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Salinity-stimulated changes in expression and activity of two carbonic anhydrase isoforms in the blue crab *Callinectes sapidus*

Laetitia Serrano, Kenneth M. Halanych and Raymond P. Henry*
Department of Biological Sciences, 101 Life Science Building, Auburn University, Auburn, AL 36849, USA
*Author for correspondence (e-mail: henryrp@auburn.edu)

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Summary

Two isoforms of the enzyme carbonic anhydrase (CA) in the blue crab gill, CasCAg and CasCAc, were identified, sequenced, and found to match the membrane-associated and cytoplasmic isoforms, respectively. The membrane-associated isoform is present in much higher levels of mRNA expression in both anterior and posterior gills in crabs acclimated to high salinity (35 p.p.t.), but expression of the cytoplasmic isoform in the posterior gill undergoes a significantly greater degree of up-regulation after exposure to low salinity (15 p.p.t.). CasCAc has the largest scope of induction (100-fold) reported for any transport-related protein in the gill, and this may be necessary to overcome diffusion limitations between gill cytoplasm and the apical boundary layer. Furthermore, the timing of the changes in expression of CasCAc corresponds to the timing of the induction of protein-specific CA activity and CA protein concentration. No changes in CA mRNA expression or activity occur in the anterior gills. The pattern of up-regulation of expression of mRNA of the α-subunit of the Na^+/K^+-ATPase is similar to that for CasCAc, and both precede the establishment of the new acclimated physiological state of the crab in low salinity. A putative 'housekeeping' gene, arginine kinase, also showed about a threefold increase in expression in response to low salinity, but only in the posterior gills. These results suggest that for studies of expression in crustacean gill tissue, a control tissue, such as the anterior gill, be used until an adequate control gene is identified.

Key words: carbonic anhydrase, ion regulation, crustacean, *Callinectes sapidus*.

Introduction

Carbonic anhydrases (CA, EC.4.2.1.1) are zinc-containing enzymes that catalyze the reversible hydration/dehydration of carbon dioxide/bicarbonate, and thus participate in a variety of biological processes that include acid–base balance, CO_2 transfer and ion exchange. Multiple CA isoforms, clearly encoded by three evolutionarily unrelated families of genes (α-CA, β-CA and γ-CA), have been identified and studied in a wide variety of animals, plants, and bacteria (reviewed in Hewett-Emmett, 2000). Their physiological roles vary according to the tissue and cell type in which they are found, their subcellular localization (e.g. cytosolic, membrane-bound, mitochondrial or secreted) and to the specific physiological or biochemical process to which they are linked (for reviews, see Dodgson et al., 1991; Henry, 1996; Geers and Gros, 2000). Despite being ubiquitously distributed throughout the animal kingdom, most of the existing work on molecular structure and phylogeny has been focused on mammalian CAs: to date, 16 different isoforms have been characterized, all of them being members of the α-CA gene family (reviewed in Esbaugh and Tufts, 2006). More recent work has shed light on the molecular structure and function of α-CA isoforms of non-mammalian vertebrates, such as fish (Peterson et al., 1997; Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004; Esbaugh et al., 2005; Boutet et al., 2006; Tohse et al., 2006) and, to a lesser extent, of invertebrates including some mollusks, worms and insects (reviewed in Hewett-Emmett, 2000). Nevertheless, little information on the molecular structure of these isoforms is available for crustaceans, a group in which CA function has been extensively studied (reviewed by Henry, 2001; Henry et al., 2003).

Euryhaline crustaceans can survive wide variations in environmental salinity by maintaining relatively high and constant hemolymph osmolality and ion concentrations, compared to those in the external medium. This process involves the active uptake of salts (primarily Na^+ and Cl^-) across the posterior gills, which are highly specialized for active ion transport (Mantel and Farmer, 1983; Gilles and Péqueux, 1985; Péqueux and Gilles, 1988; Péqueux, 1995; Taylor and Taylor, 1992). These gills also contain high levels of activity and/or expression of known ion transport proteins and transport-related enzymes such as the NaK2Cl co-transporter, the Na/H exchange protein (NHE), the Na^+/K^+-ATPase, and carbonic anhydrase (CA) (Towle and

Weihrauch, 2001; Henry et al., 2003; Luquet et al., 2005). Furthermore, the activity and/or expression of these proteins are salinity-sensitive, increasing when crabs are exposed to low salinity water (Towle et al., 2001; Genovese et al., 2005; Luquet et al., 2005; Chung and Lin, 2006; Henry et al., 2006; Li et al., 2006). Anterior gills are typically unspecialized, having thin epithelia that function primarily in diffusive gas exchange.

Some of the largest reported changes in activity (8–14-fold) in response to low salinity have been for branchial CA in two euryhaline species of crabs, the green crab *Carcinus maenas,* and the blue crab *Callinectes sapidus* (Henry, 2005). Isolation of subcellular fractions of branchial CA activity has shown the cytoplasmic pool of CA to be the most highly sensitive to low salinity. However, there are multiple CA isoforms in the gill, and membrane-associated CA is also induced by low salinity, although to a much lesser degree (2–3-fold) (Henry, 1988a; Henry et al., 2003). Recent evidence from *C. maenas* for one CA isoform strongly suggests that CA induction is under transcriptional regulation. Relative abundance of CA mRNA increased at 24 h after transfer to low salinity, and CA activity increased shortly thereafter (Henry et al., 2006).

The blue crab, *C. sapidus*, is one of the strongest osmotic and ionic regulators, having the highest rates of ion uptake and maintaining the largest ionic gradients between its hemolymph and the surrounding medium (Cameron, 1978). An essentially marine species, it may extend into brackish and full freshwater in nature and can cope with large step changes in salinity (Péqueux, 1995; Henry, 2001). CA activity is induced more quickly in *C. sapidus* than in *C. maenas*; the initial measurable increase occurs at 24 h after low salinity exposure (Henry and Watts, 2001), and the magnitude of CA induction is also greater than in other species. Because of these characteristics, the blue crab is potentially a more powerful model system in which to study CA induction and its regulation. Furthermore, a large expressed sequence tag (EST) cDNA library from the gills of *C. sapidus* has been recently produced (Coblentz et al., 2006) and is now easily accessible (a collection of almost 12 000 ESTs, representing more than 2000 putative transcripts, are available), facilitating the identification of the multiple CA isoforms within the blue crab gill. CA is also an ideal transport-related protein for this type of study, as it is a very tractable enzyme to work with: activity, protein concentration and mRNA expression can all be reliably measured, typically in the same animal.

The aim of the present study was to expand upon the mechanism and regulation of low salinity-stimulated CA induction by identifying the multiple CA isoforms in the crustacean gill and elucidating their potential role in the process of low salinity adaptation in euryhaline decapod crustaceans, using the strong hyperosmoregulating crab *C. sapidus* as model organism. Two isoforms were fully sequenced: one putative cytoplasmic form and one putative membrane-bound form. The relative levels of abundance of these two isoforms were then monitored in crabs transferred from high to low salinity for up to 28 days, a period that encompasses both the acute and acclimated stages of salinity adaptation. This report represents a coordinated study of changes in hemolymph osmolality, and CA branchial activity, mRNA expression, and protein abundance. Because other authors have employed arginine kinase as a highly expressed control transcript, we also examined its expression.

