 23 °C) in protein synthesis medium (PSM; [21]) that contained ADP (0.5 mM) and an oxidizable carbon source (5 mM succinate) but lacked amino acids, [^{3}H]leucine or BSA. This period of time is sufficient for phosphorylation of all added ADP [21]. The BSA was excluded from the initial medium to prevent frothing upon bubbling of the mixture with N_2 (see below). The pH of the initial medium was adjusted to pH 6.8, 7.5 or 8.0 with HCl or NaOH. After the aerobic preincubation, a 300 μl sample was removed for the analysis of initial levels of intramitochondrial adenine and guanine nucleotides. The vials were then transferred to a glove box pre-equilibrated with 100 % N_2, and the mitochondrial suspension was vigorously bubbled with N_2 for 5 min. This period of time was sufficient to drive off all detectable O_2 with the volumes used (< 500 μl), as assessed by a Clark-type oxygen electrode. An anoxic solution containing unlabelled amino acids, [^{3}H]leucine and BSA (fatty-acid-free; fraction V) was then added to initiate the protein synthesis assay (see [21] for final concentrations in the PSM). At designated time points, samples were removed and the incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble protein was quantified as previously described [21]. Assays under aerobic conditions were performed simultaneously with the anoxic assays.

To exclude the possibility that the observed effects of extramitochondrial pH on protein synthesis were due to pH-induced alterations in [^{3}H]leucine transport, additional experiments were performed with [^{3}H]histidine (known to enter the mitochondrion via a different carrier [22,23]). Isolated mitochondria (0.5–1.0 mg of protein/ml) were incubated in PSM containing 150 μM [^{3}H]histidine (5 μCi/nmol) in place of 60 μM [^{3}H]leucine. This concentration of histidine was determined to be saturating (results not shown). After 1 h incubations at 25 °C, [^{3}H]histidine incorporation was quantified by previously reported methods [21]. The pH of the PSM was varied between pH 6.0 and 8.0 in 0.2 pH-unit increments by titration with either HCl or NaOH.

Measurements of intramitochondrial pH

Intramitochondrial pH was estimated by using the pH-sensitive fluorescent dye BCECF by the methods of Jung et al. [24], with minor modifications. The BCECF/AM was diluted with anhydrous DMSO immediately before incubations; the final concentration of DMSO in the mitochondrial incubations was not above 0.1 %. Mitochondria were incubated at 25 mg of protein/ml of mitochondrial isolation medium (MIM; [21]) containing 10 μM BCECF/AM for 20 min at 25 °C. The AM ester of BCECF is readily taken up and hydrolysed within the matrix by an esterase, probably phospholipase A [24], and the resulting charged probe is retained within the matrix. After dye loading, the mitochondria were diluted 10-fold with ice-cold MIM and centrifuged at 9000 ***g*** for 10 min at 4 °C to wash away unhydrolysed dye. The pellet was resuspended with MIM to obtain the original stock concentration and stored on ice until use. No significant ($P \geqslant 0.46$) increase in the fluorescence intensity occurred after 1 h at 25 °C or after 3 h on ice (results not shown). Previous studies have shown that less than 10 % of the matrix BCECF is lost after 5 h on ice with loading conditions and BCECF concentrations similar to those used in the present study [24]. Mitochondrial State 3 and State 4 respiration rates, respiratory control ratios (RCRs: State 3/State 4) and P:O ratios for dye-loaded mitochondria were not significantly different ($P \geqslant 0.32$) from those of controls (results not shown).

Fluorescence was recorded with an Aminco SLM-48000 spectrofluorimeter with 4 nm slit widths and a temperature-controlled stirred cell holder maintained at 25 °C. The spectrofluorimeter was interfaced with a Dell system 325 computer using the 48 000 program supplied by Aminco. Fluorescence emission at 535 nm was monitored as a function of the excitation wavelength to determine the excitation $\lambda_{max.}$ for dye-loaded mitochondria and detergent-lysed mitochondria. The excitation maximum for BCECF-loaded mitochondria was 504 nm, whereas that of the detergent-lysed mitochondria was 500 nm (results not shown). This red shift in the spectrum for internalized dye has been previously reported for both isolated mitochondria [24] and intact cells [25].

