Trends in Comparative Biochem & Physiol
Vol. 16, 2012

Original Communication

K^+-phosphatase activity of the gill (Na^+, K^+)-ATPase from the intertidal hermit crab, *Clibanarius vittatus:* Kinetic characterization of the substrate and cation-binding sites

Daniela P. Garçon[1], Malson N. Lucena[2], Rúbia R. Gonçalves[3], Marcelo R. Pinto[3], John C. McNamara[4] and Francisco A. Leone[3,*]

[1]Departamento de Biologia Molecular, Centro de Ciências Exatas e da Natureza, Universidade Federal da Paraíba, Brasil, [2]Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, [3]Departamento de Química and [4]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, Avenida Bandeirantes, 3900. 14040-901-Ribeirão Preto, São Paulo, Brasil

ABSTRACT

The kinetic properties of the substrate and cation-binding sites of a microsomal gill (Na^+, K^+)-ATPase from the hermit crab *Clibanarius vittatus* were characterized using the synthetic substrate *p*-nitrophenylphosphate. Substrate was hydrolyzed obeying Michaelis-Menten kinetics at a maximum rate of 47.4 ± 1.9 U mg^{-1} with K_M = 1.0 ± 0.04 mmol L^{-1}. Stimulation of K^+-phosphatase activity by Mg^{2+} (V_M = 43.9 ± 1.5 U mg^{-1} and $K_{0.5}$ = 0.6 ± 0.1 mmol L^{-1}), K^+ (V_M = 48.9 ± 2.4 U mg^{-1} and $K_{0.5}$ = 3.7 ± 0.1 mmol L^{-1}) and NH_4^+ (V_M = 50.1 ± 1.7 U mg^{-1} and $K_{0.5}$ = 19.3 ± 0.7 mmol L^{-1}) showed cooperative kinetics. Stimulation by K^+ or NH_4^+ of K^+-phosphatase activity was similar, enzyme specific activity not varying noticeably in the presence of both ions. The data suggest that after binding of either K^+ or NH_4^+ to their sites, increasing concentrations of the other do not displace the first ion from its binding site. Na^+ (K_I = 16.1 ± 0.2 mmol L^{-1}), ouabain (K_I = 340.4 ± 16.1 µmol L^{-1}) and orthovanadate inhibited 70-90% of *p*-nitrophenylphosphatase activity. This is the first known kinetic characterization of K^+-phosphatase activity in a hermit crab and provides a useful tool for comparative biochemical studies of (Na^+, K^+)-ATPase activities in crustacean gill tissues.

KEYWORDS: (Na^+, K^+)-ATPase, K^+-phosphatase activity, cation binding sites, gill microsome, *Clibanarius vittatus*, hermit crab, *p*-Nitrophenylphosphate

INTRODUCTION

The (Na^+, K^+)-ATPase, found in the plasma membranes of all animal cells, underpins many homeostatic processes and is directly responsible for the asymmetrical, electrogenic counter-transport of Na^+ and K^+ that results in strong ionic gradients across their membranes [1-4]. These gradients generate transmembrane electrical potential and drive transport processes like passive water movement, active transepithelial salt, Na^+/glucose/ aminoacid/nucleotide cotransport, and cell volume regulation [2-5]. The enzyme belongs to the P_{2C} subfamily of membrane-embedded P-type ATPases whose hallmark is the formation of an acyl-phosphate intermediate during the catalytic cycle [1, 5-7]. The X-ray crystal structure of the oligomeric (Na^+, K^+)-ATPase reveals a catalytic alpha subunit and a beta subunit together with an FXYD protein [8, 9]. The active moiety of the enzyme, the αβ-complex, consists of the 110-kDa catalytic alpha-subunit and the 50-kDa beta-

*Corresponding author:
fdaleone@ffclrp.usp.br

subunit: the energy provided by ATP hydrolysis drives the counter-transport of $3Na^+$ and $2K^+$ across the cell membrane. Enzyme phosphorylation and dephosphorylation results in the transition between two main conformational states: E_1, exhibiting a high affinity for intracellular Na^+, and E_2 characterized by high affinity for extracellular K^+ [reviewed by 2-4, 8].

The (Na^+, K^+)-ATPase also hydrolyses other phosphate-donating substrates (K^+-phosphatase activity), such as *p*-nitrophenylphosphate [10-14], O-methylfluorescein phosphate [15] and acetyl phosphate [16]. While ouabain-inhibitable ATPase and K^+-phosphatase activities are strictly correlated, corresponding to different activities of the same enzyme, K^+-phosphatase activity is not yet well understood [10, 11, 14, 17-19]. Although competitive inhibition by ATP of the K^+-phosphatase activity constitutes strong evidence that p-nitrophenylphosphate (*p*-NPP) and ATP are hydrolyzed at the same site on the (Na^+, K^+)-ATPase [18, 20], a separate binding site for *p*-nitrophenylphosphatase (*p*-NPPase) activity, near the ATP binding site, also has been suggested [21, 22]. The E_2 form seems to be the main conformation involved in K^+-phosphatase activity, and enzyme phosphorylation by *p*-nitrophenylphosphate apparently stimulated by K^+ and not associated with cation transport, is also controversial [11, 23-26]. Although *p*-NPPase activity and dephosphorylation involve the hydrolysis of a phosphate bond and both are stimulated by K^+, extracellular K^+ stimulates enzyme dephosphorylation [13, 27] while intracellular K^+ stimulates K^+-phosphatase activity [13].