Materials and methods

Animals

Adult male blue crabs *Callinectes sapidus* Rathbun 1896 were obtained from commercial fishermen in Port Aransas, TX, USA, and from the Gulf Specimen Co (Panacea, FL, USA). In the laboratory, the crabs were held at 35 p.p.t. salinity in 650 L recirculating tanks equipped with biological filters. Approximate salinity was checked daily with a hand-held refractometer, and more precise measurements of osmolality (1050 mOsm kg H_2O^{-1}) were made on a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA). Water quality was monitored by measuring nitrite concentrations of all holding tanks and experimental aquaria. Crabs were fed shrimps three times per week.

Experimental protocol

Crabs that were collected from salinities above 28 p.p.t. were kept in the laboratory at 35 p.p.t. for 1 week prior experimentation. Crabs collected at lower salinities, were held at 35 p.p.t. for 3 weeks in order to ensure complete acclimation with respect to hemolymph osmolality and levels of transport-related enzymes (Henry and Cameron, 1982; Henry and Wheatly, 1988). Before beginning any experimental protocol, branchial CA activity was measured in a subset of four crabs to confirm that CA activity was at low and uniform baseline levels typically found in high-salinity acclimated animals. To follow the time course of CA induction, crabs were directly transferred to 120 L aquaria of 15 p.p.t. water equipped with undergravel biological filters. Crabs were sampled before transfer (t=0), and then at 2, 6, 12, 24, 48 h and 4, 7, 14 and 28 days after transfer. As several physiological parameters, including branchial CA activity (Henry and Kormanik, 1985), change over the molting cycle, only specimens in intermolt stage C, established by observation of the edge of the fifth pereiopod, were retained for analysis at the end of each experimental time period (Drach and Tchernigovtzeff, 1967). Hemolymph was sampled from the infrabranchial sinus at the base of the fifth pereiopod using a hypodermic 22 gauge needle mounted on a 1 ml syringe and frozen at –20°C until osmolality determination. Prior to dissection, crabs were chilled on crushed ice for 10 min. Anterior (G3) and posterior (G7) gills from the left and right sides of the crab were dissected out and used for measurement of CA activity and mRNA expression, respectively.

A second group of animals were acclimated to 35 p.p.t. and 15 p.p.t. for 7 and 14 days in order to determine the total concentration of free enzyme in gills (E_0). Anterior (gills 1–4) and posterior (gills 6–8) gills were dissected out, pooled, and

frozen in liquid nitrogen and stored at –80°C. Gill number 5 in blue crabs is a transitional gill, containing both respiratory and ion-transporting lamellae (Henry and Cameron, 1982), and so it was excluded from this study.

Osmolality

Hemolymph samples were thawed on ice, sonicated for 15 s at 25 W (Heat Systems Microson, Farmingdale, NY, USA) and centrifuged 14 000 ***g*** for 1 min to separate out clot material. Osmolality was then measured on 10 μl samples using a Wescor 5100C vapor pressure osmometer.

Carbonic anhydrase activity

Branchial carbonic anhydrase activity was measured electrometrically by the delta pH method previously described (Henry, 1991). Freshly dissected gills were homogenized in 2 ml of cold homogenization/assay buffer (225 mmol l^{-1} mannitol, 75 mmol l^{-1} sucrose, 10 mmol l^{-1} Trizma base, adjusted to pH 7.4 with 10% phosphoric acid) using an Omni TH115 hand-held homogenizer (Omni Instruments, Warrenton, VA, USA) and sonicated on ice at 25 W for 30 s (Heat Systems Microson). Homogenates were centrifuged at 10 000 ***g*** for 20 min at 4°C (Sorvall RC5-B, Wilmington, DE, USA). For the assay, 25–75 μl of supernatant were added to 6 ml of homogenization/assay buffer, refrigerated at 4°C and stirred vigorously. The enzymatic reaction was initiated by the addition of 100 μl CO_2-saturated water. The drop in pH (approximately 0.25 units) was monitored using micro pH and reference electrodes (World Precision Instruments, Sarasota, FL, USA) and a null-point pH meter. Protein concentration in the supernatant was measured by Coomassie Brilliant Blue dye binding (Bio Rad laboratories, Hercules, CA, USA). CA activity was expressed in μmol CO_2 mg $protein^{-1}$ min^{-1}.

Titration of free enzyme concentration

Changes in CA protein concentration were measured using a method previously described (Henry et al., 2006). From each pool of gills stored at –80°C (anterior gills 1–4 and posterior gills 6–8), 4 g of tissue were placed in 20 ml of cold homogenization/assay buffer (see above) and homogenized by 15 passes of a motor-driven, Teflon-glass homogenizer. The homogenate was then subjected to differential centrifugation. First, the crude homogenate was centrifuged at 1750 ***g*** (Sorvall RC5-B, Wilmington, DE, USA) for 20 min at 4°C, producing a pellet of intact cells, nuclei and large cell fragments, and a supernatant containing cytoplasm, mitochondria and microsomes. The resulting supernatant was then centrifuged at 7500 ***g*** for 20 min at 4°C (Sorvall RC5-B) to eliminate the mitochondria. Then, to separate the microsomal fraction from the cytoplasm, the supernatant was centrifuged at 100 000 ***g*** for 90 min at 4°C (Beckman L8-70M ultracentrifuge). The cytoplasmic fraction (supernatant) and the microsomal fraction (pellet, washed twice and resuspended in 2 ml cold homogenization/assay buffer) were assayed for CA activity while being titrated with increasing volumes of a 5 μmol l^{-1} acetazolamide solution (Az; a CA inhibitor). The data were transformed and graphed as a double reciprocal plot, according to the following relationship: $I_0/I=K_i/(1–I)+E_0$, where E_0 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_0 (Easson and Stedman, 1937). CA concentrations from the inhibitor plots were adjusted for differences in sample volumes used in the assay.

Total RNA purification

Total RNA was extracted from gills by phenol–chloroform extraction, using RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA). All dissecting equipment and homogenizers were cleaned with RNAse-Zap (Ambion, Austin, TX, USA) and rinsed in RNAse-free water in order to work under RNAse-free conditions. Total RNA concentration, as well as the integrity and purity of each sample were determined with a Bioanalyser 2100 (Agilent Technologies, Wilmington, DE, USA). No genomic DNA contamination was observed.

Polymerase chain reaction and specific primers

All polymerase chain reactions (PCR) mentioned in this study were carried out in a MJ Research PTC 200 thermocycler (Global Medical Instrumentation, Inc., Ramsey, MN, USA) using REDTaq™ ReadyMix™ PCR Reaction Mix kit (Sigma-Aldrich, St Louis, MO, USA). Template DNA (1 μl), 0.5 μl of each primer at 25 μmol l^{-1} and 25 μl REDTaq Ready Mix PCR reaction mix (20 mmol l^{-1} Tris-HCl pH 8.3, 100 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 0.002% gelatine, 0.4 mmol l^{-1} dNTP mix, stabilizers, 0.06 units/μl Taq DNA polymerase) were used in a final volume of 50 μl. After an initial denaturing step at 92°C for 5 min, 30 cycles of 1 min at 92°C, 1 min at 55°C and 2 min at 72°C were performed, followed by a final extension step at 72°C for 5 min. PCR products were tested for purity and molecular size by agarose gel electrophoresis (1.2% agarose in 1×TAE buffer). DNA bands were stained with Ethidium Bromide and visualized over an ultraviolet light source.

All specific primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and tested for specificity by PCR on gill cDNA and confirmed by sequencing of the resultant PCR products.

Determination of carbonic anhydrase cDNA sequences 3′end

Expressed Sequence Tags (ESTs) from blue crab gill and hypodermis normalized cDNA libraries were produced by Coblentz et al. (Coblentz et al., 2006). Clones matching contig sequences identified as carbonic anhydrase in that project and archived at the University of North Carolina at Wilmington were used to obtain full nucleotide sequences of the gill CA isoforms. Five cloned inserts were obtained and sequenced from both ends using SP6 and T7 primers. Alignment of all the sequences obtained resulted in two different contigs (ORF followed by 3′UTR) both identified as CA sequences by BLAST analysis.

