Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas* ©

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Abstract

The euryhaline green crab, *Carcinus maenas*, is a relatively strong osmotic and ionic regulator, being able to maintain its hemolymph osmolality as much as 300 mOsm higher than that in the medium when the crab is acclimated to low salinity. It makes the transition from osmoconformity to osmoregulation at a critical salinity of 26 ppt, and new acclimated concentrations of hemolymph osmotic and ionic constituents are reached within 12 h after transfer to low salinity. One of the central features of this transition is an 8-fold induction of the enzyme carbonic anhydrase (CA) in the gills. This induction occurs primarily in the cytoplasmic pool of CA in the posterior, ion-transporting gills, although the membrane-associated fraction of CA also shows some induction in response to low salinity. Inhibition of branchial CA activity with acetazolamide (Az) has no effect in crabs acclimated to 32 ppt but causes a depression in hemolymph osmotic and ionic concentrations in crabs acclimated to 10 ppt. The salinity-sensitive nature of the cytoplasmic CA pool and the sensitivity of hemolymph osmotic/ionic regulation to Az confirm the enzyme’s role in ion transport and regulation in this species. CA induction is a result of gene activation, as evidenced by an increase in CA mRNA at 24 h after transfer to low salinity and an increase in protein-specific CA activity immediately following at 48 h post-transfer. CA gene expression appears to be under inhibitory control by an as-yet unidentified repressor substance found in the major endocrine complex of the crab, the eyestalk.

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1. Introduction

Marine invertebrates, when acclimated to open ocean salinity, are osmotic and ionic conformers. Total osmotic concentration, and the concentrations of the constituent ions that make up the bulk of that total, are slightly higher in the hemolymph of the animal than they are in the surrounding medium. These values, however, are believed to be in passive equilibrium with those in the ambient seawater; and if osmotic and ionic concentrations in the seawater decrease, the concentrations in the hemolymph decrease in a parallel fashion (e.g. Florkin and Schoffeniels, 1969; Kinne, 1971). When osmoconformers are exposed to low environmental salinity they undergo hemodilution and cell swelling due to the influx of water. They respond to this challenge with a cell volume regulatory response in which the concentration of intracellular solutes is reduced, removing osmotically obligated water, and restoring the cell to near its original volume (e.g. Pierce and Amende, 1981). The ability of osmoconformers to survive in low salinity depends in their ability to reduce the pool of intracellular osmolytes. Most are stenohaline, surviving down to a salinity of approximately 18 ppt, but even the most euryhaline conformers have a lower lethal salinity in the range of 8–10 ppt (Kinne, 1971). The species that can survive salinity fluctuations from full strength seawater down to fresh water, however, are the osmotic and ionic regulators, those species that have the ability to maintain hemolymph osmotic and ionic concentrations above those in the ambient medium (e.g. Mantel and Farmer, 1983). The most commonly studied osmoregulators have been the decapod crustaceans.

With few exceptions, marine invertebrates, including crustaceans, are conformers in full strength seawater, and these species undergo at least some degree of hemodilution when exposed to low salinity. However, at a critical low salinity, typically approximately 25 ppt, osmoregulators activate a suite of cellular and molecular mechanisms that result in the activation of systemic ion uptake across the primary ion-transporting epithelium, the gills (Towle, 1997; Henry, 2001a,b). While all ions are believed to be actively transported across the gills in low salinity in these species, Na$^+$ and Cl$^-$, the two major ions that make up over 90% of the total hemolymph osmolality, have received the most study (e.g. Smith and Linton, 1971; Cameron, 1978; Riestenpatt et al., 1996). The molecular basis of osmoregulation involves the coordinated function of a number of individual transport proteins that are expressed in the gills. Na$^+$ is believed to be transported across the apical surface of the gill epithelium by one or more of the following proteins: a sodium–hydrogen exchange protein (NHE) (Towle et al., 1997), an outwardly directly V-type hydrogen ATPase coupled to an inwardly directly Na$^+$ channel (Lin and Randall, 1991; Onken and Putzenlechner, 1995), or a Na$^+/K^+/2Cl^-$ exchange protein (Riestenpatt et al., 1996). Cl$^-$ is believed to move across the apical membrane via Cl$^-$/HCO$_3^-$ or Cl$^-$/OH$^-$ exchange (e.g. Kirschner, 1979). The enzyme carbonic anhydrase (CA), localized in the branchial cytoplasm, is believed to play a support role in the general transport mechanisms of all ions by supplying H$^+$ and HCO$_3^-$ through the catalyzed hydration of respiratory CO$_2$ as it diffuses through the gills (Henry and Cameron, 1983; Henry, 1988a,b). Na$^+$ is then transported from the intracellular compartment of the gill into the hemolymph via a basolaterally localized Na$^+/K^+$-ATPase (Towle, 1984; Towle and Kays, 1986), with Cl$^-$ following passively down an electrochemical gradient.