Crustacean gills provide a selective interface between the external environment and internal milieu, constituting a multifunctional organ [28, 29]. Covered by a thin cuticle, the gill epithelium represents a selective interface between the internal and the external media across which Na^+ and Cl^- are actively absorbed from dilute environments [29-31]. In hyperosmoregulating crustaceans, the gill (Na^+, K^+)-ATPase participates in Na^+ uptake from the dilute medium to the hemolymph, showing increased activity [29, 30, 32-34].

The anomurans, including hermit crabs, mole crabs, king crabs, squat lobsters and porcelain crabs, present a broad array of body forms and functions and are among the most diverse of any decapod group [35]. Hermit crabs have adapted to occupy empty snail shells [36], a refuge dictated by the lack of calcification of their abdominal exoskeleton [37].

Clibanarius vittatus is a common intertidal species that occurs from Brazil to North Carolina [38-40]. It is a familiar inhabitant of the intertidal zone, being found frequently exposed at low tide among oyster shells and on mud flats [41] typical of estuaries. Such environments constitute ecosystems that may be physiologically stressful for their inhabitants [42]. Consequently, *C. vittatus* can be subject to large seasonal salinity changes, but may migrate offshore to avoid salinity stress [43]. Despite studies describing the distribution and basic biology of anomurans [44], osmoregulation has been less studied in hermit crabs than in other decapod crustaceans [45]. *C. vittatus* is an isoconformer at salinities of 25 ‰ and higher, and hyper-regulates below 25 ‰ salinity [46-48]. Seawater is necessary for embryonic and larval development, metamorphosis to the juvenile phase requiring 25-30 ‰ salinity [49]. Although knowledge of the histological and ultrastructural characteristics of *C. vittatus* gills is scarce [50], kinetic studies demonstrating the synergistic stimulation by K^+ and NH_4^+ of (Na^+, K^+)-ATPase suggest a significant physiological role for this enzyme in active nitrogen excretion by the gill epithelium [51].

In this study, we provide an extensive kinetic characterization of the substrate and cation-binding sites for K^+-phosphatase activity, employing a microsomal gill (Na^+, K^+)-ATPase from the hermit crab, *C. vittatus* caught at 33 ‰ salinity. The most important finding disclosed here is that K^+-phosphatase activity is not synergistically stimulated by K^+ plus NH_4^+, and neither ion can displace the other from its respective binding site.

MATERIAL AND METHODS

Material

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water and all reagents were of the highest purity commercially available. Imidazole, Hepes, *p*-nitrophenyl phosphate Tris salt (*p*-NPP), ouabain, and alamethicin were

purchased from Sigma Chemical Co (St. Louis, USA). The protease inhibitor cocktail (5 mmol L^{-1} leupeptin, 5 mmol L^{-1} antipain, 1 mmol L^{-1} benzamidine, 5 µmol L^{-1} phenyl-methane-sulfonyl-fluoride and 1 mmol L^{-1} pepstatin A) was from Calbiochem (Darmstadt, Germany). All other reagents used were of the highest quality commercially available.

Gill excision

Adult specimens of the hermit crab *C. vittatus* were collected at low tide from Araçá Beach near São Sebastião (23° 48' 86" S; 45° 24' 52" W), São Paulo State, Brazil. The crabs were transported to the laboratory in carboys containing 32 L aerated seawater from the collecting site and held in 50 L tanks containing aerated seawater from the collecting site (33 ‰ salinity, 450.0 ± 13.5 mmol Na^+ L^{-1}, 9.9 ± 0.4 mmol K^+ L^{-1}, 9.5 ± 0.2 mmol Ca^{2+} L^{-1} and 51.5 ± 0.8 mmol Mg^{2+} L^{-1}) for 2 days at 25°C and fed on alternate days with shrimp tails. For each homogenate prepared 40 to 50 hermit crabs were anesthetized by chilling in crushed ice for 5 min. After removal from the shell, the crabs were killed by destroying the cerebral and ventral ganglia, and the carapace was quickly detached. All gill pairs were then excised and transferred to ice cold homogenization buffer (20 mL/g wet tissue) consisting of 20 mmol L^{-1} imidazole, pH 6.8, 6 mmol L^{-1} EDTA, 250 mmol L^{-1} sucrose, and the protease inhibitor cocktail.

Preparation of the gill microsomal fraction

The gills were homogenized using a Potter homogenizer set at 600 rpm. After centrifuging the crude extract at 20,000 × g for 35 min at 4°C, the supernatant was placed on crushed ice and the pellet was re-suspended in an equal volume of homogenization buffer. After further centrifugation as above, the two supernatants were pooled and centrifuged at 100,000 × g for 2 h at 4°C. The resulting pellet was re-suspended in the homogenization buffer (10 mL/g wet tissue) and 0.5-mL aliquots were rapidly frozen in liquid nitrogen and stored at -20°C. Under these conditions, no appreciable changes in *p*-NPPase activity were seen after two-month's storage (fresh preparation V_M = 70.5 ± 0.5 nmol p-nitrophenolate min^{-1} mg^{-1} versus two month's freezing V_M = 68.9 ± 2.1 nmol p-nitrophenolate min^{-1} mg^{-1}). When required, aliquots were thawed, placed on crushed ice and used immediately.

Measurement of hemolymph osmolality and cation concentrations

Individual hemolymph samples (50 µL) were drawn into insulin syringes using #25-8 needles from the junction of the arthrodial membrane at the base of the last pereiopod from each of three different crabs (N = 3). Samples were stored in separate cryotubes at -20°C until processing. Hemolymph osmolality was measured in 10-µL samples using a Wescor Model 5500 vapor pressure osmometer (Logan, USA). Hemolymph Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were measured by emission spectroscopy using a Shimadzu Model AA-680 atomic absorption spectrophotometer (Kyoto, Japan) employing 10-µL hemolymph aliquots diluted 1: 2,500 in Millipore MilliQ ultrapure apyrogenic water. Data are provided as the mean ± SD of three different hemolymph samples (N = 3).

