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Protein Stability in *Artemia* Embryos During Prolonged Anoxia

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Abstract

Encysted embryos (cysts) of the brine shrimp, *Artemia franciscana*, are arguably the most stress-resistant of all animal life-history stages. One of their many adaptations is the ability to tolerate anoxia for periods of *years*, while fully hydrated and at physiological temperatures. Previous work indicated that the overall metabolism of anoxic embryos is brought to a reversible standstill, including the transduction of free energy and the turnover of macromolecules. But the issue of protein stability at the level of tertiary and quaternary structure was not examined. Here I provide evidence that the great majority of proteins do not irreversibly lose their native conformation during years of anoxia, despite the absence of detectable protein turnover. Although a modest degree of protein denaturation and aggregation occurs, that is quickly reversed by a brief post-anoxic aerobic incubation. I consider how such extraordinary stability is achieved and suggest that at least part of the answer involves massive amounts of a small heat shock protein (p26) that acts as a molecular chaperone, the function of which does not appear to require ribonucleoside di- or tri-phosphates.

Introduction

Very few adult animals, or their developmental stages, tolerate anoxia for an extended time (Hochachka and Somero, 2002). Among animals that are well adapted to surviving anoxia, the most impressive are intertidal organisms such as mussels, oysters, and various worms, but many others are known (Bryant, 1991; Hochachka *et al.*, 1993; Grieshaber *et al.*, 1994; Guppy and Withers, 1999). The response of these well-adapted animals to anoxia in the laboratory, and I assume in nature, is to reduce their metabolic rates to levels that are commonly between 1% and 10% of the aerobic level, a response that has come to be known as metabolic rate depression, or MRD (Guppy and Withers, 1999; Hochachka and Somero, 2002). The ultimate in MRD occurs in encysted embryos of the crustacean *Artemia*, which eventually reduce their overall metabolism to a rate that is not detectable, and I will consider that evidence, and its significance, in the Discussion.

Species of this genus are found in a variety of very harsh environments in all continents except Antarctica (Van Stappen, 2002). In nature their encysted embryos (cysts), the focus of this paper, encounter severe hypersalinity and air desiccation; high doses of ultraviolet radiation; varying degrees of hypoxia, including anoxia; and extremes of temperature (Clegg and Conte, 1980; Browne *et al.*, 1991; Abatzopoulos *et al.*, 2002; Tanguay *et al.*, 2004). These embryos are animal extremophiles, so it is not surprising that they exhibit a wide variety of biochemical and biophysical adaptations (Clegg and Trotman, 2002). Here I focus on their astonishing tolerance to prolonged anoxia.

Although metabolism is detectable in embryos during the first day or so of anoxia (Hand, 1997, 1998; Clegg, 1997; Hand and Podrabsky, 2000), it eventually comes to a reversible standstill, or at least is reduced to an undetectable level (see Warner and Clegg, 2001). Thus, evidence from ^{14}C-labeled amino acid incorporation and SDS-PAGE autoradiography indicated that protein synthesis stops during anoxia, and no detectable hydrolysis at the level of individual polypeptide chains was detected (Clegg, 1997; Tanguay *et al.*, 2004). However, in those studies no information was obtained about protein stability at the tertiary and quaternary levels. That is, the issue of protein unfolding and aggregation was not considered. Although the methods used in the present work do not detect subtle changes in those structures, they do reveal the occurrence of protein aggregation following unfolding (denaturation) of proteins.

Materials and Methods

Source of embryos and hatching assays

Specimens of *Artemia franciscana* from salterns in the San Francisco Bay (SFB) were purchased from San Francisco Bay Brand, Hayward, CA, as dried (activated) encysted embryos, and stored at about −10°C under 100% N_2. Embryos were placed at room temperature for 5 days before use and had a final hatching percentage close to 90%.

