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# Nuclear-Cytoplasmic Translocations of Protein p26 during Aerobic–Anoxic Transitions in Embryos of *Artemia franciscana*

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**Embryos of the crustacean *Artemia franciscana* survive continuous anoxia for periods of years, during which their metabolism comes to a reversible standstill. A question of some interest concerns the maintenance of cellular integrity in the absence of biosynthesis and an ongoing energy metabolism. The present paper continues previous work on an abundant protein (p26) that undergoes extensive intracellular translocation during aerobic–anoxic transitions, exhibits several characteristics of stress proteins, and might be involved in metabolic regulation during aerobic–anoxic transitions. Since it has been established that intracellular pH ($pH_i$) plays a major role in aerobic–anoxic transitions in this system we examined the pH-dependence of nuclear-cytoplasmic translocations of p26. In unincubated and aerobic-incubated embryos ($pH_i \geq 7.9$) p26 was located in the "soluble" fraction, whereas in anoxic embryos (pH about 6.3) roughly 50% was translocated into the nucleus as shown by immunocolloidal gold electron microscopy. These nuclear translocations also took place *in vitro,* simply by manipulating buffer pH in a physiologically appropriate fashion. Immunostaining of Western blots prepared after two-dimensional electrophoresis revealed several isoforms of native p26. The isoelectric point of the major isoform was 7.10 ± 0.05, a value close to the pH at which p26 translocation into the nucleus was first initiated *in vitro.* $^{31}$P-NMR measurements indicated that $pH_i$ was maintained at acidic levels (about 6.3) during prolonged anoxia. We also found that $pH_i$ of hydrated (0°C) but otherwise unincubated embryos was alkaline, allowing for rapid resumption of metabolism under permissive conditions. The significance of these pH-dependent translocations of p26 is discussed.**



## INTRODUCTION

The conversion of metabolically dormant biological systems into activated states, and vice-versa, commonly involves a change in intracellular pH ($\Delta pH_i$): the dormant condition is correlated with acidification and the activated state with an increase in $pH_i$ [1–4, and articles in 5]. The extreme $\Delta pH_i$ occurs in encysted embryos of the crustacean *Artemia franciscana* whose metabolic and developmental status seem to be strictly pH-dependent [6–9]. Transitions between aerobic and anoxic conditions involve a change of about 1.5 units, during which the $pH_i$ of aerobic embryos ($\geq$7.9) falls to about 6.4 during the first day of anoxia. A good case has been made for the regulation of metabolism by key enzymes exhibiting appropriate pH-dependence [10–17]. Thus, this system is an excellent model for study of the control of metabolism by $pH_i$, involving transitions between dormant and active states.

*Artemia* embryos are remarkably resistant to prolonged anoxia. While most free-living animal cells can survive anoxia for periods of hours to days [18], and some a month or so [5, 19] *Artemia* embryos experience little difficulty when undergoing at least 2 *years* of continuous oxygen lack [20]. A dramatic decrease in their metabolism accompanies the onset of anoxia [21], soon reaching a reversible standstill [see 22, 23]. How do the cells of these embryos maintain integrity in the absence of biosynthesis and without benefit of an ongoing energy metabolism? To explore this extraordinary ability we examined the proteins of anoxic embryos and found that an abundant protein of about 26,000 molecular mass undergoes extensive intracellular translocation during aerobic–anoxic transitions [24]. This protein, referred to here as p26, exhibits several features characteristic of the low-molecular-weight family of stress (heat shock) proteins, and we speculated that it might play the role of a molecular chaperone, protecting against the unfolding and aggregation of proteins during prolonged anoxia. In view of these results and the importance of $pH_i$ as a regulator in this system we examined the effects of pH on the translocation of p26 between nucleus and cytoplasm.

## MATERIALS AND METHODS

Dried encysted embryos of *A. franciscana,* purchased from San Francisco Bay Brand (Hayward, CA) were processed and stored as

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described [20]. These embryos were at the gastrula stage, consisting of about 4000 tightly packed cells (partly syncytial) surrounded by a complex shell. When incubated in seawater (SW) under aerobic conditions at 25°C, 85–90% produced viable larvae within 72 h. The studies to be presented in Fig. 6 used embryos obtained from Sanders Brine Shrimp Co. (Ogden, UT).

*Cell-free extracts.* The standard protocol involved homogenizing the embryos in buffer K (150 m*M* sorbitol, 70 m*M* potassium gluconate, 5 m*M* $MgCl_2$, 5 m*M* $NaH_2PO_4$, 0.1 m*M* phenylmethylsulfonyl fluoride, 40 m*M* Hepes at appropriate pH) using loose-fitting glass homogenizers (100 mg wet wt embryos/ml buffer K). In all cases the dried embryos were first hydrated for 24 h at 0°C in SW, referred to as "0 time" or controls. Aerobic incubations were performed at 25°C in a shaking water bath. Anoxia was imposed as described previously [20] using $N_2$ to deoxygenate SW. Anoxic incubations were carried out in 8-ml screw-capped glass scintillation vials (75 mg wet wt embryos, 7 ml anoxic SW, 1 ml $N_2$ gas phase).