A number of the individual transport proteins appear to be both tissue- and species-specific; however, the Na$^+/K^+$-ATPase, which is believed to provide the driving force for active ion uptake, and CA, which is believed to support the active transport of both cations and anions, are present in the gills of all euryhaline species. Because of these features, both enzymes are considered to be central molecular components of the systemic process of ion transport and regulation.

Among euryhaline crustacean species, the two that have received the most study are the blue crab, Callinectes sapidus and the green crab, Carcinus maenas. The green crab is a euryhaline osmotic and ionic regulator that is routinely found in estuarine waters of salinities as low as 8–10 ppt and has been acclimated to 5 ppt in the laboratory (Zanders, 1980; Siebers et al., 1982). A strong regulator, C. maenas can maintain its hemolymph osmotic concentration more than 250 mOsm above that in the medium (560 vs. 300 mOsm for crabs acclimated to 10 ppt) (e.g. Zanders, 1980). This is accomplished by high rates of Na$^+$ and Cl$^-$ influx across the gills (500–800 µmol Na$^+$ g$^{-1}$ h$^{-1}$, and 250–700 µmol
Cl$^-\text{g}^{-1} \text{h}^{-1}$) (Siebers et al., 1987a,b; Lucu, 1989) that keep the animal in positive salt balance with respect to low environmental salinity. These high rates of salt transport require high levels of activity of both the Na$^+$/K$^+$-ATPase and CA, and these values are in fact found in the posterior, ion-transporting gills of the crab (e.g. Henry et al., 2002). CA activity, in particular, undergoes an 8-fold increase in response to a change in salinity from 32 to 10 ppt, making this species a good potential model system for the study of salinity-mediated CA induction. This report reviews the current state of knowledge on the role of branchial CA in the process of low-salinity adaptation in decapod crustaceans, and it adds new data on the regulation of CA expression in the euryhaline green crab, *C. maenas*.

2. Materials and methods

2.1. Collection and maintenance of animals

Adult, male intermolt individuals of *C. maenas* were collected from the intertidal zone at the Mount Desert Island Biological Laboratory (MDIBL) during the months of June and July (average summer salinity = 32 ppt). Crabs were held in running seawater (32 ppt, 10–12 $^\circ$C) at the MDIBL for at least 48 h before use; they were fed squid twice per week and were starved for 24 h prior to use. For low-salinity acclimation, crabs were transferred directly into a 120-l recirculating tank (10 ppt, made by diluting natural seawater with deionized water produced by reverse osmosis) equipped with a 15-gallon biological filter. Crabs were not fed during the acclimation process.

2.2. Experimental protocol

For the experiment involving the use of acetazolamide (Az), a stock solution of 100-mM Az was made up in deionized water (pH adjusted to 9.0 with NaOH) and titrated to pH 8.2 with 1 N HCl. Two groups of crabs, acclimated to 32 or 10 ppt for 2 weeks, were used. A small hole was drilled in the dorsal carapace of the crab, directly above the heart, and sealed with a rubber septum and cyanoacrylate glue (Henry and Cameron, 1983). The crabs were weighed, and hemolymph volume was estimated as approximately 30% of the wet weight (Gleeson and Zubkoff, 1977). Control hemolymph samples were taken from the sinus at the base of the walking legs using a 22-ga needle and 1-ml syringe. An appropriate volume of the Az stock solution was injected to produce a circulating concentration of 1 mM. Hemolymph samples were then taken over a period of 96 h post-injection. Samples were frozen and stored at $-20^\circ$C until analysis.