Detergent-lysed mitochondria was used to generate a calibration curve relating pH to the ratio of fluorescence at the excitation maximum (500 nm) divided by that at 450 nm, an excitation wavelength that promotes a fluorescence insensitive to pH. Briefly, 100 μl of the mitochondrial suspension was placed in a 4 ml quartz cuvette containing 2.9 ml of 100 mM KCl, 20 mM Mops and 0.07 % (v/v) Triton X-100. The pH of the medium was varied from pH 6.0 to 8.5 in 0.2 pH-unit increments. The ratio of fluorescence was plotted against the pH of the medium and fitted with a third-order polynomial regression ($r^2 = 0.998$; results not shown). The matrix pH for intact mitochondria was obtained from this calibration curve. Sucrose, which was used in the assay medium for intact mitochondria, was omitted from the lysis buffer because it has been shown to increase the fluorescence ratio of BCECF in solution [24]. Because sucrose does not penetrate the mitochondrial inner membrane to a significant extent, it does not affect the fluorescence of the internalized dye [24].

Matrix pH was measured during protein synthesis assays performed under both aerobic and anoxic conditions at a final mitochondrial concentration of 0.5 mg/ml. For measurements of matrix pH during the anoxic protein synthesis assays, the PSM containing mitochondria was deoxygenated with N_2 in a glove

box. Before the cuvettes were removed from the glove box, 1 ml of mineral oil was layered over 3 ml of the anoxic mitochondrial suspension.

Measurements of intramitochondrial purine nucleotides

Mitochondria were incubated in PSM under either aerobic or anoxic conditions as described above and, at designated time points, were isolated from the PSM by the silicone-oil centrifugation method (cf. [26]). In brief, 300 μl samples (0.35–0.50 mg of protein/ml) were layered over 300 μl of silicone oil (a mixture of Dow Corning grades 550 and 710) with a specific gravity slightly higher than that of the PSM. Below the oil was a 300 μl layer of 7% $HClO_4$ containing 500 mM sucrose. Sucrose was added to the $HClO_4$ to make the specific gravity of this layer greater than that of the overlying oil. The oil and $HClO_4$ layers were pre-chilled to 0 °C in 1.7 ml microfuge tubes. After addition of samples, the tubes were immediately centrifuged at 15000 ***g*** for 2 min at 4 °C in an Eppendorf 5414 centrifuge. This procedure rapidly separates mitochondria from their medium and serves to disrupt the organelles immediately and quench metabolic reactions as the mitochondria pass into the $HClO_4$.

After centrifugation, the tubes were placed on ice and the top layer was discarded. Samples of the $HClO_4$ extract (containing nucleotides) were taken by gently inserting a pipette tip through the oil, so as not to mix the two remaining layers. The $HClO_4$ extracts were neutralized on ice with 5 M K_2CO_3, centrifuged at 12000 ***g*** for 10 min at 4 °C to remove the perchlorate salts, and stored at −70 °C for subsequent analysis. Recovery of mitochondrial protein in the $HClO_4$ pellet was typically greater than 90%. Protein content of the pellet was determined by the methods of Peterson [27]. Intramitochondrial adenine (AMP, ADP and ATP) and guanine (GMP, GDP and GTP) nucleotide concentrations were measured by HPLC utilizing weak anion exchange, by using the methods of Rees and Hand [28] with minor modifications. The mobile phase consisted of 65 mM potassium phosphate (pH 6.5)/acetonitrile (1:2, v/v). Portions (200 μl) of filtered (0.45 μm-pore) samples were injected and eluted isocratically at a flow rate of 1.75 ml/min. Adenine and guanine nucleotides were identified by co-elution with standards and quantified by integration of peak area.

Similarly to previous reports [29], homogenization of the acid-precipitated mitochondrial pellet increased the extraction of matrix purine nucleotides by 18.8 ± 3.2% (mean ± S.E.M., n = 3). Measured purine nucleotide concentrations were corrected by this percentage when pellets were not homogenized. Corrections were also made for the volume of solution trapped in the mitochondrial intermembrane space and carried into the $HClO_4$ layer, as determined with [^{14}C]sucrose by previously described methods [26]. The sucrose-permeable volume in the $HClO_4$ was 3.75 ± 0.04 ml/g of protein (mean ± S.E.M., n = 9) and did not vary as a function of pH of the medium (P = 0.47) or oxygen availability (P = 0.39) after 1 h incubation (results not shown). Assuming equilibration of the intermembrane space with bulk solution and the complete conversion of all added ADP (0.5 mM) into ATP by oxidative phosphorylation during the aerobic preincubation, a contribution of 1.87 ± 0.02 nmol of ATP/mg of protein (mean ± S.E.M., n = 9) may be attributable to ATP within the intermembrane space. This value was subtracted from the ATP values reported.

