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Evidence of metabolic microevolution of the limpet *Nacella concinna* to naturally high heavy metal levels in Antarctica

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ABSTRACT

The gastropod *Nacella concinna* is the most conspicuous macroinvertebrate of the intertidal zone of the Antarctic Peninsula and adjacent islands. Naturally high levels of copper and cadmium in coastal marine ecosystems are accumulated in *N. concinna* tissues. We aimed to study the effects of metal cations on *N. concinna* arginase in the context of possible adaptive microevolution. Gills and muscle had the highest argininolytic activity, which was concentrated in the cytosol in both tissues. Gills had the highest levels of arginase and may be involved in the systemic control of L-arginine levels. The relatively high argininolytic activity of the *N. concinna* muscular foot, with $K_M = 25.3 \pm 3.4$ mmol L^{-1}, may be involved in the control of L-arginine levels during phosphagen breakdown. *N. concinna* arginases showed the following preferences for metal cations: $Ni^{2+} > Mn^{2+} > Co^{2+} > Cu^{2+}$ in muscle and $Mn^{2+} > Cu^{2+}$ in gills. Cu^{2+} activation is a unique characteristic of *N. concinna* arginases, as copper is a potent arginase inhibitor. Cu^{2+} partly neutralized *N. concinna* arginase inhibition by Cd^{2+}, worked synergistically in muscle arginase activation by Co^{2+} and neutralized muscle arginase activation by Ni^{2+}. Mn^{2+} was able to activate muscle arginase in the presence of Fe^{3+} and Pb^{2+}. The selection of arginases that are activated by Cu^{2+} and resistant to inhibition by Cd^{2+} in the presence of Cu^{2+} over evolutionary timescales may have favored *N. concinna* occupation of copper- and cadmium-rich niches.

1. Introduction

The limpet *Nacella concinna* is found near the shore along the coast of the Antarctic Peninsula and the adjacent sub-Antarctic Islands, and it is the most conspicuous macroinvertebrate of the intertidal zone (Picken, 1980). Two populations with different morphologies and metabolic responses occur in the region. Few benthic invertebrates are able to colonize the Antarctic intertidal zone. The high annual air temperature variation (approximately 30 °C), the risk of desiccation due to air exposure, salinity fluctuations during tidal cycles, and common ice scouring in summer and ice encasement in winter render this intertidal zone one of the most extreme and stressful marine environments on the planet (Obermüller et al., 2011; Suda et al., 2015).

Although Antarctic coastal marine ecosystems are considered pristine, naturally high levels of heavy metals occur in the near-shore seawater and sediments of the Antarctic Peninsula and sub-Antarctic islands. Copper concentrations on the surface of marine sediments of King George Island (44–92 μg/g) are significantly higher than the global average copper concentration in coastal and estuarine sediments (28 μg/g) and nearly reach those of the most polluted coastal regions of the planet (93 μg/g) (Ahn et al., 1996; Santos et al., 2005). The high levels of copper probably due to the leaching of volcanic soils during glacier runoff. Southern Ocean cadmium levels are several times higher than those reported for other oceans. The deep-water upwelling has been considered to be most probable reason for cadmium displacement from sediments into the water column, providing cadmium for bioaccumulation in phytoplankton (Choi et al., 2007; Honda et al., 1987). Accordingly, some Antarctic marine organisms are well adapted to occupy niches that are naturally rich in copper and cadmium. Lo Giudice et al. (2013) showed that the increased tolerance of some Antarctic bacteria to certain heavy metals (tolerance to Cd > Cu > Zn > Hg) is closely related to their levels in marine sediments, suggesting that those bacteria are well prepared to cope with and/or have adapted to their environmental conditions.

Heavy metal bioaccumulation and biomagnification processes are involved in the food chain of Antarctic ecosystems, and at least

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two bivalves (*Laternula elliptica* and *Adamussium colbecki*) meet the criteria recommended by the "Mussel Watch" program for metal monitoring (Ahn et al., 1996; Duquesne and Riddle, 2002). Copper, manganese and lead accumulation in *N. concinna* tissues in areas subjected to glacier runoff is greater than in other locations, thus showing that the availability and bioaccumulation of certain heavy metals vary seasonally in near-shore Antarctic areas (Ahn et al., 2004; Curtosi et al., 2010; Trevizani et al., 2016). Marine mollusks are able to accumulate heavy metals and survive in polluted environments by immobilizing metals into insoluble electron-dense granules and/or forming soluble complexes with metallothioneins (Viarengo and Nott, 1993). These mechanisms reduce the intracellular concentrations of heavy metals and limit their availability to form bonds with the sulfur, nitrogen and oxygen atoms of cellular components, especially proteins and enzymes; as such bonds can lead to cellular dysfunction and death (Sharma et al., 2014; Viarengo and Nott, 1993).

Arginases are metalloenzymes that are widely distributed in the animal kingdom and depend on a divalent metal cation for full activity and structural stability. Mn^{2+} is the most common physiological cation, although Co^{2+} and Ni^{2+} may replace Mn^{2+} in activating the arginases of some organisms (Jenkinson et al., 1996; Marino et al., 2013). Conversely, Cd^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+} have been reported as potent inhibitors of arginases in mollusks (Rodrigues et al., 2009; Tormanen, 1997), fish (Srivastava and Ratha, 2013) and mammals (Munakata et al., 1976; Tormanen, 2001).