For the experiments involving branchial CA assays, nucleotide sequencing and mRNA expression, crabs were anesthetized in crushed ice for 20 min. Individual pairs of anterior (G3) and posterior (G7) gills were dissected out. For the simultaneous measurement of CA activity and mRNA expression, the gills from the left side of the crab were used in the CA assay, and the gills from the right side were used for RNA analysis. Anterior (G1-6) and posterior (G7-9) gills were pooled for the isolation of the different subcellular fractions of gill tissue. The crabs were killed by exsanguination.
To study the presence of a putative CA repressor, thought to be localized in the major endocrine complex of the crab, the eyestalk, crabs were treated with eyestalk ablation (ESA), or a combination of ESA plus injections of eyestalk homogenates. For treatment with ESA, crabs acclimated to 32 ppt were anesthetized in crushed ice, and the eyestalks were removed by making a cut where they attached to the carapace. Crabs were left on ice for 10 min to allow clotting to occur, and then they were returned to the seawater system. ESA-treated crabs were left at 32 ppt for 7 days, at which point they were anesthetized, and the gills were removed for CA assay. A second set of crabs were treated with ESA and then injected daily with homogenates of eyestalks taken from other crabs that were also acclimated to 32 ppt. A pair of eyestalks was homogenized in 500 μl of filtered (0.45 μm) seawater and centrifuged at 10 000 × g for 10 min at 4 °C (Sorvall RC5-B). An individual crab was given a daily injection of 400 μl of the supernatant, the equivalent of one pair of eyestalks per day. Injections were given over a period of 7 days, and the crabs were then anesthetized and the gills dissected out for CA assay. Sham-operated crabs were given daily injections of 400 μl of filtered seawater.

2.3. Analytical methods

2.3.1. Hemolymph osmotic and ionic concentrations

Hemolymph was thawed on ice, sonicated (25 W, 30 s, Heat Systems Microson) to disrupt the clot, and centrifuged (10 000 × g for 60 s, Fisher 235B microfuge) to separate clot from serum. Total osmolality was determined on 10 μl of serum by dew point depression using a Wescor 5100C vapor pressure osmometer. Chloride ion concentration was determined by Ag titration (Labconco chloridometer), and Na⁺ and K⁺ concentrations were measured by flame photometry (Radiometer FLM3).

2.3.2. CA activity measurements

CA activity was assayed electrophoretically using the delta pH method of Henry (1991a). For the experiments involving salinity transfers, branchial tissue from individual gills was homogenized in 2 ml of buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris, adjusted to pH 7.4 with 10% phosphoric acid) using an Omni 1000 homogenizer. Samples were centrifuged at 10 000 × g for 20 min at 4 °C (Sorvall RC5-B), and CA activity was assayed in the supernatant. Protein concentration was determined by the Coomassie blue dye binding method (Bio Rad Laboratories, Hercules, CA), and CA activity was reported as μmol CO₂ (mg protein⁻¹) min⁻¹.

In a separate procedure, pooled anterior or posterior gills (~5 gm each) were weighed and homogenized in five volumes of buffer using a motor-driven teflon-glass homogenizer (minimum of 16 complete passes) (Henry, 1991b). The homogenates were then subjected to differential centrifugation: 750 × g for 20 min; 7500 × g for 20 min, and 100 000 × g for 90 min, to produce, respectively, a cellular debris pellet, a mitochondrial pellet, and a final separation of a microsomal pellet from the cytoplasmic fraction (Henry et al., 1988; Henry, 1991b). The pellets were resuspended in buffer, and CA activity was assayed in each of the fractions as described above. The volume of each fraction was measured, and raw CA activity (μmol CO₂ ml⁻¹ min⁻¹) was multiplied by the total fraction volume, resulting in values of CA activity that could be directly compared among fractions (e.g. Henry et al., 1986).

Cytoplasmic CA activity was titrated against increasing volumes of a stock solution of 5-μM Az. The data were transformed and graphed as a double reciprocal plot (Easson and Stedman, 1937), according to the following relationship:

\[ I_o/I = K_i/(1 - I) + E_o \]

where \( E_o \) (the y intercept of the plot) is the total concentration of free enzyme, \( K_i \) is the inhibition constant, and \( I \) is the fractional inhibition of enzyme activity at an inhibitor concentration of \( I_o \).