Fluorography of mitochondrial translation products

The effects of both extramitochondrial pH and oxygen availability on the array of translation products synthesized were assessed with fluorography. [^{35}S]Methionine at 60 μM (determined to be saturating; results not shown) was used in place of 60 μM [^{3}H]leucine in the incubations (see above). At the end of the 60 min incubations, 5 vol. of PSM containing 5 mM methionine (unlabelled) was added and samples were incubated for a further 5 min at 25 °C. After centrifugation at 12000 ***g*** for 10 min (4 °C), the mitochondria were resuspended in the initial volume of PSM, containing 5 mM methionine but lacking sucrose, and incubated for 5 min at 25 °C. The sucrose was omitted from the final suspension to prevent it from precipitating upon addition of acetone. Then 4 vol. of ice-cold acetone was added, and the suspension was incubated at −70 °C for at least 4 h to precipitate protein. Samples were centrifuged at 12000 ***g*** for 10 min at 4 °C. The pellet was resuspended in SDS sample buffer [21] at approx. 2 mg of protein/ml, incubated for 4 h at 37 °C, and boiled for 2 min. Samples containing equal amounts of radioactivity (10^6 c.p.m.) were loaded into separate lanes of an SDS/Polyacrylamide gel containing urea and electrophoresed [21]. The gel was stained for total protein with 0.5% Coomassie Blue, destained overnight, and prepared for fluorography [21]. The dried gel was incubated with Kodak XAR X-ray film for 7 days at −70 °C.

Statistical analyses

Analysis of covariance (ANCOVA) was used to compare rates of mitochondrial protein synthesis among various treatments, and analysis of variance (ANOVA) was used to compare intramitochondrial adenine and guanine nucleotide concentrations (SAS Institute, Cary, NC, U.S.A.). Adjustments of α values (Bonferroni) were made when multiple comparisons were performed. Estimates given for variance among different mitochondrial preparations (see Figure legends and the text) were based on variance components obtained with the SAS VARCOMP procedure and the restricted maximum-likelihood method, using the treatment as a fixed effect and mitochondrial preparation as a random effect nested within treatments.

RESULTS

Rates of mitochondrial protein synthesis

Rates of protein synthesis were significantly lower under anoxia compared with aerobic controls for all pH values and at all time points examined (Figure 1). Similar pH profiles, however, were obtained under both aerobic and anoxic conditions, with pH 7.5 yielding the highest rates and pH 6.8 the lowest. Except for the aerobic assays at pH 7.5, the time course of incorporation was not linear. An analysis of the most linear portion of each time course (1–10 min, Table 1) indicates a rapid suppression in protein synthesis in response to either anoxia (up to 60% at pH 7.5, aerobic versus anoxic) or changes in pH (49% decrease at pH 6.8 compared with pH 7.5, aerobic). Maximal suppression of protein synthesis was 89%, as calculated over the entire 60 min period for the anoxic pH 6.8 treatment compared with the aerobic pH 7.5 treatment.

To assess the recovery of protein synthesis capacity after anoxia, rates were measured aerobically after a 30 min anoxic preincubation of the mitochondria. Similar rates (P = 0.14) were obtained before and after anoxia (193 ± 17 and 174 ± 6 pmol of leucine per mg of protein respectively; mean ± S.E.M., n = 3). Thus the effect of anoxia on mitochondrial protein synthesis was reversible upon reoxygenation.

When [^{3}H]histidine was substituted for [^{3}H]leucine in the protein synthesis assays, the pH profiles were very similar (Figure 2). A small shift in the pH optimum was observed between the

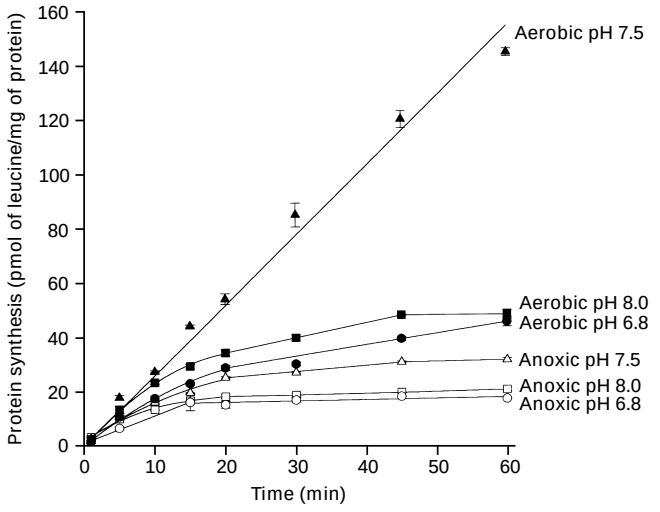

Figure 1 Effect of pH and oxygen limitation on protein synthesis rates of isolated *A. franciscana* mitochondria