5′end

The 5′ends of the CA isoforms were determined using the GeneRacer protocol (GeneRacer™ kit, Invitrogen, Carlsbad, CA, USA) designed for full-length RNA ligase-mediated amplification of 5′end (RLM-RACE). Briefly, truncated mRNA and non-mRNA were eliminated from total RNA by dephosphorylating treatment with calf intestinal phosphatase. The 5′cap structure from intact full-length mRNA was removed and a GeneRacer RNA-oligo was ligated to the 5′end. Ligated mRNAs were reverse transcribed using GeneRacer oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). The 5′end was then amplified by PCR using GeneRacer 5′ Primer and reverse gene specific primers CasCAg-Race and CasCAc-Race designed according to 3′end CA cDNA sequences described above (Table 1).

PCR products were purified from 1% agarose gel (MinElute Gel Extraction kit, Valencia, CA, USA), subcloned into a pCR®4-TOPO and transformed into chemically competent TOP10 *Escherichia coli* cells (TOPO TA Cloning® kit, Invitrogen). After selection on LB-ampicilin agar, transformed cells were screened for appropriate size inserts by PCR using forward GeneRacer 5′ Primer and reverse gene specific primers CasCAg-Race and CasCAc-Race. Recombinant plasmids were purified by QIAprep Spin Miniprep (Qiagen) and individual clones were sequenced using T7 and M13 reverse primers.

Sequencing and sequences analysis

A CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) using CEQ dye terminator chemistry was used for bidirectional sequencing. The resulting automated traces were edited with Chromas software and identified by comparison with published sequences in GenBank using the BLAST algorithm (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were produced with Multalin software (Corpet, 1988) (http://prodes.toulouse.inra.fr/multalin/) and GeneDoc software (http://www.psc.edu/biomed/gendoc/).

Quantification of mRNA expression by real-time quantitative PCR

Poly-A mRNA in 2 μg of total RNA per sample was reverse transcribed using an oligo-dT primer and Superscript II® reverse transcriptase (Invitrogen, Madison, WI, USA). As such, the samples were normalized to total RNA levels in each preparation (Bustin, 2002). The resulting cDNAs were checked by PCR and stored at –20°C. The mRNA levels were assessed by real-time quantitative polymerase chain reaction (qPCR) with a MiniOpticon Real-Time PCR detection system using iQ™ SYBR® Green Supermix kit (Bio-Rad laboratories, Hercules, CA, USA). Reactions (25 μl) were run containing 12.5 μl iQ SYBR Green mix (2×), 10.9 μl nuclease-free water, 0.3 μl of each specific primer (25 μmol l^{-1} and 1 μl template cDNA. The thermal profile consisted of an initial step at 95°C for 3 min and 35 cycles of denaturing at 95°C for 10 s and annealing–elongation at 55°C for 60 s. Each sample was analyzed in 1 μl duplicate or triplicate aliquots. A standard curve, representing the threshold cycle (C_t, cycle at which the fluorescent signal is detected) data plotted as a function of template availability (C_t *vs* $\log_{10}$ cDNA volume), was generated by serial dilution of one sample containing high CA activity. Furthermore, absence of non-specific PCR products and primer dimers was confirmed by examination of dissociation curves generated after the amplification cycles were completed (from 55°C to 95°C with a heating rate of 0.5°C every 5 s).

Specific primer pairs CasCAgF/CasCAgR and CasCAcF/CasCAcR were designed based on CasCAg and CasCAc nucleotide sequences obtained in this study to discriminate between both isoforms expression patterns (Table 1).

Na^+/K^+-ATPase α-subunit mRNA expression was studied over low-salinity adaptation using previously described primers NAKSP12F and NAKSP16R specific to ion transport α-subunit sequence (Table 1) (Li et al., 2006).

Arginine kinase (AK) mRNA expression was also

Table 1. *Nucleotide sequence of Callinectes sapidus specific primers used for amplification of carbonic anhydrase isoforms, Na^+/K^+-ATPase α-subunit and arginine kinase*

Target genes	Primer	Nucleotide sequence (5′→3′)	T_m (°C)	References
CasCAg	CasCAg-Race	AGTGCCGTAGGAACCCTTGTAGTG	60.5	This study
	CasCAgF	CGGGACAGCATGGACCTCTTCACTGG	64.6	
	CasCAgR	AGGAGCCAGGGCAGGGTTATCATT	62.3	
CasCAc	CasCAc-Race	CTCAGCGGGGTAGCAGGTG	60.5	
	CasCAcF	GTCTGGCCGTGCTGGGGATGTT	64.4	
	CasCAcR	CGCAGGGGTGGTACGACTTGAG	61.9	
NaK	NAKSP12F	GCCTCCGTGCCTCTACCTCT	61.1	Li et al., 2006
	NAKSP16R	TGGAGTTACGGCGAGTCTTAC	56.3	
AK	AKF5	CGCTGAGTCTAAGAAGGGATT	54.1	Towle et al., 2001
	AKCALLR1	CCCAGGCTTGTCTTCTTGTCC	58.0	

CasCAg and CasCAc, carbonic anhydrase isoforms; NaK, Na^+/K^+-ATPase α-subunit; AK, arginine kinase; T_m, annealing temperature.

monitored, as a putative internal control, using specific forward AKF5 and reverse AKCALLR1 primers (Table 1) previously described (Towle et al., 2001).

X-statistics

Statistics were performed using Sigma Stat version 3.1 and figures were plotted using Microsoft® Office Excel 2003.

Results

The combination of 5′ends of cDNA, obtained by cloning, and 3′ends of cDNA, obtained from the ESTs, yielded two full-length cDNA sequences found to be similar to CA sequences from the α-CA family when compared to the GenBank database using the BLAST program (Fig. 1). They were named CasCAc (denoting a presumptive cytoplasmic localization) and CasCAg (denoting the presence of a membrane glycosyl-phosphatidylinositol (GPI) link). The complete sequence of the CasCAc (GenBank EF375490) contained a 50 bp 5′ untranslated region, an 816 bp open reading frame (including the stop codon), and a 227 bp 3′ untranslated region [excluding the poly(A)-tail]. The complete sequence of the CasCAg (GenBank EF375491) contained a 108 bp 5′ untranslated region, a 927 bp open reading frame (including the stop codon), and a 518 bp 3′ untranslated region [also excluding the poly(A)-tail]. According to Kozak rules, sequences surrounding the first methionine in CasCAc and CasCAg displayed, respectively, 3 and 8 out of the 9 nucleotides expected before a translation initiation codon. Both sequences included the highly conserved puridine in position –3, and both sequences maintained the G following the ATG (Kozak, 1987). The CasCAc nucleotide sequence contained two putative *N*-glycosylation motifs (translating as Asn-anything but Pro-Thr/Ser-anything but Pro) in the coding region and a possible polyadenylylation cleavage signal (AATAAA) starting 55 bp upstream from the poly(A)-tail.