Measurement of p-Nitrophenylphosphate hydrolysis

p-NPP hydrolysis (*p*-NPPase activity) by the gill microsomal fraction was assayed continuously, at 25°C, monitoring the release of the p-nitrophenolate ion ($\varepsilon_{410\ nm\ pH\ 7.5}$ = 13,160 mol^{-1} L cm^{-1}) in a Hitachi U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions were 50 mmol L^{-1} Hepes buffer, pH 7.5, 10 mmol L^{-1} *p*-NPP, 10 mmol L^{-1} $MgCl_2$, and 15 mmol L^{-1} KCl, in a final volume of 1.0 mL. *p*-NPPase activity was also measured as above in the presence of 3 mmol L^{-1} ouabain. The difference in measured *p*-NPPase activity in the absence and presence of ouabain was considered to represent the K^+-phosphatase activity. *p*-NPPase activity was also assayed at 25ºC after 10 min pre-incubation with alamethicin (1 mg/mg protein) to demonstrate the presence of leaky and/or disrupted vesicles. Controls without added enzyme were included in each experiment to quantify the non-enzymatic hydrolysis of substrate. The initial velocities were constant for at least 15 min provided that less than 5% of substrate was hydrolyzed. Assays were performed on duplicate aliquots; each experiment was repeated using three different gill homogenates

(N = 3), and one enzyme unit (U) was defined as the amount of enzyme that hydrolyzes 1.0 nmol of *p*-NPP per minute at 25°C.

Continuous-density sucrose gradient centrifugation

An aliquot (2.9 mg protein/0.7 mL) of the (Na^+, K^+)-ATPase-rich microsomal fraction was layered into a 10 to 50% (w/w) continuous-density sucrose gradient in 20 mmol L^{-1} imidazole buffer, pH 6.8, and centrifuged at 180,000 × g and 4°C for 3 h using an Hitachi PV50T2 vertical rotor. Fractions (0.5 mL) were collected from the bottom of the tube and were assayed for protein, refractive index, *p*-NPPase activity and ouabain-insensitive *p*-NPPase activity.

Measurement of protein

Protein concentration was estimated according to [52], using bovine serum albumin as the standard.

Estimation of kinetic parameters

The kinetic parameters V_M (maximum velocity), $K_{0.5}$ (apparent dissociation constant), K_M (Michaelis-Menten constant) and the n_H value (Hill coefficient) for *p*-NPP hydrolysis were calculated using SigrafW software [53]. The curves presented are a representative of a single microsomal preparation; the kinetic parameters provided in Table 1 are calculated values and represent the mean ± SD derived from three (N = 3) different microsomal preparations. The apparent dissociation constant, K_I, for the enzyme-inhibitor complex was estimated as described by [54]. SigrafW can be freely downloaded from the site http://portal.ffclrp.usp.br/sites/fdaleone/downloads.

RESULTS

Hemolymph osmolality measured just before gill excision was 801 ± 40 mOsm/kg H_2O against a calculated ambient osmolality of 1023 mOsm/kg H_2O (in the aerated seawater from the collecting site, 33 ‰ salinity). Hemolymph Na^+ (370.0 ± 3.8 mmol L^{-1}) and Mg^{2+} (20.4 ± 0.6 mmol L^{-1}) concentrations were hypo-regulated while K^+ (9.9 ± 0.4 mmol L^{-1}) and Ca^{2+} (9.5 ± 0.2 mmol L^{-1}) were hyper-regulated against ambient concentrations.

Total *p*-NPPase activity estimated without (V_M = 71.3 ± 1.2 U mg^{-1}) and after 10 min pre-incubation of the preparation with alamethicin (V_M = 69.7 ± 0.9 U mg^{-1}) reveal that our microsomal preparation is permeable, all solutes having access to both the intra- and extracellular sites of the enzyme under the assay conditions used. The residual ouabain-insensitive *p*-NPPase activity of 26.1 ± 3.1 U mg^{-1} represents ≈36% of total *p*-NPPase activity, suggesting the presence of phosphohydrolases other than the (Na^+, K^+)-ATPase.

Sucrose density gradient centrifugation of the gill microsomal fraction revealed a single protein peak, showing *p*-NPPase activity, in the range between

Table 1. Kinetic parameters estimated for different modulators of K^+-phosphatase activity from the microsomal fraction of *Clibanarius vittatus* gill tissue.

Effector	V (U mg^{-1})	$K_{0.5}$ (mmol L^{-1})	K_M (mmol L^{-1})	n_H
p-NPP	47.4 ± 1.9	-	1.0 ± 0.04	1.1
Mg^{2+}	43.9 ± 1.5	0.6 ± 0.1	-	2.6
K^+	48.9 ± 2.4	3.7 ± 0.1	-	1.4
K^+ + 100 mmol L^{-1} NH_4^+	43.9 ± 1.1	-	-	-
NH_4^+	50.1 ± 1.7	19.3 ± 0.7	-	1.9
NH_4^+ + 15 mmol L^{-1} K^+	41.3 ± 0.8	-	-	-
		K_I (mmol L^{-1})	% Inhibition	
Ouabain		0.34 ± 0.02	70	
Ouabain + 100 mmol L^{-1} NH_4^+		0.49 ± 0.03	67	
Na^+		16.1 ± 0.2	91	

10 and 40% sucrose, while K^+-phosphatase activity focused between 23 and 35% sucrose (Fig. 1). Considerable ouabain-insensitive *p*-NPPase activity (≈36%) was found with 3 mmol L^{-1} ouabain. Given that protein recovery from the gradient was greater than 95%, the difference between total *p*-NPPase activity and ouabain-insensitive *p*-NPPase activity (≈40%) suggests the presence of phosphohydrolases other than the (Na^+, K^+)-ATPase. There was no significant loss of *p*-NPPase activity when the microsomal fraction was held at 4°C for periods as long as 6 h (data not shown).

