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Carbonic anhydrase induction in euryhaline crustaceans is rate-limited at the post-transcriptional level

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ABSTRACT

The transfer of euryhaline crustaceans from full-strength seawater to low salinity results in both a rapid up-regulation of carbonic anhydrase (CA; EC 4.2.1.1) mRNA and a slow induction of CA activity. There is a delay of several days between the two processes, which is attributed to the time required to synthesize new enzyme. These delays may also be due to limitations in the cellular uptake of Zn, which is a required post-translational active site modification to CA. To investigate these processes, the euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, were acclimated to salinities below their isosmotic points (22.5 and 25 ppt, respectively) for 7 days to activate the physiological and molecular mechanisms of osmoregulation. CA mRNA increased 90-fold in *C. sapidus* and 2-fold in *C. maenas* within 6 h; whereas it took 48 h for the initial increases in CA activity (120% and 31%), and 4 to 7 days for new acclimated levels (300% and 100%, respectively). Crabs were then transferred to lower salinities (10 and 15 ppt) to induce further CA activity and to determine if previous increases in CA mRNA reduced the time required for subsequent CA induction. Additionally, the expression of the Zn transporter ZIP1 was examined in *C. sapidus* at 35 and 22.5 ppt. In both species, prior CA mRNA elevation failed to accelerate the rate of CA induction. Levels of CA mRNA did not change in either crab following transfer from intermediate to low salinity. Taken together, these results show that the timecourse of CA induction at low salinity is not limited by the expression of CA mRNA, but by the synthesis of new enzyme from an existing pool of mRNA. No increases in ZIP1 expression occurred at low salinity, therefore these delays may be due to the limits of cellular Zn uptake.

1. Introduction

Euryhaline marine crustaceans are known to be exceptionally capable of surviving large changes in environmental salinity. These animals are isosmotic when acclimated to full strength seawater (~32–35 ppt), passively maintaining hemolymph osmolality and ionic concentrations equal to those of the surrounding medium. However, at lower salinities, usually around 25–26 ppt, these crustaceans activate ionic regulation processes that allow them to maintain their hemolymph well above ambient osmolality (see Mantel and Farmer, 1983; Pequeux, 1995; Henry et al., 2012 for reviews).

At low salinity, osmoregulating crustaceans maintain hyperosmotic hemolymph by actively pumping ions (mainly Na^+ and Cl^-) from the environment across the gills and into the hemolymph. The cellular mechanisms by which this is accomplished have been extensively studied (see Pequeux, 1995; Towle and Weihrauch, 2001; Towle et al., 2010; Henry et al., 2012; McNamara and Faria, 2012; for reviews).

There are two different classes of mechanisms of ionic uptake that have been observed in crustaceans, one in animals with high gill epithelial conductance and ion transport rates, and one in those with low gill epithelial conductance and transport rates (see Henry et al., 2012; McNamara and Faria, 2012 for reviews). In low conductance crustaceans such as the Chinese crab, *Eriocheir sinensis*, individuals spend the majority of their life cycle in fresh water. Crustaceans with high conductance gills however are typically euryhaline marine species such as the blue crab *Callinectes sapidus* and the green crab *Carcinus maenas*. These species migrate annually between full-strength seawater and the more dilute waters of the estuary (e.g., Henry, 2001; Henry et al., 2012). In the gills of these crustaceans, a $Na^+/K^+/2Cl^-$ co-transporter protein located on the apical membrane transports Na^+ and Cl^- ions from the surrounding water into specialized mitochondria-rich cells (MRC). The presence of K^+ channels on the apical and basolateral membranes results in K^+ efflux across these surfaces, establishing an electrochemical gradient (inside negative) that drives Cl^- through channels on the basolateral membrane and into the hemolymph. The basolateral Na^+/K^+-ATPase pumps Na^+ from the MRC into the hemolymph. NaCl is also absorbed from the surrounding medium via apical Na^+/H^+ and Cl^-/HCO_3^- exchange proteins. In some species this process may be electrogenic, and does not utilize counterions for exchange (Onken et al., 2003). However in other species, such as *C. sapidus* and *C. maenas* this process is electroneutral and utilizes the hydration of respiratory CO_2 by cytoplasmic carbonic anhydrase (CA) to provide H^+ and HCO_3^-,which