Homogenates were centrifuged (2°C) immediately, or after incubation at 25°C, at 1630*g* for 5 min, and the resulting pellets were washed with 200× their volume of buffer K and then restored to the original homogenate volume with buffer K. Previous work [24] has shown that the low-speed pellet consists chiefly of nuclei, yolk platelets, and shell fragments and that p26 is associated exclusively with nuclei. In general, aliquots of pellet (P) and supernatant (S) fractions were added to 2× sample buffer [26] heated at 100°C for 5 min and centrifuged at 2000*g* for 5 min. These extracts were electrophoresed [24] in 12% SDS–polyacrylamide gels. Proteins were detected with Coomassie blue-G and quantified with a densitometer (Model DNA-35, Discovery Series, from pdi, Huntington Station, NY).

*Two-dimensional gel electrophoresis and Western immunoblotting.* Embryos used for two-dimensional gel electrophoresis were hydrated in ice-cold distilled water, harvested, and homogenized in Pipes buffer: 100 m*M* Pipes-free acid, 1 m*M* $MgCl_2$, 1 m*M* EGTA, pH 6.5, using a Retsch motorized mortar and pestle. Cell-free extracts were obtained as previously described [27–29].

Isoelectric focusing (IEF) and two-dimensional (2D) gel electrophoresis were performed as described previously [30]. For IEF gels, a pH gradient of approximately 6.0 to 8.0 was established using Biolyte 3/10, 5/7, and 6/8 ampholytes (Bio-Rad) in a ratio of 1:2:2. After electrophoresis in the second-dimension gels were either stained with Coomassie blue or blotted to nitrocellulose (Schleicher & Schuell) as described by Towbin *et al.* [31]. Transfer of proteins was verified by staining blots with 0.2% Ponceau S in 4% TCA. The blots were reacted with a polyclonal antibody raised to p26 [see 24] after it was diluted 1:10,000 in Tris–HCl, 10 m*M;* NaCl, 1 *M;* Tween 20, 0.5%; pH 7.4 (HST) [32], followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (H&L), diluted 1:2000 in HST. The secondary antibody was obtained from Jackson ImmunoResearch Laboratories.

*Immunocolloidal gold electron microscopy.* Control embryos were hydrated in SW for 12 h at 0°C followed by aerobic incubation at 20°C for 2 h. Anoxic embryos had previously undergone 4 months of oxygen lack. Embryos were washed in ice-cold distilled $H_2O$, disrupted with six strokes of a glass hand-held homogenizer in ice-cold buffer K of the appropriate pH (6.5 for anoxic, 7.85 for control), and poured through a 0.15-mm mesh screen to remove shell fragments. The impermeable cuticle must be broken for fixative to enter and the shell interferes with uv light penetration necessary to polymerize the embedding resin. The resulting extracts were fixed in 2% *p*-formaldehyde + 0.1% glutaraldehyde (final concentrations in buffer K) on ice for 1 h with occasional mixing. This treatment resulted in the loss of cytoplasmic ultrastructure but intact nuclei and yolk platelets were retained.

Fixed cellular constituents were pelleted at 1800*g* for 5 min, resuspended in 30% ethanol, and incubated on ice with occasional mixing for 25 min. Pellets were serially dehydrated through 100% ethanol and infiltrated with Lowicryl K4M resin (Electron Microscopy Sciences, Port Washington, PA) with constant mixing at −20°C as follows: 60 min in 1:1 resin:ethanol, 60 min 2:1 resin:ethanol, 60 min pure resin, overnight (about 14 h) with fresh resin. Infiltrates were transferred to BEEM capsules and polymerized at −20°C under uv light. Blocks were trimmed with a glass knife and thin sections cut with a diamond knife on a Reichert-Jung Ultracut E microtome. Sections were collected on copper mesh grids and incubated as follows: 1 h in blocking buffer (9% dry milk in HST); 1 h in undiluted purified anti-p26, 3× 20 min in HST; 1 h in 15- to 20-nm colloidal gold-conjugated goat anti-rabbit IgG (Bio-Rad, Cat. No. 170-6525) diluted 1/50 in HST; 2× 10 min in HST; and 2× 10 min in distilled $H_2O$. Gold-labeled sections, either unstained or briefly stained in saturated aqueous uranyl acetate (5 min) and lead citrate (1 min), were viewed and photographed using a Zeiss CEM 902 transmission electron microscope.