The effect of increasing *p*-NPP concentrations on K^+-phosphatase activity of the gill microsomal preparation is shown in Fig. 2. Under saturating K^+ (15 mmol L^{-1}) and Mg^{2+} (10 mmol L^{-1}) concentrations, K^+-phosphatase activity increased with increasing *p*-NPP concentration over the range of 10^{-5} mol L^{-1} to 2×10^{-2} mol L^{-1} obeying Michaelis-Menten kinetics with $V_M = 47.4 \pm 1.9$ U mg^{-1} and $K_M = 1.0 \pm 0.04$ mmol L^{-1}. Excess *p*-NPP above 2×10^{-2} mol L^{-1} did not inhibit K^+-phosphatase activity (not shown). Further, the ouabain-insensitive *p*-NPPase activity was stimulated to ≈23 U mg^{-1} over the same *p*-NPP concentration range, accounting for ≈32% of phosphohydrolase activity other than the (Na^+, K^+)-ATPase (inset to Fig. 2).

Magnesium ions were essential for the K^+-phosphatase activity of the gill microsomal (Na^+, K^+)-ATPase (Fig. 3A). No activity was detected in the absence of Mg^{2+}. Under saturating *p*-NPPase (10 mmol L^{-1}) and K^+ (15 mmol L^{-1}) concentrations, increasing Mg^{2+} concentrations (from 10^{-5} mol L^{-1} to 10^{-2} mol L^{-1}) stimulated K^+-phosphatase activity to a maximum rate of $V_M = 43.9 \pm 1.5$ U mg^{-1} with $K_{0.5} = 0.6 \pm 0.1$ mmol L^{-1}. Cooperative effects ($n_H = 2.6$) were seen for the interaction of Mg^{2+} with the enzyme, but inhibition of K^+-phosphatase activity by excess free Mg^{2+} was not observed (not shown). The ouabain-insensitive *p*-NPPase activity present in the microsomal fraction was also stimulated up to 25 U mg^{-1} over the same Mg^{2+} concentration range (inset to Fig. 3A), suggesting the presence of about 35% magnesium-stimulated phosphohydrolases other than (Na^+, K^+)-ATPase.

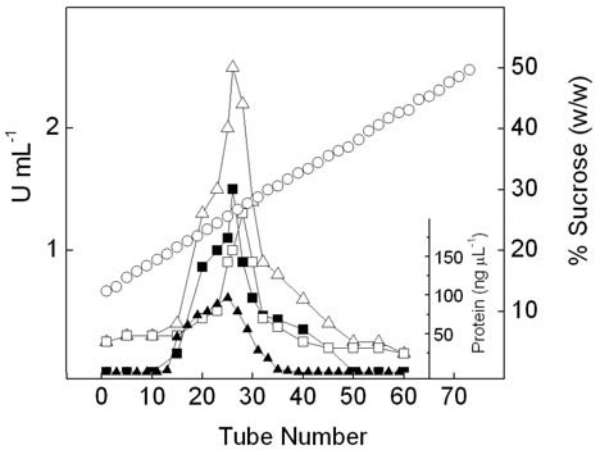

Fig. 1. Sucrose density gradient centrifugation of the microsomal fraction from *Clibanarius vittatus* gill tissue. An aliquot containing 2.9 mg protein was layered into a 10-50% (w/w) continuous sucrose density gradient in 20 mmol L^{-1} imidazole buffer, pH 6.8, and centrifuged for 3 h at 180,000×g and 4°C. Fractions (0.5 mL) were collected from the bottom of the gradient and analyzed for K^+-phosphatase activity (■), protein concentration (▲), sucrose concentration (○), total *p*-NPPase activity (Δ), and ouabain-insensitive *p*-NPPase activity (□).The experiments were repeated using at least three different gill preparations (N = 3). A representative curve obtained from one homogenate is given.

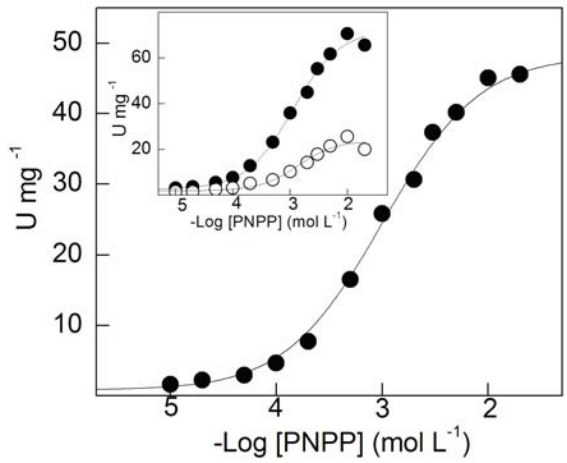

Fig. 2. Modulation by *p*-Nitrophenylphosphate of K^+ phosphatase activity in the microsomal fraction from *Clibanarius vittatus* gill tissue. Activity was assayed continuously at 25°C using 26.4 µg protein in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 10 mmol L^{-1} $MgCl_2$ and 15 mmol L^{-1} KCl in a final volume of 1 mL. Duplicate aliquots from three different gill homogenates (N = 3) were used, and a representative curve obtained from one homogenate is given. **Inset**: variation in total *p*-NPPase (●) and ouabain-insensitive *p*-NPPase (○) activities.