2.3.3. CA sequencing and RNA analysis

Total RNA from both anterior (G1-6) and posterior (G7-9) gills from crabs acclimated to 35 and 10 ppt salinity was isolated under RNAse-free conditions (Chomczynski and Sacchi, 1987) by phenol–chloroform extraction (RNAserts, Promega, Madison, WI). RNA concentrations and relative purity were measured by UV absorbance. Single stranded, complementary DNA was then produced from Poly-A mRNA in 2 μg of total RNA by reverse transcription using Superscript II reverse transcriptase (Invitrogen) and oligo dT as primer.
CA amino acid sequences from a variety of animal species were aligned using Clustal-W, and highly conserved domains were then used as the basis for designing degenerate sense and antisense primers. The putative cDNA for CA was amplified using a variety of primer combinations and PCR. Taq polymerase (Red Taq, Sigma, St. Louis, MO) was added after an initial heating to 92 °C and cooling to 60 °C. Thermal cycles of 94 °C (1 min), 45 °C (1 min) and 72 °C (2 min) were repeated a total of 30 times. Later amplifications employing non-degenerate primers were run at an annealing temperature of 55 °C rather than 45 °C.

Amplification products were separated electrophoretically on 0.8% agarose gels in 1×TBE buffer and visualized with ethidium bromide. Individual bands were excised from the gel with nuclease-free scalpels, and the DNA was extracted with a spin column kit (Qiagen). Products were directly sequenced without subcloning using an ABI 377 or 3100 automated sequencer at the Marine DNA Sequencing Center, MDIBL. Using degenerate and Carcinus-specific oligonucleotide primers for the polymerase chain reaction, we obtained two partial cDNA sequences that BLAST searches of GenBank indicated were related to CA sequences of other species.

To examine the expression of the CA mRNA in the posterior gills in response to low-salinity exposure, total mRNA was extracted from G7 of green crabs acclimated to 32 ppt and from crabs during the acute phase of acclimation (0–4 days) after being transferred to 12 ppt. Anterior and posterior gills (G3 and G7) from the opposite side of the same crabs were also dissected out, immediately frozen in liquid nitrogen, and stored at −70 °C until they could be assayed for CA activity. The change in expression of the CA mRNA was monitored using a semi-quantitative method of PCR (Towle et al., 1997). Species-specific primers were used for amplification of CA sequences; they are as follows:

81F: GGA GGA AAG CCT TGA GTG GG
82R: CCC TGA ACG TGA AGG AG

Biotin-dUTP was substituted for a portion of dTTP in the reaction mixture. Amplification proceeded under conditions in which the product abundance was directly dependent on the availability of cDNA template. Following electrophoresis, products were blotted onto nylon membranes and the biotinylated DNA was visualized with streptavidin and alkaline phosphatase (New England Biolabs Phototope System). Exposure to X-ray film indirectly revealed the relative abundance of CA mRNA that initiated the reverse transcription reaction. Arginine kinase (AK), an enzyme that does not change in either activity or expression in response to salinity, was used as an internal control (Kotlyar et al., 2000). Cycle-dependency for both CA and AK was determined (Fig. 10), and for the semi-quantitative estimation of mRNA, PCR programs of 18 and 20 cycles were used for AK and CA, respectively. Protein-specific CA activity was measured electrometrically as described above.

3. Results and discussion

3.1. CA as a critical transport enzyme

While the presence of various specific ion-transport proteins appears to be tissue- and species-specific, CA (along with the Na+/K+-ATPase) has been found to be present in significant amounts in all ion-transporting epithelia (e.g. Henry, 1984; Towle, 1984). As such, CA has always been believed to play a role in active ion uptake, especially across the gills of crustaceans; and more recently it has been characterized as one of the central components of the suite of biochemical adaptations that form the basis of the systemic mechanisms of branchial ion uptake (Henry, 2001a). The two major criteria for this are (1) salinity-sensitivity of CA expression and (2) measured disruption of ion transport and regulation when CA is inhibited by Az.