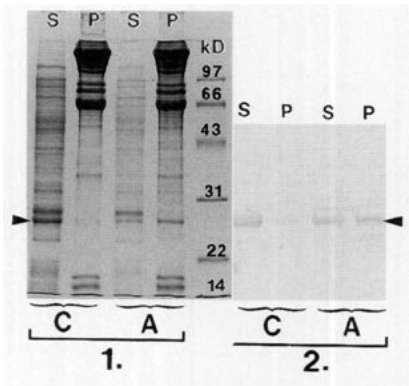


**FIG. 1.** Location of p26 (arrowheads) in extracts of 0°C, prehydrated controls (C), and embryos previously experiencing 6 months of anoxia (A). Part 1 shows the protein distributions after SDS–PAGE and Coomassie staining, and part 2 is a Western immunoblot of comparable fractions. S refers to the supernatant and P the washed pellet of homogenates centrifuged at 1630*g* for 5 min. The central lane shows molecular mass standards (kDa).

*Nuclear magnetic resonance (NMR) spectroscopy.* In this case the embryos were treated with antiformin to remove the outer layer of the shell [33]. That treatment allowed the embryos to settle rapidly (within 30 s) and pack as uniform spheres in the bottom of the NMR tube (10 mm diameter). Populations of shelled embryos exhibit varying proportions that settle, float, or are suspended, making NMR measurements difficult. Prehydrated embryos (0°C) were transferred to NMR tubes filled with anoxic 0.5 *M* NaCl. The tubes were capped and used immediately for measurement or placed on their sides for long-term anoxia prior to measurement.

$^{31}$P-NMR spectra were measured at 20°C in a General Electric (7 Tesla) spectrometer using a broadband 10-mm probe. A one-pulse experiment was carried out with a 70° (typically 20 $\mu$s) pulse, delay of 1.0 s, and 6500-Hz spectral width: 8192 digital points and 2048 transients were collected. All spectra were referenced to methylene diphosphonate (pH 8.9) as the external capillary standard, and $pH_i$ was determined from the chemical shifts of the inorganic phosphate ($P_i$) peak compared with those from a standard phosphate titration curve recorded on the same instrument. In no case did we observe more than one peak for $P_i$.

## RESULTS

Figure 1 (part 1) shows Coomassie-stained profiles of proteins in extracts of unincubated controls (C) and of embryos experiencing a prior anoxic exposure of 6 months (A). However, virtually the same result was obtained for anoxic periods ranging from 1 week to 1

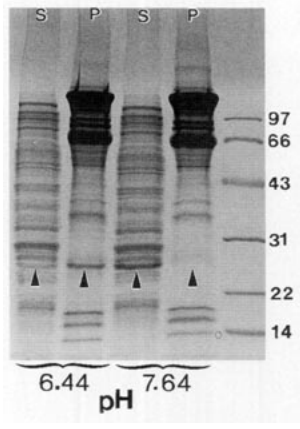


**FIG. 2.** SDS–PAGE, Coomassie profiles of proteins in extracts of control (0°C, prehydrated) embryos. The pH values refer to those of buffer K used to homogenize the embryos. After incubation at 25°C for 10 min the homogenates were centrifuged to obtain supernatant (S) and pellet (P) fractions (see legend to Fig. 1). Arrowheads mark p26, and molecular mass standards are in kilodaltons.

year [24, and unpublished results]. In these studies the buffer pH was 7.85 for controls and 6.5 for anoxic embryos since these have been shown to be approximate values *in vivo* [6–9]. The translocation of a 26-kDa protein (p26) from the supernatant to the pellet fraction of anoxic embryos was evident. Part 2 of Fig. 1 shows Western blots of these same fractions after SDS–PAGE, confirming that very little p26 was present in the pellet fraction (nuclei) of control embryos but was present in substantial amounts in pellet fractions from anoxic embryos. Virtually all of the p26 in these low-speed pellets was associated with nuclei and not yolk platelets which make up the bulk of this fraction [24, 34]. Therefore, these data indicated that roughly half of the total p26 of aerobic embryos was translocated to the nuclear fractions of anoxic embryos. We should also point out that analysis of embryos incubated *aerobically* at 25°C for 4 h produce the same results as unincubated controls [24]. Thus, the translocation of p26 is not in the developmental program of these embryos under aerobic conditions.

It is important to stress that the results shown in Fig. 1 were not due to the pH of buffer K: provided that the homogenates were kept at 2°C the same results as in Fig. 1 were obtained if control embryos were homogenized at a pH of 6.5, and anoxic ones at a pH of 7.85 (results not shown). However, if those homogenates were first incubated at 25°C *before* the supernatant and pellet fractions were prepared, a much different result was observed (Fig. 2).

In this case, control (0 time) embryos were homogenized in buffer K at pH 6.44 or 7.64 but the homogenates were incubated at 25°C for 10 min, prior to separation by centrifugation into the low-speed supernatant (S) and pellet (P) fractions (Fig. 2). When pH 6.44 was used, most of the p26 was associated with nuclei (pellet), whereas very little was found in this fraction when buffer K at pH 7.64 was used. Thus, acid pH induced a temperature-dependent translocation of p26 *in vitro,* comparable in a qualitative sense to the effects of anoxia in intact embryos (Fig. 1).