For hatching assays, groups of at least 200 embryos were placed into 20-welled plastic depression plates, each well containing 10–20 embryos in 300 µl of 0.2-µm-filtered seawater. The plates were covered, sealed with tape to prevent evaporation, and incubated in constant light at 21–24°C until hatching was complete. Adequate O_2 is present in these sealed plates since controls (embryos not exposed to anoxia) exhibited close to 90% hatching within 2 days of incubation. Previous anoxia substantially delays the hatching process (Clegg, 1997), so one must incubate long enough to ensure that hatching is complete.

Anoxia

Embryos were made anoxic with 100% N_2, using procedures described in detail previously (Clegg, 1997). Briefly, dried embryos were hydrated under anoxic conditions: about 75 mg dried weight was placed in each 8-ml screw-capped glass vial, to which was added 6 ml of 0.3 mol l^{-1} NaCl, buffered to pH 7.2 with 0.1 mol l^{-1} Na/K phosphate that had just been degassed for 4 h with the purest available grade of N_2. The 2-ml air space was displaced with N_2, and the caps were screwed tight and wrapped with several layers of Parafilm to ensure sealing and prevent loosening. Vials were then oscillated at 50 rpm for 8 h to fully hydrate all the embryos, which were then stored under laboratory conditions of light and temperature.

I note that the embryos will rapidly consume whatever traces of molecular oxygen that might be present, down to exceedingly low partial pressures that are, in effect, zero to the embryos. However, I

emphasize that it is essential to prevent extended periods of *hypoxic* metabolism since this results in limited, subsequent anoxic longevity (unpubl. obs.). Seawater should not be used for studies on anoxia in these embryos because bacteria in the shells apparently convert sulfates to sulfides that accumulate to toxic levels under anoxic conditions (see Clegg, 1997).

Analysis of p26 and artemin in previously anoxic embryos

Controls (no anoxia) and embryos that had previously undergone 1 and 4.5 years of anoxia were homogenized at ~0 °C in buffer K (5 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} NaH_2PO_4, 40 mmol l^{-1} Hepes, 70 mmol l^{-1} potassium gluconate, 150 mmol l^{-1} sorbitol, pH 7.2). Nuclei and yolk platelets were removed by low-speed centrifugation (1600 × g, 5 min, 2 °C), and the resulting supernatant was applied to a column of Sepharose CL-6B calibrated with several native proteins of known molecular mass (Clegg *et al.*, 1994). Fractions (1 ml) were obtained, reduced to equal volumes under vacuum, and analyzed by SDS-PAGE, with protein detection by Coomassie staining and p26 by Western blotting. Further details about all these methods are available (Clegg *et al.*, 1994, 1995; Clegg, 1997).

Anoxia results in the translocation of substantial amounts of the total p26 to nuclei that are moved to the pellet by low-speed centrifugation (references above, Willsie and Clegg, 2001, 2002); hence, supernatants from anoxic embryos contain visibly less p26 than those from controls. That fact should be kept in mind when evaluating the results to be presented here. Nevertheless, these low-speed supernatants contain the vast majority of the total nonyolk proteins in these embryos compared to proteins present in the low-speed pellet (Willsie and Clegg, 2001, 2002). Therefore, the studies to be presented here involve most of the physiological (nonyolk) proteins in these embryos. Although a minor aspect of this study, some results are also presented on the protein artemin, a potential RNA chaperone (Warner *et al.*, 2004).

Gel filtration

Sepharose CL-6B columns were calibrated with proteins of known native molecular mass, using 0.1 mol l^{-1} NaCl in 0.05 mol l^{-1} Tris-HCl, pH 7.2, as the mobile phase. Fractions 1–3 represent the column void volume, containing proteins and other cell components of 4 million molecular mass and higher, and fractions of greater number contain proteins of decreasing mass between that and about 20 kD (Clegg *et al.*, 1994). Native artemin and p26 in control embryo extracts elute from the column in fractions 9–12, corresponding to an average molecular mass of roughly 500 kD, in keeping with previous more precise measurements (Viner and Clegg, 2001; Warner *et al.*, 2004). Of major interest in this study was the translocation of proteins into and out of the void volume, reflecting aggregation and its reversal, respectively.