Sodium ions below 5×10^{-3} mol L^{-1} had no effect on the K^+-phosphatase activity of the gill microsomal fraction. However, as concentration increased up to 8×10^{-1} mol L^{-1}, K^+-phosphatase activity was almost completely inhibited (≈90%) (Fig. 3B). The K_I value calculated for the inhibition of *p*-NPPase activity by Na^+ is 16.1 ± 0.2 mmol L^{-1} (inset to Fig. 3B and Table 1).

The effect of K^+ and NH_4^+ on the K^+-phosphatase activity of the gill microsomal fraction of *C. vittatus* is shown in Fig. 4. Under saturating concentrations of *p*-NPP (10 mmol L^{-1}) and Mg^{2+} (10 mmol L^{-1}), enzyme activity was stimulated by increasing concentrations of K^+ (from 10^{-5} mol L^{-1} to 2×10^{-2} mol L^{-1}), following a single titration

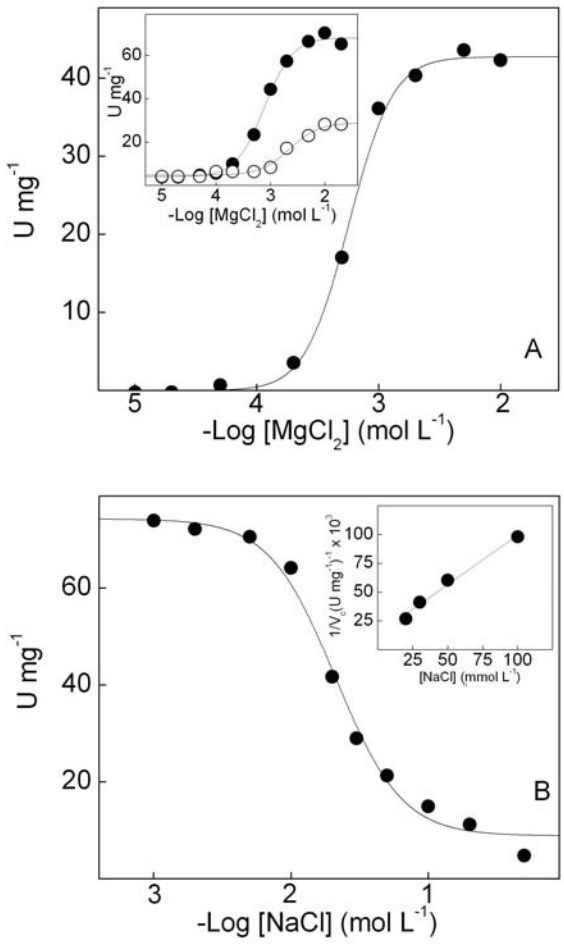

Fig. 3. Modulation by magnesium and sodium ions of K^+-phosphatase activity in the microsomal fraction from *Clibanarius vittatus* gill tissue. Activity was assayed continuously at 25°C using 26.4 µg protein in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 10 mmol L^{-1} *p*-NPP and 15 mmol L^{-1} KCl in a final volume of 1 mL. Duplicate aliquots from three different gill homogenates (N = 3) were used, and a representative curve obtained from one homogenate is given. **A**- modulation by Mg^{2+} of K^+-phosphatase activity. **Inset**: variation in total *p*-NPPase (●) and ouabain-insensitive *p*-NPPase (○) activities. **B**- Inhibition by Na^+. **Inset**: Dixon plot for K_I estimation.

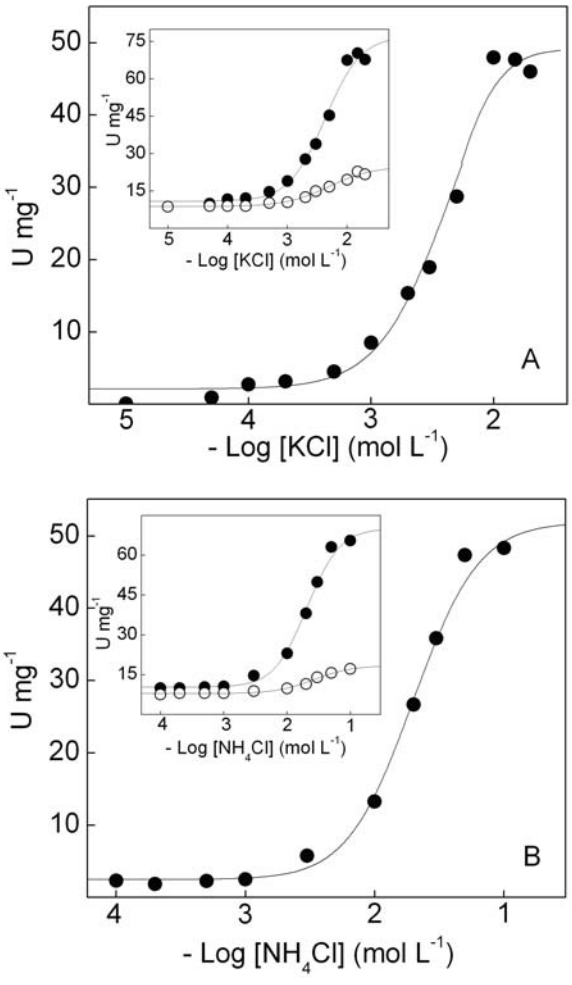

Fig. 4. Modulation by potassium and ammonium ions of K^+-phosphatase activity in the microsomal fraction from *Clibanarius vittatus* gill tissue. Activity was assayed continuously at 25°C in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 10 mmol L^{-1} *p*-NPP and 10 mmol L^{-1} $MgCl_2$, using 26.4 µg protein in a final volume of 1 mL. The experiments were performed using duplicate aliquots from three different gill homogenates (N = 3), and representative curves obtained from one homogenate are given. **A**- Effect of K^+. **B**- Effect of NH_4^+. **Insets:** variation in total *p*-NPPase (●) and ouabain-insensitive *p*-NPPase (○) activities.