After aerobic preincubation (10 min) of mitochondria in PSM containing ADP and succinate, samples were transferred at zero time to either aerobic or anoxic conditions. An amino acid mixture (0.3 mM each) containing [^{3}H]leucine (60 μM) was then added to initiate the protein synthesis assay. Data are means ± S.E.M. for three assays. On the basis of values for both aerobic and anoxic assays at pH 7.5 (60 min), variance in [^{3}H]leucine incorporation among multiple mitochondrial preparations was 8% ($n = 3$; results not shown).

two experiments, but virtually identical absolute differences between minimal and maximal rates were obtained. Maximal rates of protein synthesis were observed at approx. pH 7.7 and were nearly 6 times higher than the rates measured at pH 6.8 (the pH_i measured under anoxia *in vivo*). A recent study [8] using ^{31}P-NMR indicates that the pH_i of aerobic embryos is 7.70 ± 0.06 (mean ± S.E.M., $n = 9$). Thus mitochondrial protein synthesis *in vivo* would be operating at a pH close to the pH optimum determined *in vitro*.

Matrix pH as a function of extramitochondrial pH

Measurements of matrix pH as function of extramitochondrial pH during protein synthesis assays were performed under both aerobic and anoxic conditions at several pH values and are shown in Table 2. After a 10 min aerobic preincubation with ADP and succinate, no significant ($P \geqslant 0.20$) change in matrix pH was observed during the 1 h assays (results not shown). The ΔpH ranged from 0.31 pH unit at an extramitochondrial pH of 6.79 to 0.56 at pH 7.87. Considering that *in vivo* pH_i is acidified by $\geqslant 1$ pH unit upon oxygen deprivation [7,8] correspondingly large changes in matrix pH are likely to occur *in vivo* during anoxia. Remarkably, after 1 h of anoxia matrix pH was not significantly different ($P \geqslant 0.20$) from that of aerobic controls. These data suggest that mechanisms may exist to suppress proton leak across the mitochondrial inner membrane during anoxia. In comparison, Andersson et al. [30] have shown that ΔpH is maintained for 30 min of anoxia in rat liver mitochondria.

Intramitochondrial purine nucleotides

Under aerobic conditions, intramitochondrial ATP levels increased markedly (> 15-fold) in the first 15 min after addition of mitochondria to PSM containing ADP and succinate, and thereafter remained relatively constant during the 1 h protein

Table 1 Initial rates of protein synthesis in *A. franciscana* mitochondria as a function of oxygen availability and extramitochondrial pH

Data are expressed as pmol of leucine/h per mg of mitochondrial protein and are means ± S.E.M. for three determinations. Rates were determined as the average incorporation of [^{3}H]leucine over the first 10 min of the protein synthesis assay

	Protein synthesis rate (pmol/h per mg)	
pH	Aerobic	Anoxic
6.8	83 ± 7	44 ± 18
7.5	163 ± 11	66 ± 8
8.0	99 ± 12	45 ± 8

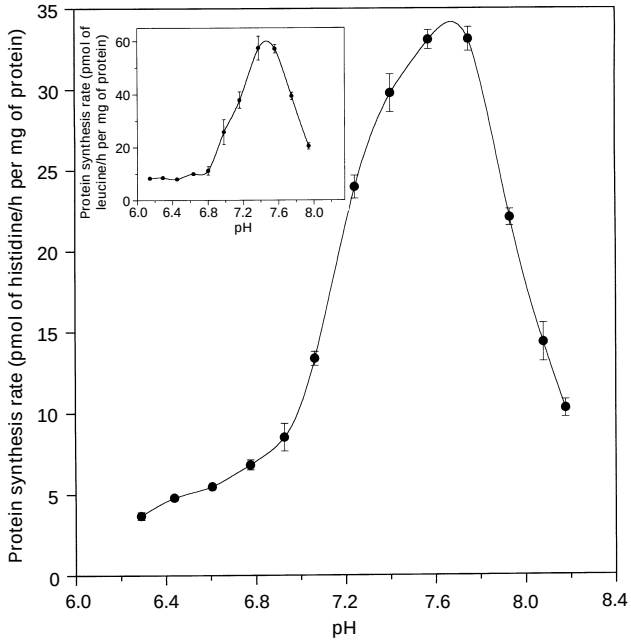

Figure 2 Effect of pH on protein synthesis rates of isolated *A. franciscana* mitochondria

Rates were assessed by monitoring the incorporation of either [^{3}H]histidine or [^{3}H]leucine (inset; after [21]) into trichloroacetic acid-insoluble proteins after a 1 h incubation at 25 °C. Data are means ± S.E.M. for three 1 h incubations.