SDS-PAGE and immunoblotting

Known volumes of low-speed supernatants and fractions from gel filtration were added to equal volumes of 2× sample buffer (Laemmli, 1970) and boiled for 5 min. Protein profiles on gels from SDS-PAGE were stained with Coomassie or transferred to nitrocellulose sheets and prepared for immunodetection using a polyclonal anti-p26 at 1:5000 for 1 h (Clegg *et al.*, 1994, 1995) as the primary antibody, and horseradish peroxidase conjugated anti-rabbit IgG (1:5000, 1 h) as secondary. Chemiluminescence was detected on blots in the Epi Chemi II Darkroom (UVP Laboratory Products) after incubation with Super Signal West Pico (Pierce, Rockford, IL).

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Anoxic viability

Three groups of embryos have been examined in this study: controls (no anoxia), and embryos that had previously experienced 1 and 4.5 years of continuous anoxia under ordinary laboratory conditions of light and temperature. Hatching in all three groups was determined as described, and the results are, for means and standard deviations: control, 89 ± 3; 1 year anoxia, 85 ± 2; 4.5 years anoxia, 34 ± 7 (also shown in Fig. 1). Three independent sets of at least 200 embryos were studied for each group to determine the standard deviations ($n = 3$).

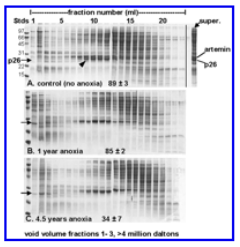

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Figure 1. The profiles of proteins in low-speed supernatants separated first on a Sepharose CL-6B gel filtration column, followed by SDS-PAGE of the resulting 1-ml fractions. An aliquot of this supernatant (super.) was applied to SDS-PAGE for reference (Fig. 1A, upper-right). Results are shown for controls (A, no anoxia) and embryos previously anoxic for 1 and 4.5 years (B and C, respectively). Artemin is the abundant protein of slightly higher molecular mass located above p26. The arrowhead indicates the location of native p26 in controls, and the horizontal arrows to the left point to the location of p26 for all fractions. Mean viability of the three groups of embryos (± standard deviations) is shown at the bottom center of each of the three gels.

Artemin and p26 in control and anoxic embryos

When encysted embryos are made anoxic, much of the p26 translocates reversibly to nuclei, whereas artemin remains in the extranuclear space, the "soluble" supernatant fraction (Clegg *et al.*, 1994, 1995; Warner *et al.*, 2004). When anoxic embryos are returned to aerobic conditions, the p26 that had translocated to nuclei quickly returns to the supernatant fraction (same references).

Figure 1 shows protein profiles from gel filtration, followed by analysis of the fractions by SDS-PAGE and Coomassie staining. In these studies the low-speed supernatants from homogenates of embryos that had not undergone anoxia (Fig. 1A, control) and those that had previously experienced anoxic bouts of 1 year (Fig. 1B) and 4.5 years (Fig. 1C) were analyzed. The numbers at the bottom-middle of each gel in Figure 1 are the mean hatching levels (± the standard deviation, SD) for these three groups of embryos. Whole supernatant for the control preparation is shown at the top right for general reference to what was applied to the gel filtration column (Fig. 1A, super.). Figure 1A also indicates the location of native artemin and p26, chiefly in gel filtration fractions 9–12, corresponding to an average molecular mass of roughly 500 kD. The horizontal arrows to the left in Figure 1A–C indicate the location of p26. As mentioned, and stressed again here, the low-speed supernatants from previously anoxic embryo extracts lack much of the total p26 because significant amounts had been translocated to nuclei under these conditions. Even so, small amounts of this protein are also present in all gel filtration fractions (Clegg *et al.*, 1994; Willsie and Clegg, 2001, 2002).

Note that the void volume (fractions 1–3) of control extracts contains very little p26, whereas void volumes from anoxic preparations contain much more of this protein (confirmed later by Western blotting) and other proteins as well. However, for the present, the principal result of Figure 1 is the marked similarity of the protein profiles of fractions 4 through 23, for controls and both anoxic samples, the exception being p26 in extracts from previously anoxic embryos.