In the green crab, branchial CA activity is uniform and low in all gills of animals adapted to 32 ppt, a salinity in which they are osmotic and ionic conformers (Fig. 1a). CA activity undergoes a salinity-dependent induction, however; and when crabs are exposed to 10 ppt there is approximately an 8-fold increase in activity in the posterior three pairs of gills. This is very similar to the selective CA induction that occurs in the posterior, ion-transporting gills of the blue crab, C. sapidus (Henry and Cameron, 1982a; Henry and Watts, 2001), and it is believed to be a common adaptive feature of all euryhaline marine crustaceans capable of osmotic and ionic regulation (Henry, 1984; Piller et al., 1995). Similar evidence was also found in euryhaline fresh water species (e.g. salinity-sensitivity of branchial CA activity in the

The increase in CA activity reported here for the green crab is of similar magnitude to that reported for other euryhaline crustaceans, but it is much larger than previously reported (1.5- to 2-fold) for *C. maenas*, given the same low-salinity exposure (Bottcher et al., 1990). The most probable reason for this discrepancy is the different CA assays used in the two studies. The pH indicator dye assay used by Bottcher et al. (1990) is relatively insensitive, and the large change in pH (2.7 units) monitored also most likely resulted in pH-inhibition of the catalyzed hydration reaction (Coleman, 1980). As a result, it is likely that both the absolute levels of CA activity and the magnitude of the CA induction were being underestimated by that assay (Henry et al., 2002).

The ability of green crabs to regulate their hemolymph osmotic and ionic concentrations is also disrupted when branchial CA activity is inhibited. *C. maenas* is a relatively strong regulator, maintaining a hemolymph-medium gradient of approximately 260 mOsm when acclimated to low salinity (10 ppt/300 mOsm) (Zanders, 1980, see also Fig. 2). Both total osmolality and the concentrations of the major hemolymph ions, Na\(^+\), Cl\(^-\) and K\(^+\), are significantly lowered in crabs acclimated to 10 ppt after being given an injection of 1-mM Az (Fig. 3). Osmolality and ionic concentrations were significantly lowered by 6 h post-injection, and they remained depressed through 96 h. Az injection had no effect on hemolymph osmolality or on the concentrations of the major ions in green crabs acclimated to 32 ppt (Fig. 3). This is not surprising, as at this salinity *C. maenas* is an osmotic and ionic conformer, and its hemolymph...
lymph ion concentrations are maintained passively in equilibrium with those in the surrounding seawater. This response is similar to that reported in previous studies of other euryhaline crab species (e.g. blue crabs), and it supports the general idea that CA is physiologically important in all crustaceans that are capable of ion regulation. Treatment of blue crabs with Az had no effect on hemolymph osmotic and ionic concentrations in crabs acclimated to high salinity (28 ppt) but resulted in a dose-dependent depression of these values in crabs acclimated to low salinity (8 ppt) (Henry and Cameron, 1983; Henry, 1988b). Branchial ion transport in the fresh water crayfish, *Astacus leptodactylus*, has also been shown to be Az-sensitive (Ehrenfeld, 1974), as it has ion transport in blue crabs acclimated to fresh water (Cameron, 1979). Inhibition of branchial CA activity inhibits the active uptake of Na$^+$ and also results in a stimulation of Cl$^-$ efflux, so that the combined effect is to put the animal into negative salt balance (i.e. more salts were being lost to the medium by diffusion than were being taken up by the gills) (Ehrenfeld, 1974; Cameron, 1979; reviewed by Henry, 2001a). Furthermore, inhibition of branchial CA with Az in blue crabs acclimated to high salinity and acutely transferred to low-salinity results in 100% mortality by 48 h post-transfer, as a result of the breakdown of the ionic regulatory process (Henry and Cameron, 1982b).

These results, and the conclusions that branchial CA was involved in the general ion uptake mechanism of the gills, were called into question by a study on ion transport in *C. maenas* that reported no effect of 100-$\mu$M Az on the influx of either Na$^+$ or Cl$^-$ in isolated, perfused gills of the green crab (Bottcher et al., 1991). The discrepancy in the results and conclusions of these studies probably lies in the way Az was used in the two sets of experiments. Az is slow to permeate into cells; a circulating concentration of 1 mM in the hemolymph of blue crabs produces full branchial CA inhibition only after 4 h (Henry and Cameron, 1983); Bottcher et al. (1991) perfused the gills of green crabs with 100-$\mu$M Az for only 20 min, a concentration and incubation time that most likely did not result in full inhibition of branchial CA.

### 3.2. Properties of salinity-mediated CA induction

CA has one of the highest turnover numbers of any enzyme known (Maren, 1967), and because of this, it is rarely, if ever, considered to be the limiting step in the biochemical and physiological processes in which it is involved. CA is also typically expressed in excess of what is needed to meet the needs of the processes that it supports. For these reasons, the salinity-mediated induction of CA activity is an interesting system to study for two reasons: (1) it is the largest documented change in CA expression in the animal kingdom and (2) it is one of the few examples of a specific environmental factor (salinity) directly altering CA expression. Also, different CA isoforms are found in different subcellular fractions (Henry, 1988b, 1996), and not every isoform responds to environmental changes in the same way.