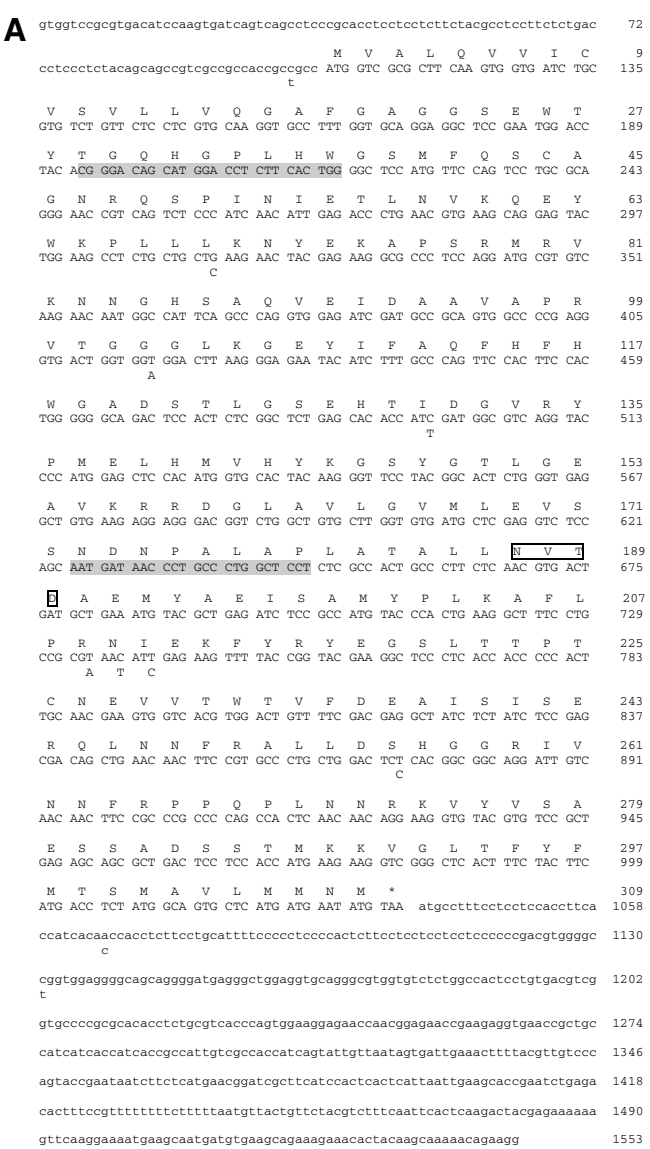

Fig. 1. Nucleotide and deduced amino acid sequences of CasCAg (A) and CasCAc (B) cDNA from *Callinectes sapidus* gills (GenBank EF375491 and GenBank EF375490, respectively). Both sequences are numbered at the end of each line. The grey shading corresponds to forward and reverse specific primers used to performed qPCR. Asterisks indicate stop codon. Putative *N*-glycosylation motifs are boxed. Putative polyadenylylation signal is underlined.

Only one putative *N*-glycosylation motif was identified in the coding region of CasCAg.

Two variations in the nucleotide sequence were noted in the CasCAc isoform, and ten variations were present in the CasCAg. Except for three variations in CasCAg (one in the 5′UTR and two in the 3′UTR), all variations were found in open reading frames and were in the third codon position. As none of them led to amino acid substitution, and as most of them were found in two or more sequenced products, they are unlikely to be *taq* errors. It is possible that variations in cDNA sequence could have existed in the different individual organisms from which the tissues for sequencing were taken.

The deduced amino acid sequences for CasCAc and CasCAg were different in length (271 and 308 amino acid residues, respectively) and shared only 30% of identity (Fig. 2). Both sequences were submitted to DGPI analysis (Kronegg and Buloz, 1999): CasCAg was diagnostic as a GPI-anchored protein with an N-terminal signal (Met^{1} to Ala^{18}), a potential cleavage site at 284 (Asp^{284}-Ser^{285}-Ser^{286}) and the presence of hydrophobic and hydrophilic tails. The amino acid sequences of CasCAc and CasCAg were aligned and compared with previously characterized α-CA sequences from invertebrates and vertebrates (Fig. 2). The α-CA peptides are highly variable both in length and in sequence, even though some features essential to the catalytic mechanism appear to be common to nearly all family members. Among the presented enzymes, 15 out of the 36 active site residues are identical. The three zinc-binding histidine residues annotated His-94, His-96, His-119 in HosCAII, and the proton-binding network annotated Glu-106 and Thr-199 in HosCAII are conserved. All but CasCAc, AngCA, HosCAIII and HosCAV have the highly conserved histidine residue implicated as a proton shuttle group defined as His-64 in human CAII.

The hemolymph osmolality of blue crabs fully adapted to 35 p.p.t. seawater was slightly hyperosmotic to that of the seawater (1112±22 *vs* 1050 mOsm kg H_2O^{-1}) (Fig. 3), as is the typical case for marine osmotic conformers (Henry, 2001). After transfer to low salinity (15 p.p.t./450 mOsm kg H_2O^{-1}), the hemolymph osmolality sharply decreased during the initial 12 h post-transfer (from 1110 to 820 mOsm kg H_2O^{-1}) and by 24 h stabilized at around 780 mOsm kg H_2O^{-1}, corresponding to the new acclimated level. After 28 days, the hemolymph osmolality value was 285 mOsm kg H_2O^{-1} higher than the 450 mOsm kg H_2O^{-1} ambient medium.

The CA activity in G3 and G7 of crabs acclimated to 35 p.p.t. was low (approximately 155 μmol CO_2 mg $protein^{-1}$ min^{-1}) and not significantly different (P=0.39, t-test, Fig. 4). CA activity in G3 showed no significant variation during the 28 days following the transfer to low salinity (P<0.05, ANOVA, Tukey).

Low salinity exposure resulted in significant induction of CA activity in G7. The initial increase (77%, P<0.001, t-test) occurred at 24 h after transfer, and the activity continued to increase through 7 days, at which time it reached the maximum value of 2296±203 μmol CO_2 mg $protein^{-1}$ min^{-1}, corresponding to an approximate 14-fold increase compared to the initial value. CA activity in G7 remained significantly high (above 1500 μmol CO_2 mg $protein^{-1}$ min^{-1}, P<0.001, ANOVA, Tukey) throughout the remainder of the 28 day experimental time course.

Expression of CasCAg mRNA was not significantly different (P=0.49, t-test) between G3 (0.12±0.05, N=6) and G7 (0.16±0.03, N=8) in crabs acclimated to 35 p.p.t. (Fig. 5A). There were no significant changes in G3 mRNA expression at any times after low salinity transfer (P>0.995, ANOVA, Tukey). Changes in expression of CasCAg mRNA in G7 were pulsatile. There was an initial increase in CasCAg mRNA relative expression of approximately 3.5-fold and 5.5-fold at 2 h and 6 h post-transfer, respectively (t=6 h: P<0.001, ANOVA, Tukey). Expression then started to decrease at 12 h to reach a value at 24 h that was not significantly different from the 35 p.p.t. controls. There was a second increase in CasCAg mRNA expression starting at 48 h followed by two time periods (4 days and 28 days after transfer) at which expression decreased. By the end of the 28 days acclimation, the level of CasCAg mRNA (0.46±0.17) was threefold higher, but not significantly different compared to the level in crabs acclimated to high salinity (P=0.85, ANOVA, Tukey).

In crabs acclimated to 35 p.p.t., CasCAc mRNA levels in G7 were low but still 15-fold higher than in G3, in which this isoform was barely detectable (0.006±0.002, N=8 and 0.0004±0.0002, N=6, respectively, P<0.05, t-test) (Fig. 5B). Low salinity exposure induced a dramatic increase in G7 CasCAc mRNA expression, occurring from 2 h to 12 h (100-fold increase, P<0.001, ANOVA, Fisher LSD). Thereafter, throughout the duration of the experimental time course, CasCAc mRNA levels remained elevated relative to the 35 p.p.t. controls, with one sharp peak of expression occurring at 48 h (1.60±0.32, N=12) and a slight decrease at 4 days (0.51±0.05, N=13). By contrast, no significant changes were measured in G3.