Given that the manipulation of pH *in vitro* allowed p26 translocation to nuclei, we asked whether it was released from nuclei by their subsequent exposure to alkaline pH. To evaluate this (Fig. 3) we first homogenized unincubated embryos in buffer K at pH 6.44, translocating p26 to nuclei (see Fig. 2). After washing at pH 6.44, the pellet was resuspended in buffer K, but this time at a pH of 7.64, incubated at 25°C for 10 min, and centrifuged as usual. The results showed that a substantial amount of p26 was indeed released from the nuclei into the supernatant by the use of buffer K at alkaline pH (Fig. 3). That result was consistent with the location of p26 and the $pH_i$ values in intact controls (alkaline) and anoxic (acid) embryos [6, 7].

Previous work showed clearly that p26 is associated with purified nuclei from anoxic but not aerobic embryos [24] but it did not prove that the protein was located *within* nuclei of anoxic embryos. To evaluate this issue further we carried out immunocolloidal gold labeling of nuclei obtained from aerobic and anoxic embryos (Fig. 4). Although the ultrastructural integrity of preparations obtained by this method was not impressive, the results indicated clearly that p26 was indeed found within the nuclei of anoxic but not aerobic embryos (Fig. 4). Quantitative estimates of ultrastructural observations are given in Table 1. We consider the low level of labeling observed in nuclei from aerobic embryos to be background.

To examine the possibility that pH-dependent translocations of p26 observed *in vitro* (Fig. 2) were a simple consequence of isoelectric precipitation we performed

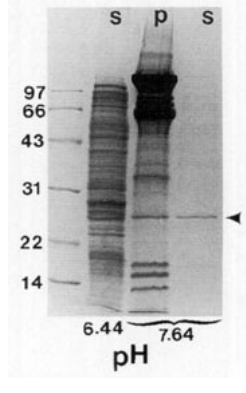


**FIG. 3.** Translocation of p26 (arrowhead) from the low-speed pellet (P) *in vitro.* The left lane shows the low-speed supernatant (S) from control (0°C, prehydrated) embryos homogenized in buffer K, pH 6.44, incubated for 10 min at 25°C. The washed pellet from this extract was resuspended in buffer K at pH 7.64, incubated for 10 min at 25°C, and then centrifuged again to obtain the pellet (P, central lane) and supernatant (S) shown in the lane to the right.