curve to maximum rate of V_M = 48.9 ± 2.4 U mg^{-1} and $K_{0.5}$ = 3.7 ± 0.1 mmol L^{-1} (Fig. 4A). Site-site interactions (n_H = 1.4) were observed suggesting the presence of multiple binding sites for K^+. The stimulation of ouabain-insensitive *p*-NPPase activity to ≈ 20 U mg^{-1}, representing 30% of total *p*-NPPase activity, over the same K^+ concentration range, also suggests the presence of phosphohydrolases other than the (Na^+, K^+)-ATPase (inset to Fig. 4A). Under saturating concentrations of *p*-NPPase (10 mmol L^{-1}) and Mg^{2+} (10 mmol L^{-1}), K^+-phosphatase activity is also stimulated by increasing NH_4^+ concentrations (from 5×10^{-4} to 100 mmol L^{-1}) to a maximum rate of V_M = 50.1 ± 1.7 U mg^{-1} and $K_{0.5}$= 19.3± 0.7 mmol.L^{-1} (Fig. 4B). Cooperative kinetics (n_H = 1.9), suggesting multiple binding sites for NH_4^+ was also observed. As for K^+, ammonium ions also stimulated the ouabain-insensitive *p*-NPPase activity up to 18 U mg^{-1} (inset to the Fig. 4B), suggesting the presence of about 25% phosphohydrolases other than (Na^+, K^+)-ATPase. The similar values estimated for maximum stimulation of K^+-phosphatase activity by K^+ or NH_4^+ is unusual.

The effect of both K^+ and NH_4^+ on the modulation of K^+-phosphatase activity in the gill microsomal fraction of *C. vittatus* is shown in Fig. 5. Assays carried out under saturating *p*-NPP (10 mmol L^{-1}) and Mg^{2+} (10 mmol L^{-1}) concentrations revealed that in the presence of 100 mmol L^{-1} NH_4^+, increasing K^+ concentrations (from 10^{-5} mol L^{-1} to 5×10^{-2} mol L^{-1}) did not displace bound NH_4^+ (Fig. 5A) and maximal K^+-phosphatase activity is 43.9 ± 1.1 U mg^{-1}. Unexpectedly, the modulation of K^+-phosphatase activity by NH_4^+ in the presence of 15 mmol L^{-1} K^+, under the same saturating concentration conditions, resulted in an activation profile quite similar to that described above, and K^+-phosphatase activity remains constant at V_M = 41.3 ± 0.8 U mg^{-1} (Fig. 5B). Under these conditions, there is no apparent modulation of one ion by the other, although K^+-phosphatase activity is considerably inhibited by NH_4^+ or K^+ concentrations greater than 10^{-2} mol L^{-1}. Ouabain-insensitive *p*-NPPase activity was not stimulated by the two ions over the same concentration ranges used (insets to Fig. 5A and B).

The effect of a wide range ouabain and orthovanadate concentrations on *p*-NPPase activity

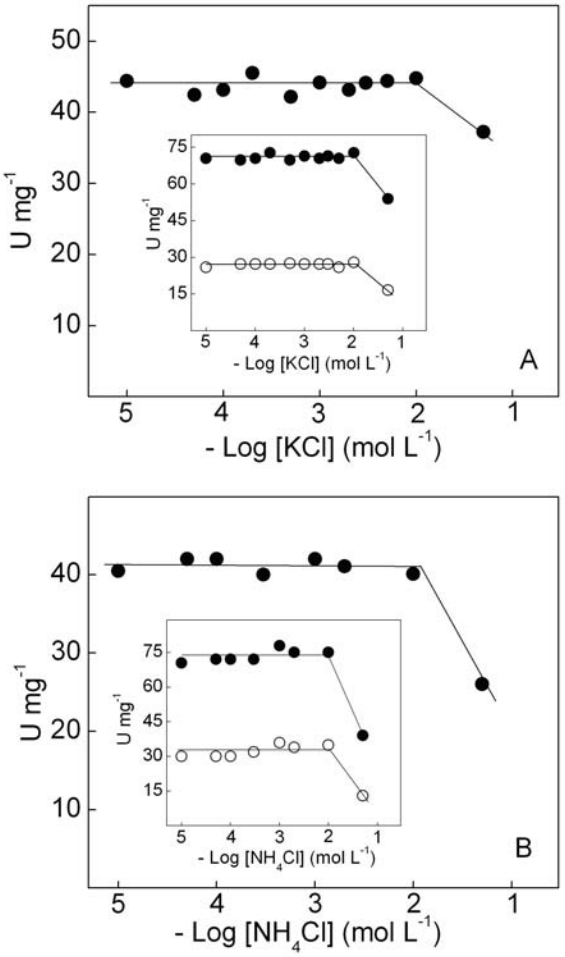

Fig. 5. Effect of both ammonium and potassium ions on K^+-phosphatase activity of the microsomal fraction from *Clibanarius vittatus* gill tissue. Activity was assayed continuously at 25°C in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 10 mmol L^{-1} *p*-NPP and 10 mmol L^{-1} $MgCl_2$, using 26.4 µg protein in a final volume of 1 mL. The experiments were performed using duplicate aliquots from three different gill homogenates (N = 3), and representative curves obtained from one homogenate are given. **A**- 100 mmol L^{-1} NH_4Cl. **B**- 15 mmol L^{-1} KCl. **Insets:** variation in total *p*-NPPase (●) and ouabain-insensitive *p*-NPPase (○) activities.