Table 2 Matrix pH as a function of extramitochondrial pH and oxygen availability after a 1 h protein synthesis assay using isolated mitochondria from *A. franciscana* embryos

Data are means ± S.E.M. for three determinations at each pH. Variance in matrix pH among multiple mitochondrial preparations was < 1% ($n = 3$; results not shown).

	Matrix pH	
Extramitochondrial pH	Aerobic	Anoxic
6.79	7.10 ± 0.02	7.09 ± 0.01
7.49	8.05 ± 0.01	8.05 ± 0.01
7.87	8.43 ± 0.01*	8.45 ± 0.01

* Because the fluorescence intensity of BCECF changes little in response to pH above pH 8.3, the absolute value is less reliable in this range.

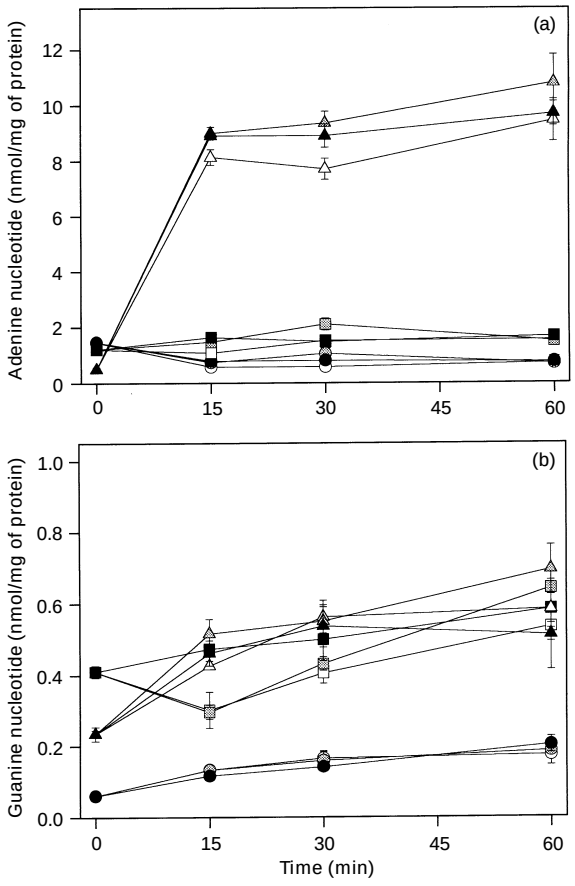

Figure 3 Effect of extramitochondrial pH on intramitochondrial levels of adenine (a) and guanine (b) nucleotides during protein synthesis in isolated mitochondria from *A. franciscana* embryos under aerobic conditions

To initiate the assay, freshly isolated mitochondria were added directly to PSM (containing 0.5 mM ADP and 5 mM succinate). Circles denote respective monophosphates, squares diphosphates, and triangles triphosphates. White symbols represent pH 6.8, stippled symbols pH 7.5, and black symbols pH 8.0. Data are means ± S.E.M. for at least three assays.