Salinity-mediated CA induction in crustacean gills has been localized specifically to the cytoplasmic pool of CA activity, the subcellular fraction that is believed to be involved in ion transport (Henry, 1988a,b). The pattern of subcellular distribution in the gills of *C. maenas* turns out to be very similar to that in the only other crustacean species studied, *C. sapidus*. For green crabs acclimated to 32 ppt, anterior and posterior gills had approximately the same levels of CA activity in each of the subcellular fractions. The initial pellet (cellular debris), produced by low-speed centrifugation, contained the highest levels of CA activity (Fig. 4). CA activity in the microsomal and cytoplasmic fractions were approximately equal, and both made up less than 10% of the total activity in the gill homogenate. The distribution of branchial CA in green crabs acclimated to 10 ppt was altered primarily in the posterior gills, in which there was a 5-fold increase in activity compared to posterior gills from crabs at 32 ppt (Fig. 5). There was no comparable increase in anterior gills. Most of the measured increase could be traced to two fractions: cellular debris and cytoplasm. The increase in cytoplasmic CA activity was almost 20-fold, and this fraction made up approximately 20% of the total branchial CA activity. In contrast, the microsomal fraction, although it did increase 3-fold over values found at 32 ppt, still made up only approximately 5% of the total CA activity of the gill (Fig. 5). CA activity associated with the cellular debris pellet has been shown to be mostly soluble (cytoplasmic) CA that is loosely associated with the pellet and easily removed (Henry et al., 1988), so the subcellular fraction of CA activity that is induced in response to low salinity appears to be localized primarily to the cytoplasmic pool.

In general, it is believed that the cytoplasmic pool of CA accounts for the overwhelming major-
Fig. 4. Schematic representation of the procedure for differential centrifugation and separation of the subcellular fractions of the anterior (AG) and posterior (PG) gills of *C. maenas* acclimated to 32-ppt salinity. Values for CA activity represent total activity for each fraction (μmol CO₂ ml⁻¹ min⁻¹ multiplied by the fraction volume in ml). Values in parentheses indicate percent recovery for each step. Values are the means of duplicate assays.

...ivity of the total branchial CA activity, and it is this pool of enzyme that is induced in response to low salinity (Henry, 1988a,b), and the data reported here for green crabs support this conclusion. The pattern of localization was originally reported to be different in green crabs. In homogenates of posterior gills (G7-9) of *C. maenas*, CA activity was reported to be highly concentrated in the microsomal fraction; this putative membrane-associated CA was reported as constituting between 75 and 94% of the total branchial CA activity in crabs acclimated to 10 and 30 ppt, respectively (Bottcher et al., 1990). Since this subcellular pool of CA had been previously described as being important in CO₂ mobilization at the gills (Henry, 1987), it was suggested that branchial CA in *C. maenas* was more important in gas exchange and acid–base balance than in ion regulation. This was the opposite situation of what had been previously found for the subcellular distribution of CA activity in the gills of the blue crab, in which 90–95% of the total branchial CA activity was localized to the cytoplasmic pool (Henry, 1988a), and the distribution for *C. maenas* reported here.

The differences in the results between the study of Bottcher et al. (1990) and the data reported here can most likely be traced to alterations in the procedure of the differential centrifugation used in the two studies. For the early work on the blue crab, and for the work reported here, three centrifugation steps were used. The first step, a low-speed centrifugation, was used to separate intact cells, nuclei and large cell fragments (cellular debris). This fraction has been shown to possess a high level of CA activity that is only loosely associated with the pellet and that is most likely cytoplasmic in origin (e.g. Henry et al., 1988). This step was omitted in the previous work on *C. maenas*, and instead, only one centrifugation step, a single spin of 100 000 × g, was used to separate the microsomal from the cytoplasmic fractions (Bottcher et al., 1990). This most likely resulted in the single pellet being made up of a combination of cellular debris, mitochondria, and microsomes,
Fig. 5. Schematic representation of the procedure for differential centrifugation and separation of subcellular fractions of anterior (AG) and posterior (PG) gills of *C. maenas* acclimated to 10-ppt salinity. Values for CA activity represent total activity for each fraction (μmol CO₂ ml⁻¹ min⁻¹ multiplied by the fraction volume in ml). Values in parentheses indicate percent recovery for each step. Values are the means of duplicate assays.

with the high levels of CA activity coming from the cellular debris. Thus, the microsomal contribution to the total branchial CA activity was overestimated.