of the microsomal fraction is shown in Fig. 6. Under optimal concentrations of *p*-NPP (10 mmol L^{-1}), Mg^{2+} (10 mmol L^{-1}), and K^+ (15 mmol L^{-1}) or NH_4^+ (100 mmol L^{-1}), increasing ouabain concentrations up to 7 mmol L^{-1} inhibited almost 70% of the *p*-NPPase activity (Fig. 6A). The inhibition pattern corresponds to that of a single binding site model,

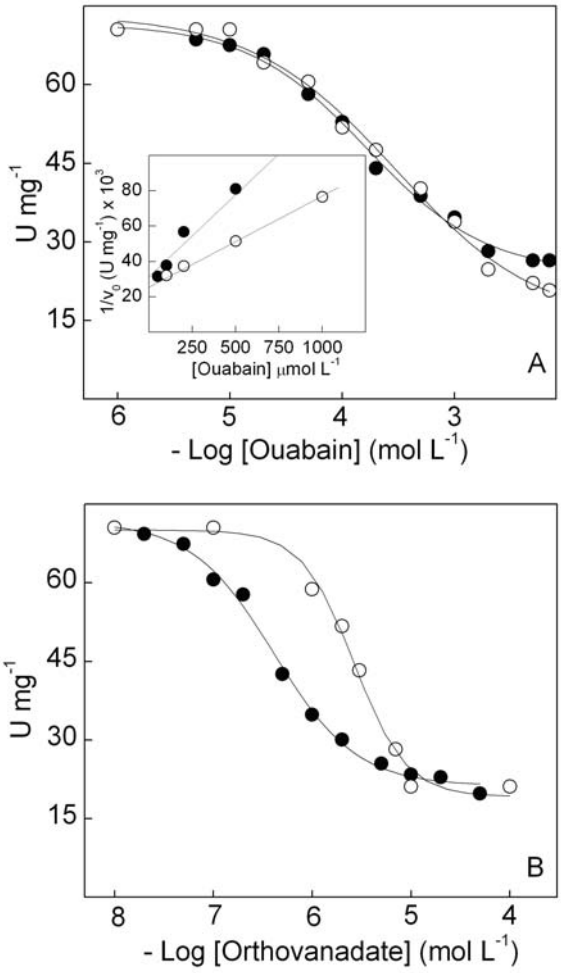

Fig. 6. Inhibition by ouabain and orthovanadate of total *p*-NPPase activity in the microsomal fraction from *Clibanarius vittatus* gill tissue, in the presence and absence of ammonium ions. Activity was assayed continuously at 25 °C in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 10 mmol L^{-1} *p*-NPP, 15 mmol L^{-1} KCl and 10 mmol L^{-1} $MgCl_2$, using 26.4 µg protein in a final volume of 1 mL. The experiments were performed using duplicate aliquots from three different gill homogenates (N= 3), in the absence (●) and presence (○) of 100 mmol L^{-1} NH_4Cl. Representative curves obtained from one homogenate are given. **A**- Ouabain. **Inset**: Dixon plots for K_I for ouabain. **B**- Orthovanadate.

and the calculated apparent K_I value (ouabain concentration at which activity is 50% inhibited) was 340.4 ± 16.1 µmol L^{-1} (inset of Fig. 6A). When *p*-NPPase activity was assayed as above but with additional 100 mmol L^{-1} NH_4^+ a similar profile was found and the K_i value increased to 494.8 ± 28.3 µmol.L^{-1} (inset of Fig. 6A). The K^+-phosphatase activity of the gill microsomal fraction was also inhibited by about 70% with orthovanadate concentrations up to 0.01 mmol L^{-1} (Fig. 6B) and was time-dependent (data not shown). In the presence of additional 100 mmol L^{-1} NH_4^+, the orthovanadate inhibition pattern is considerably modified.

Table 1 summarizes the kinetic parameters for the modulation of the K^+-phosphatase activity in the microsomal fraction by K^+, NH_4^+ and Mg^{2+} ions and *p*-NPP, and inhibition by Na^+, ouabain and orthovanadate.

DISCUSSION

In this investigation, we provide an extensive kinetic characterization of the modulation by *p*-NPP, K^+, Na^+, Mg^{2+} and NH_4^+, and inhibition by ouabain, of gill microsomal (Na^+, K^+)-ATPase activity in the striped hermit crab, *C. vittatus*. The most important conclusion of this study is that stimulation by K^+ or NH_4^+ of K^+-phosphatase activity is similar, with little alteration in enzyme specific activity in the presence of both ions. Our findings also suggest that after the binding of either ion to its site, increasing concentrations of the other do not displace the first ion.

The single protein peak coincident with *p*-NPPase and ATPase activities and with the 100-kDa immunoreactive band confirm earlier data for *C. vittatus* [51], suggesting that K^+-phosphatase activity may be a convenient tool for use in comparative studies of gill (Na^+, K^+)-ATPase [18, 19, 55-57]. However, the complete mechanism of *p*-NPP hydrolysis clearly remains to be elucidated [14] since, in the absence of Na^+, K^+ apparently activates the catalytic reaction at cytoplasm-exposed sites that correspond to the Na^+ binding sites [11, 24].