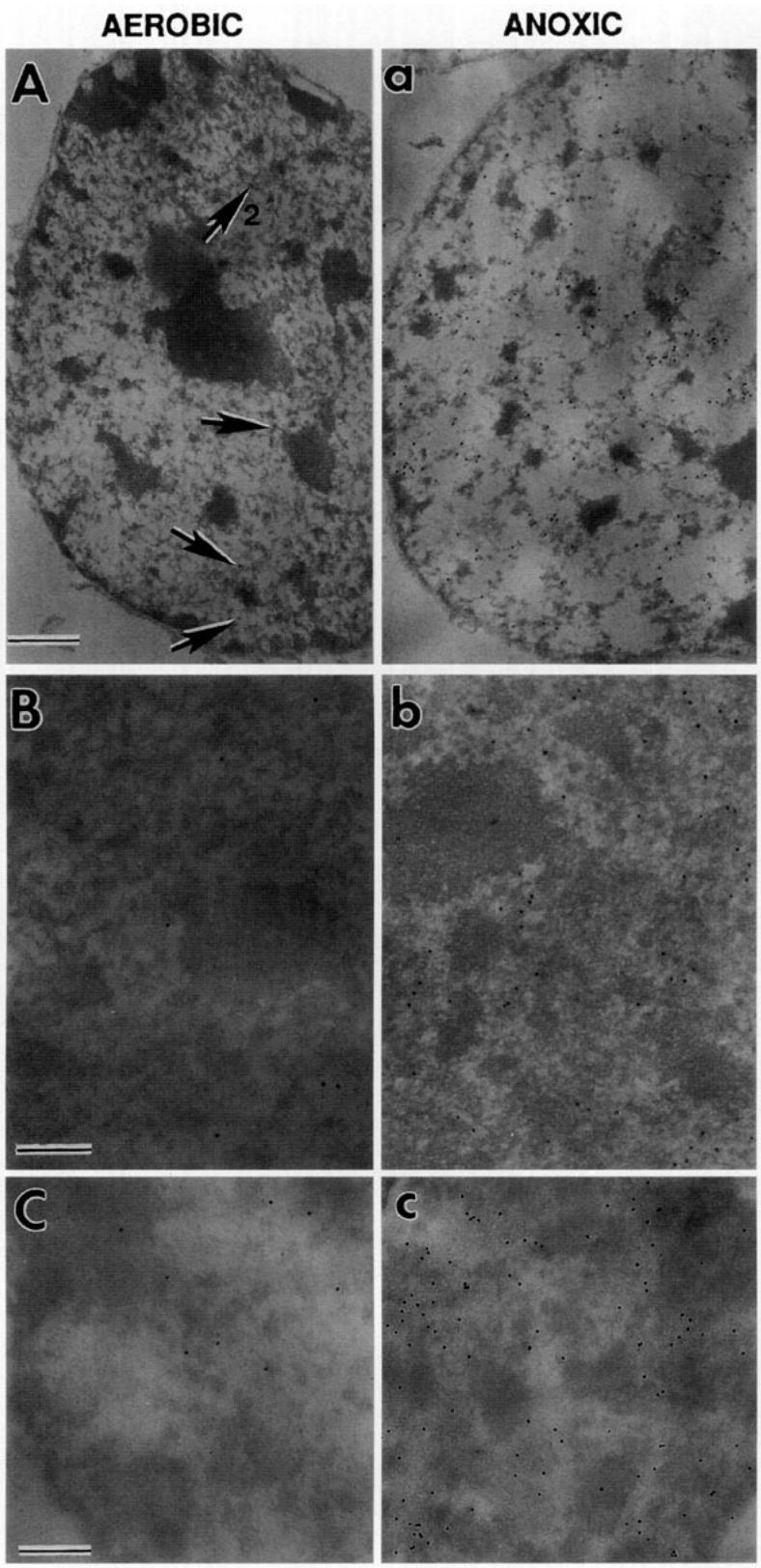


**FIG. 4.** Immunocolloidal gold labeling of p26 in nuclei from control, aerobic (2 h) and anoxic (4 months) embryos. Bars in A,a are 0.70 $\mu$m and 0.35 $\mu$m in B,b and C,c. Arrows in part A show the 5 gold particles (total) present in nuclei from controls that are otherwise not easily seen.

**TABLE 1**

Immunocolloidal Gold Labeling of the 26-kDa Protein (p26) in Nuclei of Aerobic and Anoxic Embryos

| Conditions | Number of nuclei | Number of gold particles |
|---|---|---|
| Aerobic | | |
| Stained | 12 | 3.1 ± 2.5 |
| Unstained | 7 | 1.4 ± 1.2 |
| Total | 19 | 2.5 ± 2.3 |
| Anoxic (4 months) | | |
| Stained | 8 | 29 ± 15 |
| Unstained | 4 | 25 ± 7 |
| Total | 12 | 29 ± 12 |

*Note.* The number of gold particles was determined in a field of 1.8 $\mu m^2$. Three counts were made on each nucleus section, at different locations, and averaged. Data represent mean ± SD for the number of nuclei specified.

two studies. In the first case, a pH titration of the location of p26 was carried out *in vitro*. These studies employed the same conditions as those used earlier (Fig. 2) except that a broader range of pH was employed (Fig. 5). At pH 7 and above, very little p26 was associated with nuclei. As the pH fell below 7, however, increasing amounts of p26 were translocated to the pellet, presumably into nuclei (Fig. 4). The total amount of p26 in these homogenates was divided almost equally between nuclei and supernatant at a pH between 6 and 6.5, a result that was in general agreement with those from extracts of anoxic embryos (Fig. 1), whose $pH_i$ was in this range [6, 7].

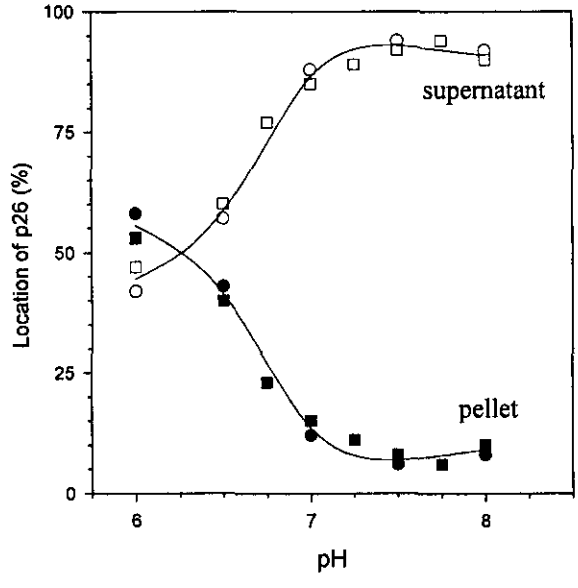


**FIG. 5.** Distribution of p26 in fractions from control (0°C, prehydrated) embryos homogenized in buffer K at the pH values indicated. Homogenates were incubated at 25°C for 10 min before centrifugation. The relative amount of p26 in the two fractions was determined by densitometry of SDS–PAGE gels. Squares and circles represent data from two independent studies.