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serve as counterions for Na^+ and Cl^- uptake, respectively, in low salinity (Cameron, 1979; Henry and Cameron, 1982, 1983; Henry et al., 2003). There has been some debate as to whether the presence of the $Na^+/K^+/2Cl^-$ co-transporter obviates a role for CA in ionic uptake, however a large a body of evidence in both *C.maenas* and *C. sapidus* (localization, salinity-sensitivity, and physiological responses to inhibition of CA — reviewed by Henry et al., 2012), confirms the central role of CA and points to the potential for multiple NaCl uptake mechanisms that operate concurrently.

When crustaceans are acclimated to full-strength seawater and are in a state of osmoconformity, the aforementioned processes are physiologically silent, being activated only upon exposure to salinities below 26 ppt (Henry, 2005). This includes the up-regulation of the transport proteins responsible for ion regulation. The Na^+/K^+-ATPase and CA are perhaps the two best-studied molecules regarding the activation of ionic regulatory processes (see Henry, 2001; Towle and Weihrauch, 2001; Henry et al., 2012 for reviews), with increases in specific activity of these enzymes being documented in many different euryhaline marine crustaceans (Towle et al., 1976; Henry and Cameron, 1982; Holliday, 1985; Wheatly and Henry, 1987; Corotto and Holliday, 1996; Mañanes et al., 2000; Henry et al., 2002, 2003; Roy et al., 2007; Tsai and Lin, 2007). These elevations occur in the posterior, ion-regulating gill pairs and are typically absent in the anterior, respiratory gills (e.g., Henry, 1984; Towle, 1984).

Low salinity-stimulated increases in both CA and Na^+/K^+-ATPase activity are preceded by the up-regulation of the mRNAs encoding these proteins (Luquet et al., 2005; Jayasundara et al., 2007; Serrano et al., 2007; Serrano and Henry, 2008). Like salinity-stimulated CA and Na^+/K^+-ATPase activity, these gene activation events occur in the posterior ion-regulating gills and are absent in anterior gills. These data strongly suggest that the increases in activity of these enzymes at low salinity are the direct result of gene activation and subsequent synthesis of new protein. In most crustacean species studied, the increases in gene expression occur rapidly and are quite dramatic (Luquet et al., 2005; Jayasundara et al., 2007; Serrano et al., 2007; Serrano and Henry, 2008). For example, in the *C. maenas* and *C. sapidus*, the up-regulation of CA mRNA following low salinity transfer increases by 10-fold after 6 h and 100-fold after 24 h (Serrano et al., 2007; Serrano and Henry, 2008).

Despite the thoroughness with which CA gene activation and rises in enzyme activity have been studied in the last decade, several unanswered questions still exist pertaining to the molecular mechanism of CA induction. Some evidence suggests that there are molecular limitations that dictate the speed at which CA activity can increase. Despite the rapid onset of CA mRNA upregulation, increases in CA activity in *C. sapidus* and *C. maenas* occur relatively slowly, taking 24 – 96 h for the initial increase. Furthermore, in both species, CA activity continues to rise from 4 to 7 days following the initial increase. This is independent of the levels of CA mRNA, as gene expression does not rise further after the initial 6–24 h upregulation, suggesting that post-transcriptional processes rate-limit CA induction (Henry and Cameron, 1982; Henry and Watts, 2001; Henry et al., 2002, 2003; Henry, 2005; Serrano et al., 2007; Serrano and Henry, 2008). Other proteins have shown the same pattern of rapid mRNA expression and slower increases in activity (e.g., UPC1, a protein used in thermogenesis of mammalian brown adipose tissue) (Nedergaard and Cannon, 2013). However, some proteins show rapid induction: ornithine decarboxylase activity can be up-regulated within minutes (Watts et al., 1996), suggesting that there may be regulatory limits other than protein synthesis. It may be that the delays in CA activity induction are the result of limitations in Zn transport. CA requires a Zn atom to be bound to the active site in order to perform catalysis (McCall et al., 2000). In order to bind Zn to the large cytoplasmic pool of CA, it presumably must be extracted from food in the gut, transported to the gills, and taken into the MRC. Cellular uptake is very likely accomplished via the action of Zip proteins. These proteins have been implicated as the primary mechanism for zinc transport and regulation in yeast, plants, *Drosophila*, and mammals (Guerinot and Eide, 1999; Liuzzi and Cousins, 2004; Mathews et al., 2005). There are currently no published studies which have examined the possibility of CA limitation via Zn.