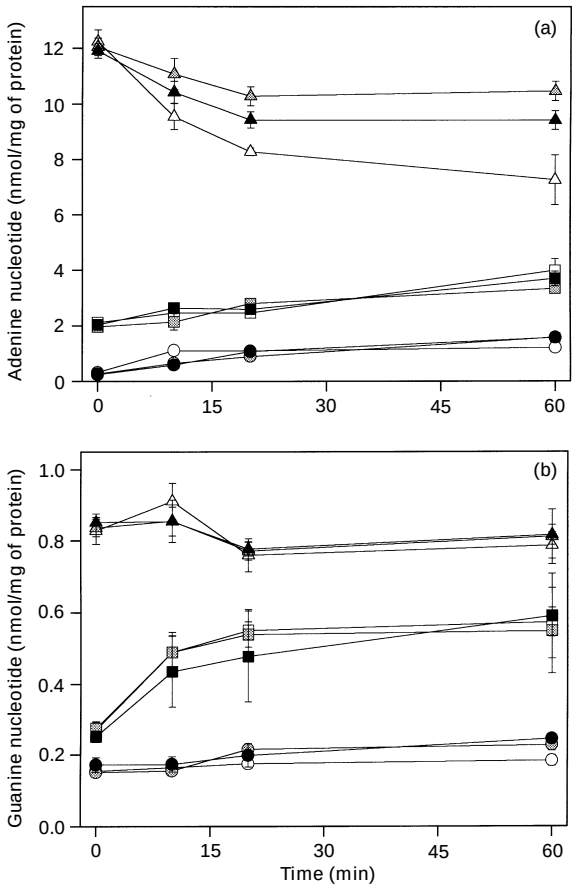

Figure 4 Effect of extramitochondrial pH on intramitochondrial levels of adenine (a) and guanine (b) nucleotides during protein synthesis in isolated mitochondria from *A. franciscana* embryos under anoxic conditions

Mitochondria were aerobically preincubated (10 min) with ADP and succinate before the onset of anoxia. Circles denote respective monophosphates, squares diphosphates, and triangles triphosphates. White symbols represent pH 6.8, stippled symbols pH 7.5, and black symbols pH 8.0. Data are means ± S.E.M. for at least three assays.

synthesis assays (Figure 3a). It is important to note that in these aerobic experiments zero time represents the first point at which mitochondria were provided with succinate and ADP since their isolation. High rates of net ADP and ATP accumulation have been previously reported for rat liver mitochondria, and such increases presumably involve the ATP(ADP)-Mg/P_i carrier [31]. Except for the initial (15 min) increase ($P < 0.0001$) in ATP with all pH treatments, no significant changes ($P \geqslant 0.10$) in intramitochondrial adenine nucleotides (AMP, ADP or ATP) were observed as a function of extramitochondrial pH or during the 1 h time course of protein synthesis. Similar levels of total matrix adenine nucleotides have been reported for rat liver mitochondria [26]. Both GMP and GTP levels increased during the first 15 min, but thereafter remained relatively constant and did not vary as a function of pH ($P \geqslant 0.09$) (Figure 3b). GDP concentrations did not vary significantly with time of incubation or pH ($P > 0.39$), except for a significant difference ($P = 0.02$) between pH 7.5 and 8.0 at 15 min. Because matrix purine nucleotide concentrations varied little as a function of pH, other factors must be responsible for the change in rates of mitochondrial protein synthesis observed in response to pH.

During the 1 h protein-synthesis assays under anoxia, intramitochondrial ATP levels decreased ($P \leqslant 0.01$), whereas AMP, ADP and GDP levels increased ($P \leqslant 0.01$) for all pH treatments (Figures 4a and 4b). GTP and GMP levels remained unaltered ($P > 0.05$) during this period. After 1 h of anoxia, concentrations of both adenine and guanine nucleotides were not different among the pH treatments, except for higher ATP concentrations at pH 7.5 versus pH 6.8 ($P = 0.03$). Total (mono-, di- and triphosphate) adenine and guanine nucleotide pools did not differ among pH treatments ($P > 0.05$), either before or after 1 h of anoxia. Thus no net loss of intramitochondrial purine nucleotides was observed during 1 h of anoxia.

Both ATP/ADP and GTP/GDP ratios declined during the 1 h protein synthesis assays under anoxia. ATP/ADP (all pH treatments pooled) declined from 5.90 ± 0.11 to 2.50 ± 0.38 (mean ± S.E.M., $n = 3$), and GTP/GDP declined from 3.16 ± 0.11 to 1.41 ± 0.03. Variance among mitochondrial preparations in both ATP/ADP and GTP/GDP ratios was $< 1\%$ ($n = 2$; results not shown). To determine whether the decrease in protein synthesis rates during anoxia could be a result of the lowered energy status of mitochondria, we measured rates of mitochondrial protein synthesis and levels of matrix purine nucleotides in the presence of (1) an ATP-regenerating system (5 mM phosphoenolpyruvate, 10 units of pyruvate kinase/ml)