CasCAg was the more highly expressed isoform in either G3 or G7 at any of the sampling times during the experiment (Fig. 6). In crabs acclimated to 35 p.p.t. salinity, CasCAg was expressed at levels 9000 and 500 times greater than CasCAc in G3 and G7, respectively. After transfer to 15 p.p.t., the ratio of CasCAg to CasCAc transcript fell to between 2450 and 147 in G3 and between 2 and 17 in G7, indicating that CasCAc was the isoform with the highest degree of salinity sensitivity.

Results from titrating both cytoplasmic and microsomal CA fractions of homogenates from pooled anterior and posterior gills from crabs acclimated to 35 p.p.t. and transferred to 15 p.p.t. are summarized in Table 2. The K_i values were in the range of 3–7 nmol l^{-1}, which is within the range of previously reported values for crustacean CA (e.g. Henry et al., 2003; Henry et al., 2006). In crabs acclimated to 35 p.p.t., the protein concentrations (E_0) of both CA isoforms were in the same value range when comparing AG with PG, and CAg was the most abundant isoform in both gills. After transfer to 15 p.p.t., E_0 of both CA isoforms almost doubled in anterior gills, but with the small sample size it is not possible to say whether this increase is real. In posterior gills, however, a large (45-fold) increase in

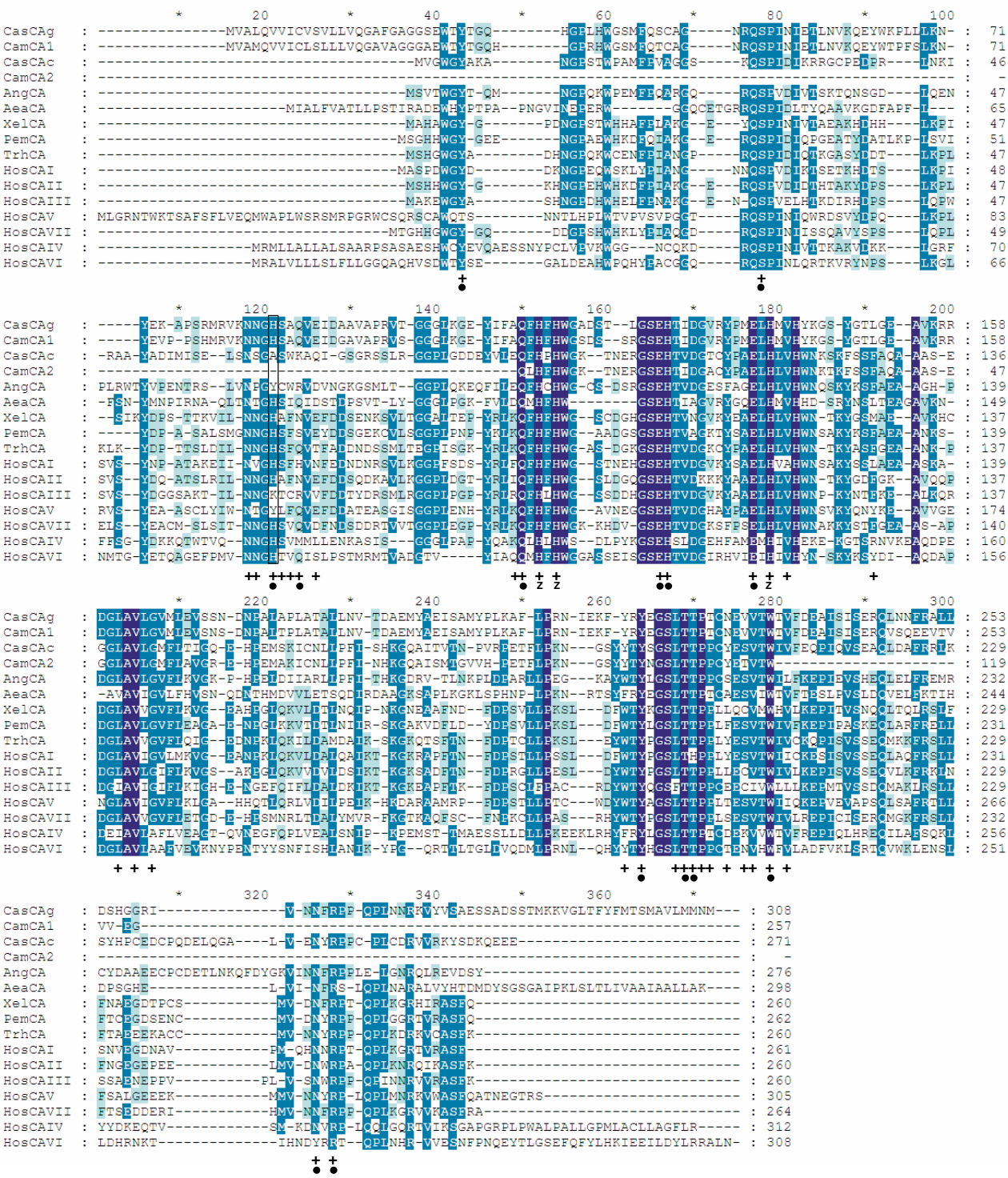

Fig. 2. Multiple alignment of translated amino acid sequences of *Callinectes sapidus* CasCAg and CasCAc isoforms (GenBank EF375491 and GenBank EF375490, respectively) with partial CA isoforms sequences from the crab *Carcinus maenas* (CamCA1 and CamCA2) (Henry et al., 2003) and with other well-characterized α-CA isoforms published in GenBank from invertebrates such as the insects *Aedes aegypti* (AeaCA: AF395662) and *Anopheles gambiae* (AngCA: DQ518576) and from vertebrates such as the batrachia *Xenopus laevis* (XelCA: BC042287), the agnatha *Petromyzon marinus* (DQ157849), the teleostei *Tribolodon hakonensis* (TrhCA: AB055617) and the mammal *Homo sapiens* (HosCAI: M33987; HosCAII: NM000067; HosCAIII: NM005181; HosCAIV: NM000717; HosCAV: L19297; HosCAVI: NM001215; HosCAVII: BC033865). Intensity of shading indicates degree of similarity: dark background: 100% agreement; medium dark background: >50% agreement; light background: >30% agreement. Symbols under sequences: + indicate the 36 putative active site residues; z indicates the zinc-binding histidine residues; black circles localize the active-site hydrogen bond network; and the most common proton shuttle group is boxed (Hewett-Emmett and Tashian, 1996).

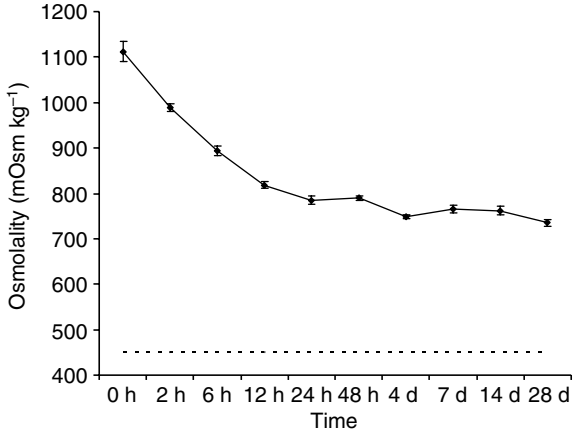

Fig. 3. Hemolymph (solid line) and diluted seawater (broken line) osmolality (mOsm kg H_2O^{-1}) for *Callinectes sapidus* acclimated to 35 p.p.t. (t=0 h) and transferred to 15 p.p.t. for 28 days (d). Values are means ± s.e.m., N=4–12.