Because gel filtration *precedes* SDS-PAGE in these studies, the irreversible denaturation and aggregation of proteins will be revealed by this approach. But the overall similarity of these three profiles indicates that such an outcome did not occur (see Discussion).

Figure 2 shows Western blots of void-volume fractions of extracts from controls and previously anoxic embryos. The results are for two independent studies, indicating the degree of variation one can expect. However, the cogent result is that the void volume from controls contains no detectable p26, whereas substantial p26 is seen in these fractions (1–3) from anoxic embryos.

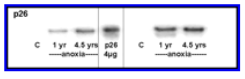

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Figure 2. Western blots of extracts from controls (C) and from embryos previously incubated anoxically for 1 and 4.5 years. Each of these lanes represents void volumes, the combined fractions 1–3 (Fig. 1A–C). Duplicates are shown (to the left and right of p26) to indicate variability, along with pure p26 in the center lane.

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Further analysis is described in Figure 3. In this case, samples of controls (C) are compared to those from embryos previously anoxic for 1 year, and for 1 year followed by an aerobic incubation of 2 h (1 yr+). This figure compares void volumes (fractions 1–3) and fractions 9–12 that contain most of the native p26 and artemin (Fig. 1A). Figure 3A shows Coomassie staining of the abundant artemin and p26, and part B shows the result of Western immunoblotting for identification of these proteins. As seen previously in Figure 1, the amount of p26 in the control void volume is negligible, but is abundant in these fractions of extracts of embryos that previously experienced 1 year of anoxia. However, when the latter embryos were first given an aerobic incubation of 2 h and then analyzed the same way, little if any p26 was detected in the void volume. Similarly, fractions 9–12 from control embryos contained the usual amounts of artemin and p26 (also seen in Fig. 1A), although little p26 was detected in these fractions in extracts from previously anoxic embryos (1 yr). But when those anoxic embryos were first incubated aerobically for 2 h (1 yr +), abundant p26 was detected in fractions 9–12, the region where native p26 is found.

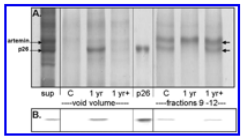

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Figure 3. Coomassie staining (A) and Western immunoblotting (B) of extracts from controls (C) and from embryos immediately after 1 year of anoxia (1 yr) and from embryos that were anoxic for 1 year, followed by an aerobic incubation of 2 h at 23°C prior to analysis (1 yr+). Void volume refers to pooled fractions 1–3 from gel filtration and SDS-PAGE. Pooled fractions 9–12 represent the location of native structures of most of the p26 and artemin in these preparations.

Taken together, these results show that the movement of p26 from fractions 9–12, the location of its native molecular mass, to the void volume takes place under anoxic conditions, and that the reversal takes place when anoxic embryos are returned to aerobic conditions. I will consider reasons for this behavior of p26, one of which involves molecular chaperoning.

Discussion

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Previous work on these embryos indicated that they eventually bring their overall metabolism to a reversible standstill when placed under anoxia (Clegg, 1997; Clegg and Trotman, 2002; Tanguay *et al.*, 2004). Although a minuscule change in some of the guanosine nucleotides has been observed, its detection requires incubation periods of years (Warner and Clegg, 2001). One cannot prove the absence of a rate by experiments at physiological temperatures. Nevertheless, the case has been made (references above) that if these anoxic embryos are metabolizing at all, the rate is so slow as to be irrelevant in any reasonable context of what the term "metabolism" means (Clegg and Trotman, 2002).