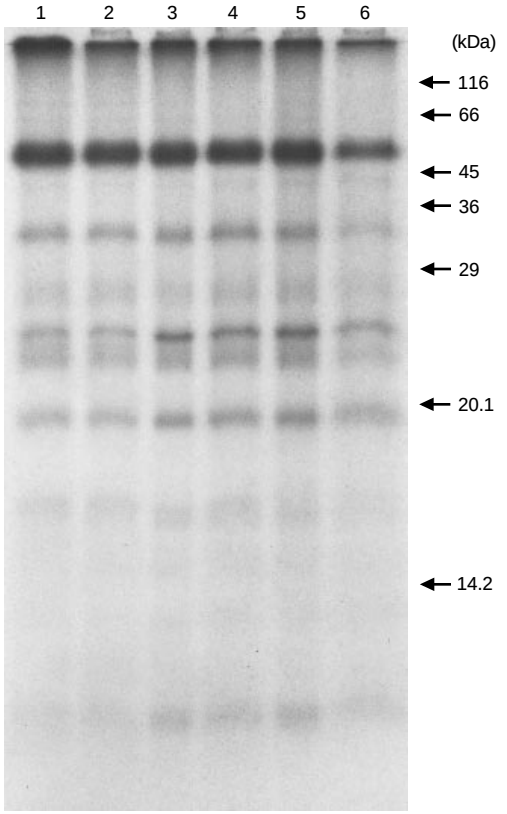

Figure 5 Effect of pH and oxygen limitation on the array of translation products synthesized by isolated *A. franciscana* mitochondria

To facilitate the direct comparison of mitochondrial translation products from different treatments, equal amounts of radioactivity (10^6 c.p.m.) were loaded from each treatment. The origin is at the top of the Figure. Positions of molecular-mass standards (kDa) are indicated on the right-hand side. Lanes: 1, aerobic pH 6.8; 2, anoxic pH 6.8; 3, aerobic pH 7.5; 4, anoxic pH 7.5 ; 5, aerobic pH 8.0; 6, anoxic pH 8.0.

and (2) 1 mM ATP added at the onset of the anoxic assay. Rates of mitochondrial protein synthesis were not enhanced ($P = 0.65$) with either treatment compared with controls (assayed at 15 min intervals for 1 h; results not shown). However, the addition of ATP maintained the ATP/ADP ratio at the aerobic value ($P = 0.28$) to the end of the 1 h anoxic assay. Thus we can rule out any influence of adenine nucleotide changes on rates of protein synthesis during anoxia. Neither treatment influenced GTP/GDP ratios ($P > 0.05$).

Mitochondrial translation products

No qualitative differences were observed in the array of mitochondrial translation products synthesized as a function of either oxygen availability or extramitochondrial pH (Figure 5). On the basis of the apparent molecular masses, the three largest mitochondrial translation products correspond to COX subunits I, III and II respectively. Definitive identification of these translation products awaits antibody production and immunoprecipitation.

DISCUSSION

In this study, we examined the direct effect of anoxia and changes in extramitochondrial pH on rates of mitochondrial protein synthesis *in vitro*. In response to anoxia, there was a rapid (within 5 min) and severe suppression of protein synthesis rates at all pH values examined, which was fully reversible upon reoxygenation. Because there was also a decrease in the intramitochondrial energy status during anoxia, we attempted to rescue protein synthesis with an ATP-regenerating system or with the addition of 1 mM ATP at the onset of anoxia. However, identical rates of protein synthesis were observed. Whereas addition of ATP at the onset of anoxia maintained the matrix ATP/ADP ratio at aerobic values, neither treatment stabilized the GTP/GDP ratio during the 1 h anoxic assays. Thus, although changes in adenine nucleotides had no influence on rates of protein synthesis during anoxia, we cannot rule out the possibility that the decrease in GTP/GDP ratios contributes to the suppression of protein synthesis during anoxia.

The sensitivity of at least cytoplasmic protein synthesis to changes in GTP/GDP ratio is well documented [32–34]. Decreases in GTP/GDP ratio can inhibit polysome aggregation, because GDP is a competitive inhibitor of the Met-$tRNA_i \cdot GTP \cdot eIF\text{-}2$ initiation complex [32] and of several elongation factors [34]. However, it is appropriate to note that, in the present study, the anoxia-induced changes in mitochondrial GTP/GDP occurred across a range where there would be no effect on protein synthesis on the basis of data obtained for the cytoplasmic compartment [33]. Furthermore, the rapid suppression in protein synthesis in response to anoxia occurred before substantial changes in purine nucleotides were observed, which suggests that other factors are likely involved in this anoxia-induced suppression (e.g. molecular oxygen itself).