CAc E_0 resulted after 7 days of low salinity acclimation compared to the lesser 1.5-fold in CAg E_0. After 2 weeks, the CAc concentration was even more enhanced, reaching a 65-fold induction.

In crabs acclimated to 35 p.p.t., Na^+/K^+-ATPase α-subunit (NaK) and arginine kinase (AK) mRNA levels were fourfold significantly higher (P<0.05, t-test) in G7 (0.15±0.04, N=7 and 0.12±0.04, N=5, respectively) than in G3 (0.04±0.01, N=5 and 0.03±0.01, N=5, respectively) and they remained relatively stable in G3, exhibiting no significant variation (P>0.2, ANOVA, Fisher LSD) (Fig. 7 and Fig. 8). Six hours after the crabs were transferred to 15 p.p.t. Na^+/K^+-ATPase mRNA expression in G7 had significantly increased by fourfold (P<0.001, ANOVA, Fisher LSD). Then, the NaK level remained significantly higher (fluctuating between 3 and 4.8-times) in G7 of crabs acclimated to low salinity than in G7 of crabs acclimated to 35 p.p.t.. This pattern of expression was similar to that seen for CasCAc in response to low salinity. After transfer to 15 p.p.t., AK expression in G7 exhibited a progressive increase from 6 h (0.23±0.04, N=5, P<0.05, ANOVA, Fisher LSD) to 7 days (0.40±0.05, N=5, P<0.001, ANOVA, Fisher LSD) then it decreased at 28 days.

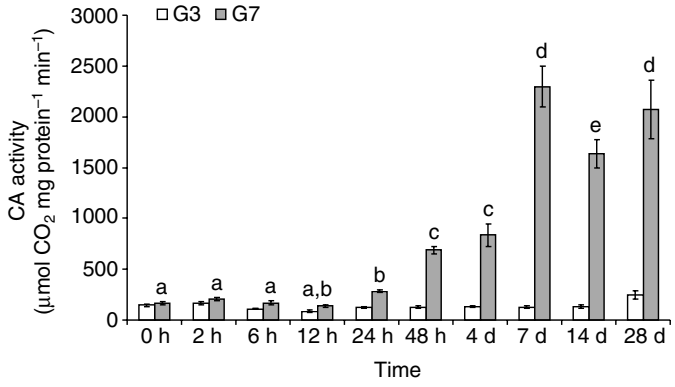

Fig. 4. CA activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G3, white bars) and posterior (G7, dark bars) gills of *Callinectes sapidus* acclimated to 35 p.p.t. (t=0) and at various times after being transferred to 15 p.p.t. Values are means ± s.e.m., N=6–18. Letters above bars indicate statistical comparisons within G7 across the time course: two columns not sharing at least one same letter are statistically different (P<0.05, two-way ANOVA, Tukey's *post-hoc* test). There were no significant differences among G3.

Discussion

This report presents the first full sequence identity for two distinct isoforms of CA in crustacean gills. The cytoplasmic isoform (CasCAc) shows strong identity with the CA Type II (cytoplasmic) isoform of other species (primarily vertebrates), and the CasCAg isoform can be identified as a CA Type IV (membrane-associated) isoform by the presence of a GPI-anchor motif (Figs 1, 2). These data confirm the presence of two distinct isoforms in two separate subcellular compartment of the gill – the cytoplasm and the membrane. The presence of these isoforms had only been documented up until now by the

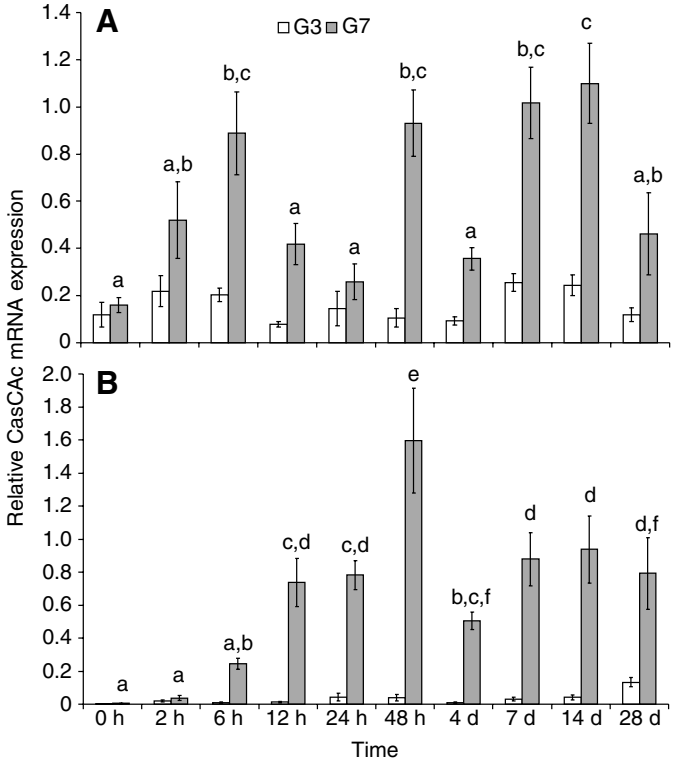

Fig. 5. Relative expression of CasCAg (A) and CasCAc (B) mRNA in anterior (G3, white bars) and posterior (G7, black bars) gills of *Callinectes sapidus* acclimated to 35 p.p.t. (t=0) and at various times after being transferred to 15 p.p.t. Values are means ± s.e.m., N=4–14. Letters above bars indicate statistical comparisons within G7 across the time course: two columns not sharing at least one same letter are statistically different (P<0.05, two-way ANOVA, Tukey's and Fisher LSD's *post-hoc* tests). There were no significant differences among G3.

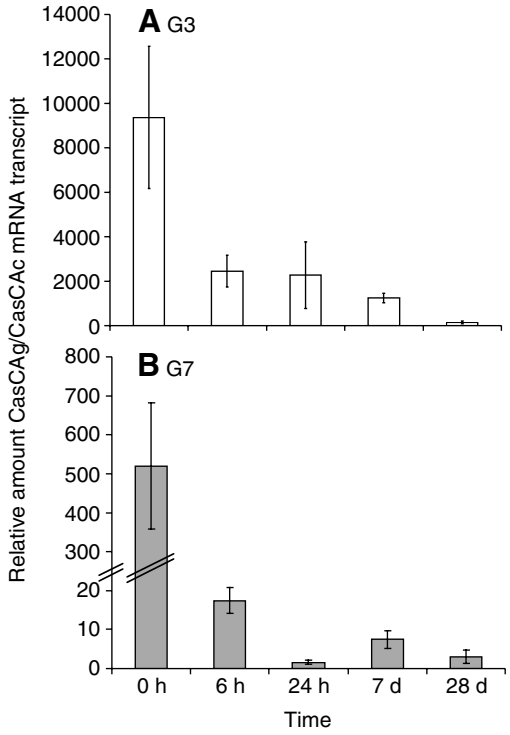

Fig. 6. Relative amounts of CasCAg and CasCAc mRNA transcripts in (A) anterior (G3) and (B) posterior (G7) gills of *Callinectes sapidus* acclimated to 35 p.p.t. (t=0) and at various times after being transferred to 15 p.p.t. Values are means ± s.e.m., N=4–5.