Another remaining question in salinity-stimulated CA induction is the cause of CA activity increases following multiple instances of environmental dilution. For example when osmoregulating crabs are acclimated to progressively lower salinities (e.g. 25 then 15 ppt) the degree of CA activity induction is progressively greater in the lower salinities (Henry and Watts, 2001; Henry, 2005). It is currently not known if these increases in enzyme activity result from enhanced gene expression or if they come about by increased translation of the CA message to new protein from the same pool of mRNA.

In this study we examined these questions by acclimating both *C. sapidus* and *C. maenas* to high salinity and then subjecting them to sequential salinity transfers: first to an intermediate salinity below their isosmotic points in order to activate CA expression and induction, and then to a lower salinity in order to stimulate further CA induction. Our goals were twofold; first, to determine if the delay in CA induction could be reduced by prior activation of the induction mechanism; and second, to determine if the elevations in CA activity at the lowest salinities were accompanied by increases in CA gene expression or if they were simply the result of increased protein synthesis from the same pool of mRNA. Additionally we investigated the gene expression levels of ZIP1, a member of the Zip Zn-transporting family, in *C. sapidus* at high and low salinity to examine the possible role of Zn as a limiting-factor in CA induction.

2. Materials and methods

2.1. Collection and maintenance of animals

Adult, intermolt, blue crabs, *Callenectes sapidus* were obtained from commercial watermen in East Point, FL, USA, packed in wet burlap, placed in 45 L coolers, and transported to Auburn University. Crabs were held in 570 L recirculating aquaria equipped with biological filters (oyster shell and sand) at 24 °C and ambient photoperiod. Crabs were held at 35 ppt for at least 3 weeks prior to experimentation to ensure that CA activity and expression were at baseline levels (Henry and Watts, 2001; Jillette et al., 2011). Blue crabs were fed daily on shrimp, salinity was monitored with a conductivity meter (YSI 30 1, Yellow Springs, OH, USA) and adjusted with distilled water or concentrated brine made with artificial sea salts (Instant Ocean Reef Crystals, Blacksburg, VA, USA). Water quality was checked by monitoring nitrite concentrations (Dry Tab, Mansfield, MA, USA).

Adult, intermolt green crabs, *C. maenas* L., were collected by hand from the intertidal zone, and by trap from the subtidal zone of Frenchman's Bay along the shoreline of the Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, ME, USA. Green crabs were held at MDIBL in 400 L fiberglass tanks equipped with filtered, running seawater (32–33 ppt salinity and 11–12 °C). Crabs were fed a combination of mussels and squid every other day but were starved for a minimum of 48 h prior to use. Salinity was checked with a hand-held refractometer, and water quality was monitored by measuring nitrite concentrations of all holding tanks and experimental aquaria (Dry Tab).

2.2. Experimental protocol

C. sapidus and *C. maenas* were acclimated to full strength seawater (35 ppt for *C. sapidus*, 32 ppt for *C. maenas*) for 3 weeks prior to being used in any experiment. For *C. sapidus* one set of crabs was transferred to 22.5 ppt. CA activity and relative gene expression were measured in six crabs at each time point: 0 (35 ppt acclimated), 6, 12, 24, 48, 96 h, and 7 days following that transfer. To test the effects of previously elevated CAc expression on the induction of CA activity, a second set of crabs was acclimated to 22.5 ppt for 1 week (used as 0 h) and