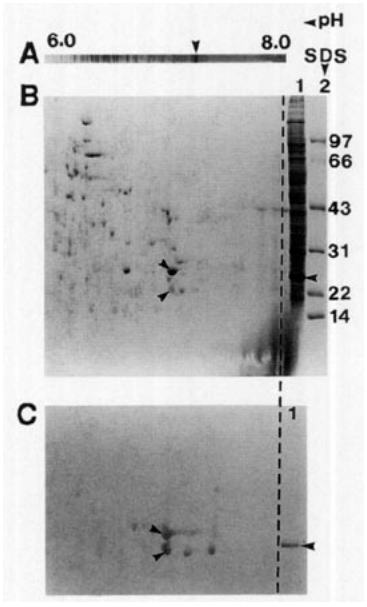


**FIG. 6.** Isoelectric point determination and isoform composition of p26. Cell-free supernatants were electrophoresed on IEF gels with a pH gradient of 6.0–8.0 (A), followed by electrophoresis on 12.5% SDS–polyacrylamide gels which were stained with Coomassie blue (B). Two-dimensional gels were blotted to nitrocellulose and stained with antibody to p26 (C). Cell-free samples were also added to gels (lane 1 in B and C) for concurrent one- and two-dimensional electrophoresis. Lane 2, part B, shows low-molecular-weight standards × $10^{-3}$. The unlabeled arrowheads indicate p26. The arrowheads labeled pH and SDS indicate, respectively, the directions of isoelectric focusing and SDS–polyacrylamide gel electrophoresis.

The second approach used to evaluate the mechanism for the pH-dependent translocation of p26 involved determination of its isoelectric point. Electrophoresis of *Artemia* cell-free supernatants on IEF gels yielded numerous bands with a major protein focusing at pH 7.1 (arrowhead in Fig. 6A). Protein samples enriched in p26 by standard purification techniques also gave a major band at pH 7.1 upon IEF (not shown). Application of IEF gels to SDS–polyacrylamide slabs and subsequent electrophoresis gave many spots upon staining with Coomassie blue (Fig. 6B). One large spot (upper arrowhead in Fig. 6B) aligned with the major band on the applied IEF gel and migrated closely with a major band resolved by one-dimensional electrophoresis (lane 1, Fig. 6B). This spot was shown to be about 26 kDa by comparison to molecular weight markers (lane 2, Fig. 6B). The major band on one-dimensional SDS–polyacrylamide gels usually migrated slightly differently than the large spot on 2-D gels because it was very difficult to have all samples enter the separating gel at the same time. In the case shown here the major band migrated slightly further in the one-dimensional gel. Upon blotting to nitrocellulose after 2-D electrophoresis and immunostaining with antibody to p26, a series of spots arranged in two families of slightly different size, and somewhat variable in composition,

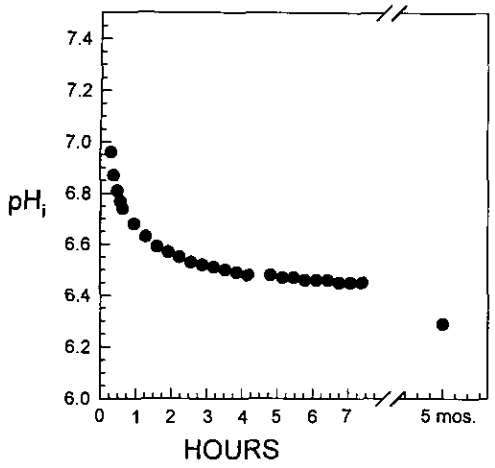


**FIG. 7.** $^{31}$P-NMR measurements of intracellular pH (pH$_i$) in control (0°C, prehydrated) embryos after transfer to anoxic 0.5 *M* NaCl. Acquisition of adequate spectra took about 15 min for the first pH$_i$ measurement; therefore, initial pH$_i$ in control embryos entering anoxia was more alkaline than revealed by these data. The same NMR tube used for the first 7 h was stored on its side for 5 months (21–24°C) in the dark before pH$_i$ was measured again.

was observed (arrowheads, Fig. 6C). These spots corresponded to proteins observed in Coomassie blue-stained 2-D gels (arrowheads, Fig. 6B). Upon immunostaining of blots from one-dimensional gels, however, it was most common to see only one major band (lane 1, Fig. 6C). A faint lower band was sometimes apparent, although it did not stain as strongly as the protein on blots from 2-D gels (not shown). To limit the possibility that the lower-molecular-weight isoforms originated from the larger proteins by proteolysis, the experiments were repeated in the presence of a proteolytic enzyme inhibitor cocktail [29], yielding the same result. Also, when tubulin, a protein very sensitive to proteolytic enzymes [28] was immunostained on blots prepared under these conditions no degradation was observed (results not shown).