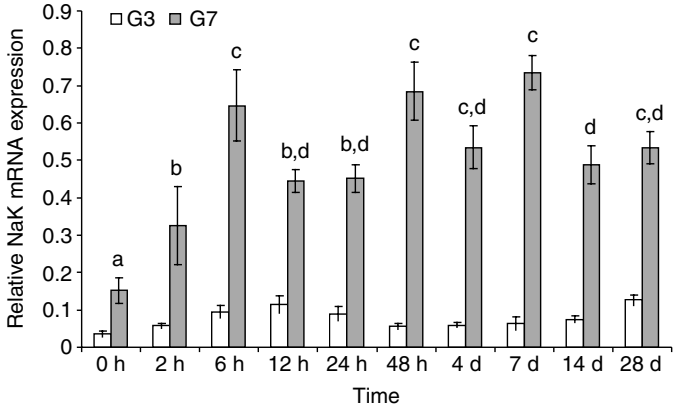

Fig. 7. Relative Na^+/K^+-ATPase α-subunit (NAK) expression in anterior (G3, white bars) and posterior (G7, black bars) gills of *Callinectes sapidus* acclimated to 35 p.p.t. (t=0) and at various times after being transferred to 15 p.p.t. Values are means ± s.e.m., N=4–14. Letters above bars indicate statistical comparisons within G7 across the time course: two columns not sharing at least one same letter are statistically different (P<0.05, two-way ANOVA, Fisher LSD's *post-hoc* test). There were no significant differences among G3.

presence of CA activity in the cytoplasmic and microsomal fractions of gill homogenates (Henry, 1988a).

This study also presents data on the different responses of the two isoforms to changes in environmental salinity that link their regulation and expression to their specific physiological roles in the gill. Relative to the other major gill CA isoform, CasCAg, the CasCAc isoform is expressed in extremely low levels in crabs acclimated to 35 p.p.t., being nearly undetectable in both anterior and posterior gills. Cytoplasmic CA is believed to be the isoform that is directly involved in osmotic and ionic regulation (Henry, 1988a; Henry, 1988b), and so it is not surprising to find very low levels of expression in the gills when the crab is acclimated to a salinity at which it is an osmotic and ionic conformer, and at which the major ion transport mechanisms are silent.

The degree of salinity-sensitivity of the cytoplasmic isoform, however, was very large. The relative abundance of CasCAc mRNA in the posterior, ion-transporting gills, increased by 100-fold in the initial 12 h after transfer from 35 to 15 p.p.t. This is the largest magnitude of induction reported for any transport-related protein in the gills of euryhaline crustaceans, and this could be a conservative estimate. A recent study on another euryhaline species of crab, *Chasmagnathus granulatus*, reported that the relative abundance of the $Na^+/K^+/2Cl^-$ co-transporter mRNA increased a maximum of 20-fold, and that of the Na^+/K^+-ATPase α-subunit increased a maximum of 55-fold in crabs given a salinity transfer from 30 to 2 p.p.t. (Luquet et al., 2005). That was a much larger magnitude salinity transfer

Table 2. *Concentration and inhibition constant of cytoplasmic CA and GPI-linked CA in anterior and posterior gills of Callinectes sapidus acclimated to 35 ppt and transferred to 15 ppt for 7 days and 14 days*

		E_0			K_i			R^2		
		35 p.p.t.	15 p.p.t. 7 days	15 p.p.t. 14 days	35 p.p.t.	15 p.p.t. 7 days	15 p.p.t. 14 days	35 p.p.t.	15 p.p.t. 7 days	15 p.p.t. 14 days
AG	CAc	9.96	17.31	16.75	6.19	4.59	3.67	0.9898	0.9906	0.9922
	CAg	17.55	29.67	27.81	6.22	5.36	5.17	0.9788	0.9865	0.9837
PG	CAc	2.67	123.57	176.02	7.13	5.86	3.51	0.9978	0.9934	0.9774
	CAg	23.52	37.00	71.19	6.64	7.36	5.40	0.9841	0.9932	0.9800

E_0, enzyme concentration; K_i, inhibition constant; CAc, cytoplasmic CA; CAg, GPI-linked CA; AG, anterior gill; PG, posterior gill.
R^2, value of the linear regression obtained after transformation of the data. See 'Titration of free enzyme concentration' in the Materials and methods section for details.

than was used on blue crabs in this study (35–15 p.p.t.); therefore, for comparable salinity transfers, the degree of cytoplasmic CA mRNA induction could be much greater. The relative abundance of the Na^+/K^+-ATPase α-subunit in the posterior gills of blue crabs increased by only fivefold by comparison (Fig. 7), but in this species the relative levels of expression in crabs acclimated to 35 p.p.t. were already high. High levels of expression of the Na^+/K^+-ATPase most likely represent the molecular basis for correspondingly high levels of the Na^+/K^+-ATPase activity in gills in crabs at high salinity (Henry et al., 2002). Another recent study reported a 2.5-fold increase in Na^+/K^+-ATPase expression in posterior gills of blue crabs but only after 5 days of low salinity (10 p.p.t.) exposure (Lovett et al., 2006a). The differences in baseline levels of expression and activity between CA and the Na^+/K^+-ATPase at high salinity, and the different degrees of salinity sensitivity, further support the idea that the two proteins are regulated differently in response to low salinity exposure.

Salinity-mediated CA induction appears to be under transcriptional regulation. The timing of the induction of CA activity was most closely correlated with that of CasCAc mRNA. Increases in CasCAc mRNA and CA activity occurred as stair-step patterns that were slightly out of phase. The initial increase in CasCAc mRNA occurred first, at 6 h post-transfer and peaked at 48 h, while the initial measurable increase in CA activity occurred at 24 h post-transfer and peaked at 7 days. The pattern of induction of CA activity was similar to that already reported (Henry and Watts, 2001). After reaching peak values, both mRNA and activity remained high for the remainder of the experimental time course. CasCAc mRNA levels were elevated first in response to low salinity exposure, and synthesis of new CA protein followed thereafter, giving rise to higher levels of CA activity. In support of this relationship, the concentration of cytoplasmic CA protein in posterior gills was approximately 60-fold higher in crabs after 7 days exposure to low salinity. This is a similar pattern to that seen in another euryhaline species of crab, *Carcinus maenas* (Henry et al., 2003; Henry et al., 2006), suggesting that transcriptional regulation of CA induction may be a common mechanism in euryhaline crustaceans. In contrast, there does not appear to be any mechanism of CA induction in stenohaline, strictly osmoconforming crabs, such as *Cancer irroratus* (Henry and Campoverde, 2006).

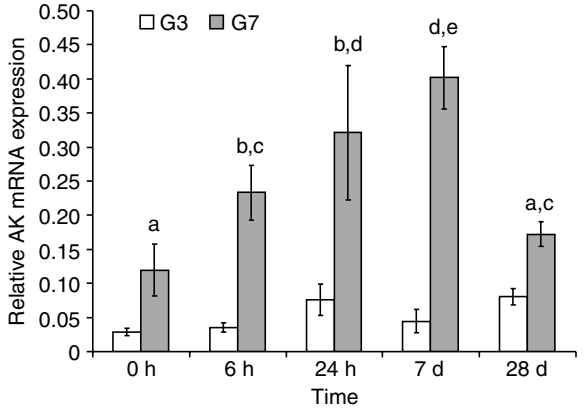

Fig. 8. Relative arginine kinase (AK) mRNA expression in anterior (G3, white bars) and posterior (G7, black bars) gills of *Callinectes sapidus* acclimated to 35 p.p.t. (t=0) and at various times after being transferred to 15 p.p.t. Values are means ± s.e.m., N=4–5. Letters above bars indicate statistical comparisons within G7 across the time course: two columns not sharing at least one same letter are statistically different (P<0.05, two-way ANOVA, Fisher LSD's *post-hoc* test). There were no significant differences among G3.