Interestingly, even though the quantitative level of CA activity and its contribution to the total activity of the gill were originally grossly underestimated in *C. maenas*, the same degree of increase in CA activity in the cytoplasmic fraction was seen in response to low-salinity acclimation in all the studies involving either *C. sapidus* or *C. maenas*. For *C. sapidus*, the increase in CA activity in the posterior gills was approximately 13-fold for the cytoplasmic fraction and 50% for the microsomal fraction (Henry, 1988a, 1991a), and the increase for *C. maenas* was approximately 18- and 3-fold for the cytoplasmic and microsomal fractions, respectively, reported here (Figs. 4 and 5), and 13- and 3-fold, respectively, reported by Bottcher et al. (1990).

There are two conclusions that can be drawn from the above review of new and existing data

Fig. 6. Double reciprocal inhibitor titration plots of CA activity from the cytoplasmic (open circles) and microsomal (closed circles) fractions of the posterior (G7-9) gills of *C. maenas* acclimated to 12-ppt salinity. Regression lines, determined by the method of least squares, are as follows: cytoplasm (S3): $y = 2.71x + 21.3$, $r = 0.97$; microsomes (P3): $y = 2.49x + 11.3$, $r = 0.99$. Points represent the means of duplicate assays.
Fig. 7. Partial nucleotide and predicted amino acid sequences of CA-1 amplified from the gills of C. maenas. The 9-amino acid sequence shown in bold was identified by hydrophobicity analysis.

on branchial CA in euryhaline crustaceans. First, the subcellular distribution of the enzyme and the physiological effects of CA inhibition via Az are consistent with its having a central role in the support of the general ion-transport process in the gills of all euryhaline species examined so far. Second, and perhaps more interesting, is that CA activity is labile and highly sensitive to environmental salinity, and the increase in CA activity in response to low salinity appears to be a central
feature of the transition from osmotic and ionic conformity to regulation.

3.3. CA induction is regulated at the transcriptional level

The large increase in CA activity in response to low-salinity exposure could be a result of regulatory processes operating at either the transcriptional or translational level. CA induction could be the end result of gene activation and de novo synthesis of new enzyme, or the increase in activity could result from the activation of a large pool of pre-existing CA. Salinity-mediated CA induction occurs on the order of days, depending on species (Henry and Wheatly, 1988; Henry and Watts, 2001; Henry et al., 2002), and that time course is more consistent with gene activation and protein synthesis, but direct evidence has been lacking. One way to approach this question is to see if differences in CA activity correspond to similar differences in enzyme concentration.

For the green crab, results from titrating both the cytoplasmic and microsomal fractions of gill homogenates from animals acclimated to 10 ppt indicated that the induction in CA activity was a result of an increase in CA concentration. The direct comparison of free enzyme concentration in the two fractions showed that cytoplasmic CA was present in roughly twice the concentration as microsomal CA (Fig. 6); and when the dilution factor was taken into account for the differences in total volumes of the two fractions (~10 ml for cytoplasm and 2 ml for microsomes), the difference in total concentration in the gill was approximately 9-fold. While this is larger than the approximate 4.5-fold difference in activity between the two fractions (Fig. 5), it strongly suggests that differences in activity are due to differences in enzyme concentration.

The mechanism of CA induction was confirmed by more direct studies on the expression of the CA gene. The sequence designated as CA-1 is likely nearly complete, consisting of 810 nucleotides with an open reading frame coding for a 257-amino acid protein (Fig. 7). Although the 5’ end of the cDNA may be complete, the 3’ end is not. Hydrophobicity analysis of the CA-1 amino acid sequence revealed a 9-amino acid sequence near the N-terminus (LSLLLVQGA) that could potentially be a transmembrane domain, possibly serving as a signal sequence targeting the protein to a specific subcellular region. However, no trans-
Fig. 9. Multiple alignment of CA-1 and CA-2 partial amino acid sequences from *C. maenas* (present study) with CA sequences identified in *Tribolodon hakonensis* (Acc. AB055617.1), *Anopheles gambiae* (Acc. AAAB01008807), *Aedes aegypti* (Acc. AF395662.1), and *Homo sapiens* (BC011949.1). Intensity of shading indicates degree of similarity.
membrane domains were identified in the remaining portion of the protein, supporting our suggestion that CA-1 may represent the isoform that is present in the highest level of expression, the cytoplasmic isoform.