The role of arginases in specific invertebrate physiological processes has not been well studied. The removal of L-arginine formed during mollusk phosphagen breakdown typically occurs through octopine synthesis in a reaction catalyzed by the enzyme octopine dehydrogenase. However, the muscular foot of the gastropod *Helix pomatia* hydrolytically removes L-arginine in a reaction catalyzed by arginase (Wieser and Platzer, 1983). Similarly, the muscular foot of *N. concinna* accumulates no octopine during phosphagen breakdown (Pörtner et al., 1999), although the mechanism by which L-arginine is removed remains unclear. Arginase tissue distribution varies among mollusks, and high levels of arginase have been found in the gill (Carvajal et al., 1984, 1994), muscular foot (Carvajal et al., 1988) and kidney (Rodrigues et al., 2009). High arginase levels in non-ureotelic animal tissues have been associated with the control of L-arginine levels, as L-arginine is a substrate of several metabolic pathways (Jenkinson et al., 1996).

To our knowledge, no reports of Cu^{2+}-activated arginases have been published. Whereas, Cd^{2+} may replace Mn^{2+} in reactions catalyzed by mollusk arginases, although the low activating capacity of Cd^{2+} represents a great catalytic disadvantage for the reaction (Carvajal et al., 1988, 1994). High copper and cadmium levels in the *N. concinna* gut indicate that the uptake pathway for these metals is essentially enteric. However, there is a lack of robust data about copper and cadmium bioaccumulation in microphytobenthos, which are the most likely food source for *N. concinna* (Ahn et al., 2004, 2002). Data about the tolerance of *N. concinna* physiological systems to heavy metals is scarce. Nevertheless, high levels of cadmium dissolved in seawater may deteriorate the *N. concinna* digestive gland epithelium, thereby compromising the digestion and absorption of dietary components (Najle et al., 2000).

The present study aimed to examine the tissue distributions, subcellular locations and kinetic properties of *N. concinna* arginases in the context of protein-based energy metabolism. We also assessed the effects of metal cations, especially Cu^{2+} and Cd^{2+}, on the argininolytic activity of *N. concinna*, with the goal of further understanding the occupation of niches rich in these metals.

2. Materials and methods

2.1. Animal sampling

N. concinna specimens were collected from the Keller Peninsula intertidal zone (62°05'28.9" S/58°24'21.3" W), Admiralty Bay, King George Island, South Shetlands Archipelago, Antarctica (Fig. 1). The limpets were picked by hand from the rocks between January and March 2011, transferred immediately to aquaria at the Brazilian Antarctic Station Comandante Ferraz and kept in seawater, where they were acclimatized to a thermo-saline condition of 0 °C and 35 psu for 48 h. The experiments were conducted using limpets with shell length 32–45 mm. Limpet captures and experimental procedures were authorized by the Environmental Management Group of the Brazilian Ministry of the Environment.

2.2. Preparation of tissues extracts and subcellular fraction

The freshly excised foot muscles, gills and remaining pooled soft tissues of 5 limpets were homogenized in 20 mM HEPES buffer (containing 5 mM K_2HPO_4, 1 mM trimethylamine N-oxide and 250 mM sucrose at pH 7.4) at ratios of 1 g to 5 mL, 1 g to 10 mL and 1 g to 10 mL, respectively. The homogenates were sonicated for 30 s and centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatants were used to study the effects of substrate (L-arginine) concentration and metal cations on arginase activity.

Gills, foot muscles and a pool of other soft tissues from 10 limpets were used to determine the tissue levels of arginase. Tissue homogenates, prepared as described above, were centrifuged at 2500*g* for 10 min. Aliquots of the supernatant were used to determine the arginase and total protein levels. The remaining gill and muscle supernatants were then centrifuged at 12,000*g* for 10 min. The supernatants from that step were used to quantify the total arginase activity in the cytosolic fraction. Pellets containing mitochondria were resuspended in homogenization buffer and centrifuged at 12,000*g* for 10 min; this procedure was then repeated twice. After the final centrifugation, the pellets were resuspended in 500 μL HEPES buffer, sonicated for 30 s to disrupt the mitochondrial membranes and centrifuged for 10 min at 12,000*g* (Casey and Anderson, 1982). The supernatants were used to determine the total arginase activity in the mitochondrial fraction. All of the preparative steps were conducted at 4 °C.

2.3. Analytical methods

Tissue and subcellular arginase levels were determined in a reaction mixture containing 20 mM glycine buffer, 100 mM L-arginine and 1 mM $MgCl_2$ at pH 9.5. L-arginine saturation curves were established in 20 mM HEPES buffer containing 1 mM $MnCl_2$ and L-arginine concentrations ranging from 0 to 200 mM at pH 7.4. The effects of metal cations were studied in reaction mixtures containing 20 mM HEPES buffer, 30 mM L-arginine and 1 mM metallic cation salt ($HgCl_2$, $Zn(CH_3COO)_2$, $CoCl_2$, $Cu(CH_3COO)_2$, $NiCl_2$, $CdCl_2$, $Pb(CH_3COO)_2$, $FeCl_3$ or $MnCl_2$) at pH 7.4. Baseline argininolytic activity was determined in a reaction mixture without metal cation addition. The reaction was started by adding aliquots of gill homogenate (10 μL, with protein concentrations ranging from 2.6 to 5.3 mg/mL) or muscle homogenate (40 μL, with protein concentrations ranging from 4.3 to 7.3 mg/mL). All kinetic analyses were conducted at 0 °C. The reaction was stopped by adding HCl to a final concentration of 0.375 mM. L-ornithine formed in the reaction was quantified using the method described by Chinard (1952). Spectrophotometric measurements were performed in a FLUOstar OPTIMA microplate reader at 520 nm. Enzymatic activity was expressed in nmol L-ornithine formed per minute (mU). Total protein was determined using the bicinchoninic acid (BCA)