Both oxygen limitation [35–38] and anoxia-induced changes in redox potential (see discussions in [39,40]; reviewed in [38]) are known to affect gene expression at both the translational and transcriptional levels in some organisms. For example, in the yeast *Saccharomyces cerevisiae* synthesis of mitochondrial-encoded subunits of COX is suppressed in response to oxygen deprivation both *in vitro* [41,42] and *in vivo* (reviewed in [35,38,43]). Oxygen is also required for the assembly of specific mitochondrially and cytoplasmically synthesized subunits [42]. Translational regulation of mitochondrial-encoded COX subunits in response to oxygen limitation has been shown, in part, to be exerted by nuclear-encoded translational activators that bind to specific mitochondrial mRNAs and regulate the association with the small ribosomal subunit (reviewed in [43,44]). As these activators become depleted during anoxia, translational rates are suppressed [44]. Haem, which requires oxygen for its synthesis [45], also influences the expression of many genes that are similarly regulated by oxygen (reviewed in [40]). Although direct modulation of mitochondrial translation by haem levels or redox state has not been documented, haem is necessary for the accumulation, processing and assembly of yeast cytochrome oxidase subunits [46]. At present, the precise mechanism by which oxygen limitation influences mitochondrial gene expression is unclear. The effects of oxygen limitation and anoxia-induced changes in redox potential on components of the mitochondrial translational machinery warrant further study.

In a previous study [21], we found that protein synthesis rates in isolated mitochondria from *A. franciscana* embryos were acutely sensitive to changes in extramitochondrial pH; an 80 % decrease in the rate of protein synthesis occurred when pH was lowered from the optimum of pH 7.5 to 6.8, the pH_i measured under anoxia. The manner by which changes in extramitochondrial pH affect mitochondrial protein synthesis is not known. One of the objectives of the present study was to determine if the decrease in protein synthesis in response to changes in extramitochondrial pH could result from variations in intramitochondrial purine nucleotide concentrations. Intramitochondrial

adenine and guanine nucleotide concentrations did not differ as a function of extramitochondrial pH during a 1 h protein synthesis assay under aerobic conditions, with the exception of higher GDP levels at 15 min for pH 8.0 compared with pH 7.5. Thus other factors must be responsible for the change in mitochondrial protein synthesis rates observed in response to alterations in extramitochondrial pH.

Another way in which pH could indirectly affect the rate of mitochondrial protein synthesis is by altering the transport of amino acids into the matrix. If alterations in pH decreased the transport of the radiolabelled amino acid used to assess rates of protein synthesis, so that it became rate limiting, protein synthesis rates would decrease. Therefore we repeated the pH profile for protein synthesis, using [^{3}H]histidine in place of the [^{3}H]leucine that was used in a previous study [21]. We chose histidine primarily because it relies on a different mitochondrial transporter from that for leucine [22,23]. In addition, histidine's low endogenous level within the matrix [21] permits the achievement of a high specific radioactivity, and its moderate mole percentage in COX (bovine) [47] ensures sufficient incorporation in the protein synthesis assay. The pH profiles for protein synthesis obtained with either [^{3}H]leucine or [^{3}H]histidine, as well as [^{35}S]methionine (K. E. Kwast and S. C. Hand, unpublished work), were similar. If pH influences amino acid transport, the effect spans multiple transporters to a similar degree.

In *A. franciscana* embryos, levels of selected mitochondrial mRNA (*COX1*) do not decrease substantially in response to either anoxia or aerobic acidosis *in vivo* over several hours (I. Hardewig, T. J. Anchordoguy, D. L. Crawford and S. C. Hand, unpublished work). Given that large changes in matrix pH ($\sim$ 1.3 pH units; Table 2) occur when the extramitochondrial pH is acidified across the relevant physiological range observed during anoxia *in vivo*, we suggest there may be a direct effect of pH on some component of the translational machinery within the matrix. The large change in intramitochondrial proton concentration could serve as a very strong signal within this organelle. To our knowledge, no studies to date have examined pH-sensitive translational components in the mitochondrion.

Finally, the effect of both oxygen limitation and pH on the array of mitochondrial translation products was investigated. A previous study, which examined the synthesis of mitochondrial proteins in anaerobically grown yeast in the presence of cycloheximide, showed that anoxia does not differentially inhibit the synthesis of specific mitochondrial translation products, but rather resulted in an overall down-regulation of mitochondrial protein synthesis [42]. Similarly, our fluorograph revealed no differential inhibition of specific mitochondrial translation products as a function of either oxygen partial pressure or extramitochondrial pH. It appears that *in vitro* there is an overall suppression of the capacity for translation within the mitochondrion in response to either anoxia or changes in pH.

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