The current study has focused on the stability of native proteins in these anoxic, "ametabolic" embryos. We know from previous work that the proteins of anoxic embryos are not turning over at a rate sufficient to be detected over periods of years using radioactive amino acids (Clegg, 1997). As mentioned, that research did not consider protein stability at the tertiary and quaternary levels, which is obviously a consideration of major importance. Very preliminary work (Tanguay *et al.*, 2004) suggested that most proteins were stable at all levels of structure during anoxia. The present study supports those findings since the overall profiles of soluble proteins in these embryos are very similar, whether they have experienced 4.5 years of continuous anoxia or no anoxia at all (Fig. 1). Indeed, that is the case even though the overall hatching level of previously long-term anoxic embryos was reduced by a factor of about 2.5. In other words, even embryos that do not hatch maintain the integrity of their proteins although they are, in effect, dead (Clegg and Trotman, 2002). That is, such stability apparently does not require viability or an ongoing overall metabolism.

These anoxic embryos appear to be exceptions to a central paradigm of cell biology, namely, that a constant and substantial flow of free energy is required to maintain cellular structure and function (including the requirement for macromolecular turnover); these major endergonic processes in cells are apparently not in evidence in long-term anoxic *Artemia* embryos. A question then arises—what are the mechanisms that enable protein stability in the absence of turnover and free-energy supply? There are probably a number of such mechanisms, including the presence in these embryos of very large amounts of trehalose and glycerol, "compatible solutes" that could be involved with protein stabilization (Clegg and Trotman, 2002; Hochachka and Somero, 2002). Even the stoppage of metabolism might involve processes that stabilize proteins against unfolding. The present study has implicated participation of the molecular chaperone p26, and I focus on that possibility next.

Much previous work documents the importance of p26 in these embryos (for example, Clegg *et al.*, 1994, 1995; Liang *et al.*, 1997; Liang and MacRae, 1999; Crack *et al.*, 2002; Clegg and Trotman, 2002; MacRae, 2003; Sun and MacRae, 2005; Qiu *et al.*, 2006; Sun *et al.*, 2006; Villeneuve *et al.*, 2006). In the absence of stress, all of the p26 is in the cytoplasm and presumably not associated with cytoplasmic elements. Upon encountering stresses such as high temperature or anoxia (or when the diapause embryo forms) about half of the p26 is translocated into nuclei (see Willsie and Clegg, 2002) and perhaps other sites such as mitochondria (unpubl. obs.). These translocations are apparently initiated by stress-induced acidification within the embryos (Clegg *et al.*, 1994, 1995; Willsie and Clegg, 2001, 2002). That intracellular pH regulates anoxia-induced metabolic transitions was first shown by Crowe and colleagues in the early 1980s (see Busa and Crowe, 1983) and confirmed many times since then (see Hand, 1997, 1998; Hand and Podrabsky, 2000). However, the mechanisms that lead to acidification, and its reversal after anoxia ends, have escaped description for almost 25 years. In two elegant studies, Covi and Hand (2005) and Covi *et al.* (2005) showed that V-ATPase is of direct and central importance in these proton transactions. The mechanism of the pH-induced translocation of p26 into and out of nuclei is not known, but it does not seem to require a source of free energy in the form of nucleotides (see Clegg and Trotman, 2002). This translocation process requires further study.

Translocated p26 is believed to function as a molecular chaperone for nuclear proteins, including lamins—major nuclear proteins—probably in association with hsp70 (Willsie and Clegg, 2002). Importantly, no indication of a requirement for ribonucleoside di- or tri-phosphates for p26 chaperoning can be found in a variety of *in vitro* studies (Liang *et al.*, 1997; Viner and Clegg, 2001).

Comparing the protein profiles of extracts from embryos after 1 year and 4.5 years of anoxia (Fig. 1), one is hard-pressed to conclude that they are appreciably different, considering the limited resolution of this system of gel filtration (for native proteins) followed by SDS-PAGE (for their subunits). Thus, it should be recognized that the resolving power of this approach would not reveal modest changes in tertiary and quaternary structure, only the aggregation that resulted from the initial unfolding of polypeptide chains. In view of the inherent instability of most globular proteins (Somero, 1995; Hochachka and Somero, 2002) and the lack of detectable protein turnover in these embryos (Clegg, 1997; Tanguay *et al.*, 2004), one can assume that at least some globular proteins are indeed unfolding, but that this does not lead to irreversible aggregation. Thus, it seems plausible that the chaperoning activity of p26 is involved. Further rationale for that interpretation follows.