The timing of induction of both CA and the Na^+/K^+-ATPase corresponds to the timing of the establishment of the new, acclimated physiological state of the crab after transfer to low salinity. These changes occur rapidly. In the transition from osmoconformity to osmoregulation, hemolymph osmotic concentration in blue crabs becomes stabilized by 24 h post-transfer. The transition to osmotic and ionic regulation is preceded by the up-regulation of two of the central transport-related proteins required for active ion transport, which is the mechanistic basis for hemolymph osmotic and ionic regulation.

There also appears to be a difference in the baseline levels and the magnitude of up-regulation of cytoplasmic *vs* membrane-associated proteins. The membrane-associated proteins appear to be expressed at higher levels in high salinity, but the cytoplasmic proteins appear to have a greater degree of 'inductive scope' (magnitude of difference between baseline and maximal levels of expression). For CA, the levels of mRNA for the membrane-associated isoform, CasCAg, are as much as 2–4 orders of magnitude higher than those for CasCAc in crabs at 35 p.p.t. The differences can be linked to isoform-specific physiological function. The membrane-associated isoform is believed to function in the mobilization of hemolymph HCO_3^- to molecular CO_2 in order to facilitate CO_2 excretion across the gill (Henry, 1987). The blue crab is highly active, regardless of salinity, and it is possible that the relatively high abundance of CasCAg mRNA is needed to maintain the levels of the membrane-associated CA protein necessary to provide the degree of catalytic activity required for branchial CO_2 excretion. The degree of salinity-mediated induction in CasCAg, however, is small by comparison (about 5- *vs* 100-fold for CasCAc), and this also corresponds to a smaller degree of CA activity induction in the membrane *vs* cytoplasmic fractions of the gill (about 2- *vs* 14-fold) (Henry, 1988a). Induction of the membrane-associated isoform is most likely a result of the proliferation of the population of ion-transporting 'chloride cells' that takes place in the posterior gills in response to low salinity exposure. This cell type is characterized by a highly infolded basal membrane (Compere et al., 1989), where CasCAg is localized, and the area that this cell type occupies in the lamellae of the posterior gills of the blue crab expands significantly after transfer to low salinity (Neufeld et al., 1980; Lovett et al., 2006b). So, essentially, there is more membrane-associated CA because there is simply more basolateral membrane in the gill lamellae at low salinity.

Interestingly, the timing and degree of the induction of the single CA isoform reported previously for *C. maenas* (Henry

et al., 2006) is most similar to that of CasCAg in *C. sapidus*. That isoform, which was termed CAI (Henry et al., 2003), was expressed in higher amounts than a second isoform, termed CAII, and so was presumed to be the dominant, cytoplasmic form. At that time, only partial nucleotide sequences were available for the CA isoforms from *C. maenas*. More complete sequence data and relative levels of expression have indicated that in *C. maenas* the CAI isoform is most likely membrane-associated, and CAII is the cytoplasmic form (R.P.H. and L.S., unpublished data), and as result of this new information a more thorough investigation of both *C. maenas* isoforms is under way.

From the limited data in this and two related studies, it appears that transport-related proteins have different degrees of induction depending on their subcellular localization. Specifically, cytoplasmic CA mRNA has a 2–20-fold larger degree of induction than that of any of the membrane-associated proteins. This may be related to potential diffusion limitations within the gill itself. The boundary layer on the inside of the apical surface of the gill acts as a separate fluid compartment, and the large excess of CA activity, a result of the high degree of CasCAc mRNA induction, may be necessary to maintain a large enough intracellular gradient of H^+ and HCO_3^- to drive their diffusion into the boundary layer, especially when ion transport is stimulated by low salinity. Cytoplasmic CA activity in gills of crabs acclimated to 35 p.p.t. is still present in approximately 1000-fold excess over what is needed to meet the ion transport needs of crabs in low salinity (Henry, 2001), and yet it is induced in this subcellular compartment by up to 15-fold. This suggests that the excess catalytic enhancement of the cytoplasmic CO_2 hydration reaction is needed to prevent intracellular diffusion limitations of H^+ and HCO_3^-. Conversely, trans-membrane ion transport takes place within the confines of the boundary layer where presumably, smaller changes in transport protein expression would be effective.

As discussed above, for crabs acclimated to 35 p.p.t., the relative levels of CasCAg mRNA are between 500 and 10 000 times higher than that for CasCAc (in G7 and G3, respectively). Despite this large difference in expression, cytoplasmic CA activity is high, being just about equal to that of membrane-associated CA. The reason behind this may be the low turnover rate of the CA protein in the gill. Once synthesized, cytoplasmic CA appears to have an extended biological half-life. For *C. sapidus* or *C. maenas* acclimated to low salinity and transferred back to high salinity, high levels of CA protein concentration and activity persist for 3–4 weeks, while mRNA levels decline rapidly (N. Jillette and R.P.H., unpublished data).

One observation that should be noted is that traditionally accepted 'housekeeping' genes (i.e. genes that are expressed constitutively and do not change in response to the treatment) were not applicable to the posterior gill of the blue crab. Actin expression has already been reported to change in response to low salinity (Lovett et al., 2003). Furthermore, arginine kinase, whose activity and expression do not change in the posterior gills of *C. maenas*, increased by threefold after 7 days of low salinity exposure (Fig. 8). AK activity has also been shown to double under similar conditions (Kotlyar et al., 2000). The posterior gill of the blue crab is a metabolically active tissue, and the rate of oxygen uptake and the concentrations of oxidative enzymes both increase as a result of low salinity exposure (Pillar et al., 1995), so it is not surprising to find that the expression of metabolic genes such as AK also increases. At the current time there is no known gene in the blue crab gill that is completely unresponsive to environmental salinity. This is not surprising, given the physiological, biochemical and ultrastructural changes that the posterior gill undergoes in response to low salinity. Ion-transporting gills in both invertebrates and vertebrates undergo complete re-modeling in response to salinity changes, including cellular differentiation and proliferation, membrane and cytoskeletal re-organization, and up-regulation of a variety of transport and transport-related proteins (Perry, 1997; Luquet et al., 2002; Evans et al., 2005). On the other hand, neither the activity nor the expression of the proteins examined in this study was significantly affected by salinity in the anterior gills. It may therefore be more accurate in crustaceans to use the anterior gills as a control tissue. Anterior and posterior gills are anatomically similar, homologous tissues that differ only in their functional responses to environmental salinity. This pattern appears to hold for other euryhaline species of crustaceans as well. There were no changes in CA activity, or CA or AK mRNA expression in anterior gills of *C. maenas* in response to low salinity (Henry, 2006) and no change in expression of the V-type H^+-ATPase or the $Na^+/K^+/2Cl^-$ co-transporter in anterior gills of *Pachygrapsus marmoratus* (Spanings-Pierrot and Towle, 2004). In another species, *Chasmagnathus granulatus*, expression of both the co-transporter and the Na^+/K^+-ATPase changed in posterior and anterior gills, but the changes in the anterior gills were much smaller, and they were not consistently correlated with low salinity exposure. But at this time it appears that using the anterior gill as a control tissue is more reliable than trying to identify a control gene.

In summary, two isoforms of CA have been identified and sequenced in the blue crab gill that are different in cellular localization, physiological function, expression and regulation. Changes in expression of the cytoplasmic isoform appear to be the basis for low salinity-mediated induction of CA activity, and the cytoplasmic isoform displays the largest inductive scope of any known transport-related protein.

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