The involvement of pH$_i$ in aerobic–anoxic transitions is, as mentioned, a well-documented phenomenon in this system [6–9]. However, those studies described neither pH$_i$ in embryos undergoing long-term anoxia (many months), nor did they evaluate pH$_i$ in zero time controls; that is, hydrated (0°C) but otherwise unincubated embryos. Therefore, we measured pH$_i$ in control embryos transferred to anoxic SW, and after 5 months of continuous oxygen lack (Fig. 7). As observed by others previously [6–9], pH$_i$ fell rapidly as the embryos entered anoxia. In fact, the decrease in pH$_i$ was so rapid that we were not able to determine its initial value since about 15 min was required to collect sufficient NMR data. We assume, on good grounds [6–9], that the initial pH$_i$ was $\geq$7.9. Therefore, these results indicated that the pH$_i$ of 0 time control embryos, being alkaline, allows them to rapidly resume metabolism under permissive conditions without altering pH$_i$. Importantly, we also found that pH$_i$ was maintained at an acidic level as anoxia continued (5 months), a result that is consistent with the long-term control of metabolism in anoxic embryos by acidic pH$_i$.

## DISCUSSION

We begin by brief review of previous findings on p26 [24]. This protein is a major constituent of *Artemia* embryos, making up 10–15% of the total non-yolk protein. That study also indicated that p26 in control (0 time) and aerobic embryos exists chiefly as a molecule of about 500 kDa and is located almost totally in the "soluble" fraction (105,000*g*, 1 h). In contrast, extracts from anoxic embryos were shown to exhibit a very broad distribution of p26 when analyzed by gel filtration, ranging in molecular mass from about 25,000 to greater than 4 million. It is possible that this broad distribution in anoxic embryos is due, in part, to the aggregation of variable numbers of p26 subunits. However, there also is evidence that the interaction of p26 with a wide variety of proteins is involved in this distribution. Finally, it has been shown that p26 is specific to the encysted embryo, not being found in appreciable amounts in any other stage of the life cycle [24]. Although the functions of p26 remain to be determined, its abundance and aforementioned characteristics indicate that it is certain to play an important role in the metabolism and development of *Artemia* embryos. Therefore, it seemed important to evaluate the behavior of p26 under varying conditions of pH, the rationale coming from abundant evidence that intracellular pH regulates metabolism during aerobic–anoxic transitions in this system [6–17].

The translocation of p26 into nuclei that occurs in embryos during anoxia (Figs. 1 and 3) can also be carried out *in vitro* simply by manipulating buffer pH (Figs. 2 and 5). The pH-dependence of nuclear translocation *in vitro* is consistent with the pH$_i$ of intact aerobic and anoxic embryos as measured by $^{31}$P-NMR [6, 7, and Fig. 7].

Having shown that p26 is translocated to nuclei *in vitro* simply by manipulation of pH we found that the reverse translocation is also achieved by use of alkaline pH (Fig. 3). While we believe that these *in vitro* studies reflect events that occur in the intact embryo, we accept the caveat that must be applied to any work involving cell disruption. Nevertheless, the finding that p26 translocations can be controlled *in vitro* simply by the use of an appropriate pH (Figs. 2, 3, and 5) allows for the further detailed study of these processes in the future. For example, it will be interesting to compare transcription in isolated nuclei that contain p26 with those lacking this protein.

The isoelectric point (p*I*) of the native form of p26 from control (aerobic) embryos is 7.1 $\pm$ 0.1 for the main

isoforms (Fig. 6). Our pH titrations on extracts from comparable embryos indicate that translocation of p26 into nuclei is initiated at buffer pH below this same value (Fig. 5). Although the mechanism by which pH influences nuclear translocation remains to be determined, it is probably not a simple isoelectric precipitation of p26 since this protein actually enters nuclei (Fig. 4). It is interesting that entry of p26 into nuclei begins at a pH approximating the p*I* of p26. Perhaps a change in the charge of p26, enhanced by further reduction in $pH_i$ as anoxia continues, is involved in the nuclear translocations we observe. Thus, recognition of this protein by nuclear pores, or movement through them, could be influenced by the charge characteristics of p26.

Since the low-speed pellets contain large amounts of yolk platelets we should mention the work of Utterback and Hand [35], who found that protein release/retention by isolated yolk platelets is strongly pH-dependent. Although we see little indication of that (Figs. 1–3) major differences between their buffer composition and ours, as well as the duration of incubation (theirs was 40 min, ours 10 min) make direct comparisons difficult. In any event, the findings presented here on p26 translocation seem unaffected by the presence of yolk platelets.

As mentioned earlier in this paper, p26 shares a number of characteristics in common with the low-molecular-weight family of stress proteins [see 36] and determination of the amino acid sequence of p26 is in progress to further evaluate this relationship. The possibility that p26 could be a protective molecular chaperone in anoxic embryos is currently under study.