A second partial CA sequence was identified and designated as CA-2 (Fig. 8). Multiple alignment of the two partial CA sequences from Carcinus gills revealed similarities to CA sequences published for other arthropods and even to a human CA isoform (Fig. 9).

Specific oligonucleotide primers were designed for semi-quantitative PCR analysis that would differentiate between CA-1 and CA-2, and these were used in an analysis of CA mRNA expression in response to low-salinity exposure. The results of this experiment were conclusive. There was a large increase in the expression of CA mRNA in G7 by 24 h after transfer to 12 ppt (Fig. 11), and this level of expression persisted through the 4-day time course of the experiment. There was no corresponding increase in CA mRNA in anterior gills (G3); in fact, it appeared that CA expression in these gills was actually reduced in low salinity (Fig. 10), and the expression of AK also did not change. The initial increase in protein-specific CA activity in G7 occurred at 48 h post-transfer, immediately after the increase in mRNA expression (Fig. 11), and continued to increase toward acclimated levels through 4 days. There was no change in CA activity in anterior (G3) gills. The timing and coordination of changes in CA mRNA and activity indicate that CA induction occurs through the synthesis of new enzyme, a process that is stimulated by low salinity and results in an increase in gene expression in the posterior, ion-transporting gills only.

3.4. CA induction is under inhibitory control

Recent work, in print primarily as preliminary reports, has indicated that CA induction is under inhibitory control of a repressor substance found in the major endocrine complex of the crab, the eyestalk. When green crabs, acclimated to 32 ppt,
Fig. 12. CA activity (μmol CO₂ (mg protein⁻¹) min⁻¹) in anterior (G-3) (left-hand bars) and posterior (G-7) (right-hand bars) gills of C. maenas. (a) Control crabs acclimated to 32-ppt salinity. (b) Crabs acclimated to 32 ppt and injected with filtered seawater once a day for 7 days. (c) Crabs acclimated to 32 ppt and treated with ESA and left for 7 days. (d) Crabs acclimated to 32 ppt, treated with ESA and given an injection of eyestalk extract once a day for 7 days. Mean±S.E.M. (N=5–8). T=12 °C.

were treated with ESA, CA activity in the posterior gills (G7) increased by 50% over 7 days (from ~200 to 300 μmol CO₂ (mg protein⁻¹) min⁻¹), while there were no changes in CA activity in anterior gills (Henry et al., 2000; see also Fig. 12). However, when crabs acclimated to 32 ppt and treated with ESA were then given daily injections over a 7-day period of the supernate of homogenates of eyestalks taken from other crabs at 32 ppt, the ESA-stimulated CA induction was abolished (Fig. 12). Injections of filtered seawater had no effect on CA activity in either anterior or posterior gills.

Furthermore, ESA appeared to potentiate the normal low-salinity-mediated CA induction. In crabs acclimated to 32 ppt, treated with ESA, and then transferred to 12 ppt, CA activity in the posterior gills increased to a greater degree (≈20%) than in untreated crabs given the same salinity transfer. In the stenohaline crab, Cancer irroratus, however, CA induction did not occur in any gills in response to low-salinity (18 ppt) exposure, and ESA had no effect on branchial CA activity in those crabs at either high or low salinity (Henry et al., 2000). These early results indicate that there is a substance in the eyestalk that acts as a repressor of CA expression, keeping the levels of CA activity in the gills low in animals acclimated to high salinity. When the eyestalks are removed, a degree of CA induction occurs even in the absence of a low-salinity stimulus. The effects of this putative repressor are removed during acclimation to low salinity, resulting in an increase in CA gene expression and the observed high degree of CA induction. This idea is further supported by the fact that injections of extracts of homogenates of eyestalks from crabs acclimated to high salinity reduce CA induction by approximately 50% in intact crabs and by 70% in crabs treated with ESA (Henry, 2001b). The specific localization of the repressor within the eyestalk, its chemical nature and composition, and the nucleotide sequence of the gene that encodes it are currently under investigation.

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