Analysis reveals significant amounts of p26 in the void volume of embryos that had previously experienced a year of anoxia (Figs. 1B and 3), but not in controls (Figs. 1A and 3). That result is consistent with p26 binding to unfolding proteins, to aggregates that are large enough (greater than 4 million equivalent molecular mass) to enter the void volume, or to both entities—an interpretation in keeping with what is known about molecular chaperones (Morimoto *et al.*, 1994; Arrigo and Müller, 2002; MacRae, 2003; Kültz, 2005; Sun and MacRae, 2005). But if those anoxic embryos are first given a 2-h aerobic incubation and then analyzed as above, p26 disappears from the void volume and resumes its native molecular mass (fractions 9–12 in Fig. 3). Although no direct evidence was obtained for this interpretation, such results are precisely those expected for p26 chaperoning. An alternative interpretation would be that p26 undergoes self-aggregation and is then chaperoned back to its native state by itself or by some other molecular chaperone, such as hsp70. That possibility seems unlikely to me, but cannot be excluded by current results. However, hsp70 requires ATP to function, and there is adequate evidence that the amount of ATP is very low and that turnover is not occurring in long-term anoxic embryos. In addition, the levels of this stress protein are much lower than those of p26 in these embryos. Finally, if p26 is not actively chaperoning, then one must advance an alternative explanation for the observed exceptional stability of embryonic proteins during anoxia.

I believe the interpretation proposed here is the most plausible way to account for the lack of substantial and irreversible protein aggregation over 4.5 years of anoxia. In further support is the evidence that p26 does act as a molecular chaperone *in vivo*. Thus, when bacteria were transfected with the p26 gene and this protein was expressed, the bacteria exhibited significantly greater thermal tolerance (Liang and MacRae, 1999; Sun *et al.*, 2006), and similar results were obtained using human 293H cells in culture (Villeneuve *et al.*, 2006). Also, green monkey kidney cells were protected from the damaging effects of

hydrogen peroxide when pure p26 was directly introduced into these cells by means of the BioPORTER reagent (Collins and Clegg, 2004). Finally, Ma *et al.*, (2005) found that human 293H cells were better able to survive dehydration when transfected with p26, although it is also possible that p26 plays a role in reducing apoptosis under these conditions (Villeneuve *et al.*, 2006).

We recently turned our attention to the function of artemin (Fig. 1), a protein first described by De Herdt *et al.* (1979) and later by De Graaf *et al.* (1990). This ferritin-like protein (Warner *et al.*, 2004) is present in amounts comparable to p26, and both proteins appear and disappear at about the same time in development (Jackson and Clegg, 1996; MacRae, 2003). Artemin is worth mentioning here because there is evidence, albeit it somewhat indirect (Warner *et al.*, 2004), that artemin might be a protective RNA chaperone, a subject of increasing interest (Lorsch, 2002; Henics, 2003). Should this prove to be correct, it means that these embryos have acquired dual protection of macromolecules: p26 to protect proteins, and artemin to do the same for RNA.

A unique feature of p26 and artemin in terms of stress proteins is that they are synthesized as an integral part of the developmental program of these embryos and not in response to environmental stress *per se* (see Jackson and Clegg, 1996; Liang and MacRae, 1999; Clegg and Trotman, 2002). It is as if these syntheses pre-meditate stresses that these robust embryos may encounter soon, or decades after they are produced and released into their harsh environment. This scenario is yet another example of the remarkable features of the encysted embryo of *Artemia*, and its repertoire of biochemical and biophysical adaptations (reviewed by Clegg and Conte, 1980; Clegg and Trotman, 2002).

Are the massive levels of these stress proteins sufficient to account for the extraordinary tolerance to anoxia exhibited by these embryos? Although possible, it seems likely that factors in addition to these stress proteins are involved in the remarkable tolerance of these embryos to prolonged anoxia and other stresses that rapidly kill nonadapted cells.

Acknowledgments

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Footnotes

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