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## REFERENCES

1. Busa, W. B., and Nuccitelli, R. (1984) *Am. J. Physiol.* **246,** R409–R438.
2. Busa, W. B. (1986) *Annu. Rev. Physiol.* **48,** 389–402.
3. Madshus, I. H. (1988) *Biochem. J.* **250,** 1–8.
4. Kurkdjian, A., and Guern, J. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40,** 271–303.
5. Hochachka, P. W., Lutz, P. L., Sick, T., Rosenthal, and van den Thillart, G. (Eds.) (1993) Surviving Hypoxia, CRC Press, London.
6. Busa, W. B., Crowe, J. H., and Matson, G. B. (1982) *Arch. Biochem. Biophys.* **216,** 711–718.
7. Busa, W. B., and Crowe, J. H. (1983) *Science* **221,** 366–368.
8. Drinkwater, L. E., and Crowe, J. H. (1987) *J. Exp. Zool.* **241,** 297–307.
9. Crowe, J. H., Crowe, L. M., Drinkwater, L. E., and Busa, W. B. (1987) *in* Artemia Research and Applications (Declair, W., Moens, L., Slegers, H., Jaspers, E., and Sorgeloos, P., Eds.), Vol. 2, pp. 19–40, Universa Press, Wetteren, Belgium.
10. Carpenter, J. F., and Hand, S. C. (1986) *J. Comp. Physiol. B.* **156,** 451–459.
11. Hand, S. C., and Carpenter, J. F. (1986) *Science* **232,** 1535–1537.
12. Hofmann, G. E., and Hand, S. C. (1990) *Am. J. Physiol.* **258,** R1184–R1191.
13. Hofmann, G. E., and Hand, S. C. (1992) *J. Exp. Biol.* **164,** 103–116.
14. Kwast, K. E., and Hand, S. C. (1993) *Am. J. Physiol.* **265,** R1238–R1246.
15. Hofmann, G. E., and Hand, S. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8492–8496.
16. Slegers, H. (1991) *in* Artemia Biology (Browne, R. A., Sorgeloos, P., and Trotman, C. N. A., Eds.), pp. 37–73, CRC Press, London.
17. Drinkwater, L. E., and Clegg, J. S. (1991) *in* Artemia Biology (Browne, R. A., Sorgeloos, P., and Trotman, C. N. A., Eds.), pp. 93–117, CRC Press, London.
18. Bryant, C. (ed.) (1991) Metazoan Life Without Oxygen, Chapman & Hall, New York.
19. Storey, K. B., and Storey, J. M. (1990) *Quart. Rev. Biol.* **65,** 145–174.
20. Clegg, J. S. (1994) *J. Exp. Zool.* **270,** 332–334.
21. Hand, S. C., and Gnaiger, E. (1988) *Science* **239,** 1425–1427.
22. Hontario, R., Crowe, J. H., Crowe, L. M., and Amat, F. (1993) *J. Exp. Biol.* **178,** 149–159.
23. Clegg, J. S. (1993) *J. Comp. Physiol. B* **163,** 48–51.
24. Clegg, J. S., Jackson, S. A., and Warner, A. H. (1994) *Exp. Cell Res.* **212,** 77–83.
25. Clegg, J. S., and Jackson, S. A. (1989) *in* Cellular and Molecular Biology of Artemia Development (Warner, A. H., Bagshaw, J., and MacRae, T., Eds.), pp. 1–16, Plenum, New York.
26. Laemmli, U. (1970) *Nature* **227,** 680–685.
27. Rafiee, P., MacKinley, S. A., and MacRae, T. H. (1986) *Biochem. Cell Biol.* **64,** 238–249.
28. Zhang, J., and MacRae, T. H. (1992) *Biochem. Cell Biol.* **70,** 1055–1063.
29. Campbell, E. J., MacKinley, S. A., and MacRae, T. H. (1989) *J. Cell Sci.* **93,** 29–39.
30. MacRae, T. H., and Ludueña, R. F. (1984) *Biochem. J.* **219,** 137–148.
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
32. Birkett, C. R., Foster, K. E., Johnson, L., and Gull, K. (1985) *FEBS Lett.* **187,** 211–218.
33. Nakanishi, Y. H., Iwasaki, T., Okigaki, T., and Kato, H. (1962) *Annot. Zool. Jpn.* **35,** 223–233.
34. Vallejo, C. G., Perona, R., Garesse, R., and Marco, R. (1981) *Cell Differ.* **10,** 343–356.
35. Utterback, P. J., and Hand, S. C. (1987) *Am. J. Physiol.* **252,** R774–R781.
36. Arrigo, A.-P., and Landry, J. (1994) *in* The Biology of Heat Shock Proteins and Molecular Chaperones (Morimoto, R. I., Tissières, A., and Georgopoulos, C., Eds.), pp. 335–374, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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