Hemolymph osmolality and cation concentration

Clibanarius vittatus appears to be a modest hyporegulator in seawater, since hemolymph osmolality (801.0 ± 40.1 mOsm/kg H_2O at 33 ‰ salinity) is hyposmotic to ambient (1023 mOsm kg H_2O). Crabs acclimated to 45 ‰ salinity are also hyposmotic (1102.5 ± 22.1 mOsm/kg H_2O, [58]) to ambient (1395 mOsm/kg H_2O). At 33 ‰

salinity, hemolymph K^+ and Ca^{2+} are hyper-regulated well above their respective ambient concentrations while Na^+ and Mg^{2+} are strongly hyporegulated.

Modulation of gill K^+-phosphatase activity *p*-NPP

The specific *p*-NPPase activity (47.4±1.9 U mg^{-1}) of the gill (Na^+, K^+)-ATPase is 3 times less than its ATPase activity [51]. The ratios between the two activities for the enzyme from gill tissue of *Macrobrachium olfersi* [18] and *M. amazonicum* [56, 59], *Xiphopenaeus kroyeri* [34], *Callinectes danae* [19] and *Cancer pagurus* [55] are ≈2.5: 1. Given that for the vertebrate enzyme ratios are around 6:1 [10, 17], such lower ratios may be characteristic of the crustacean enzyme. The affinity (K_M = 1.00 ± 0.04 mmol L^{-1}) of the *C. vittatus* enzyme for the synthetic substrate *p*-NPP is similar to that of other crustaceans, between 1.0 and 2.5 mmol L^{-1} [18, 19, 55-57, 60] and for vertebrate tissues [61, 62].

The single family of sites revealed for *p*-NPP hydrolysis by the *C. vittatus* gill enzyme, like that from *M. olfersi* [18], *C. pagurus* [55], and vertebrate tissues [61, 62], contrasts with the biphasic curve seen for ATP, shows both high- and low-affinity binding sites, as demonstrated in *C. danae* [63], *C. vittatus* [51], *Dilocarcinus pagei* [64] and *M. amazonicum* [59]. The characterization of high affinity ATP-binding sites is controversial [65, 66] since maximum hydrolysis rates for such sites are just 10% of total activity [11, 19, 51, 59, 67]. Thus, the absence of high-affinity sites for K^+-phosphatase activity in *C. vittatus* gill tissue is consistent with the negligible hydrolytic activity detected below 10^{-5} mol L^{-1} *p*-NPP.

Elevated (Na^+, K^+)-ATPase activity might be expected for *C. vittatus*, given its intertidal habitat susceptible to freshwater influence and ample tidal and seasonal salinity changes [38, 39, 43, 68]. However, *C. vittatus* exhibits low (Na^+, K^+)-ATPase [51] and K^+-phosphatase activities (present study) compared to the *C. danae* enzyme [19]. This may be a consequence of the fact that *C. vittatus* inhabits gastropod shells and is protected against the metabolic consequences of ambient salinity fluctuations [41, 69] since variation in shell water osmolality is attenuated [70].

Modulation by Mg^{2+} of gill K^+-phosphatase activity

Magnesium ions are essential for both the ATPase and K^+-phosphatase activities of (Na^+, K^+)-ATPases from various sources [11, 18, 19, 24, 25, 56, 57, 71]. According to [72] there is at least one separate Mg^{2+}-binding site that is important for both ATPase and *p*-NPPase activities. The $K_{0.5}$ value 0.6 ± 0.1 mmol L^{-1} calculated for stimulation by Mg^{2+} of the K^+-phosphatase activity is similar to those for *C. pagurus* [20], *C. danae* [19], *M. olfersii* [18, 56], *M. amazonicum* [57] and the vertebrate enzyme [10, 61]. With the exception of the Michaelis-Menten behavior seen for the *C. pagurus* enzyme [55], steady state kinetics performed for various crustacean species reveal that stimulation of the enzyme by Mg^{2+} exhibits site-site interactions [18, 19, 56, 57, Garçon *et al.*, this paper].

Inhibition by Na^+ of gill K^+-phosphatase activity

The *C. vittatus* gill enzyme does not hydrolyze *p*-NPP in the presence of Na^+ alone, a characteristic noted by others [11]. However, at saturating K^+ concentrations, K^+-phosphatase activity is strongly inhibited (≈90%) by high Na^+ concentrations [18, 19, 56, 60], apparently the result of competition between Na^+ and K^+ ions for the cytosolic sites [11]. The E_2 form of the (Na^+, K^+)-ATPase which is not associated with ion transport or enzyme phosphorylation during the catalytic cycle, is the main conformation involved in *p*-NPP hydrolysis [11, 24-26]. Curiously, Na^+ does not affect stimulation of the K^+-phosphatase activity by K^+ in *M. olfersi* [18], contrasting with *C. danae* [19], *C. pagurus* [20], 21 ‰-acclimated *M. olfersii* [56], *M. amazonicum* [57], and *Electrophorus electricus* electric organ [73]. Sodium ions appear to constitute an allosteric effector of enzyme stimulation by K^+ ions given that Na^+ concentration affects both the Hill coefficient (n_H) and $K_{0.5}$ values for stimulation by K^+ [18].

Inhibition by ouabain and orthovanadate

Microsomal preparations of crustacean gill tissues are usually contaminated by neutral [74] and/or acid and alkaline phosphatases [75] and Ca^{2+}-ATPase [76]. To ascertain the true contribution of

the (Na^+, K^+)-ATPase in crustacean microsomal preparations it is imperative to estimate inhibition by both ouabain and orthovanadate. Ouabain inhibited *C. vittatus* gill K^+-phosphatase activity with an apparent K_I of 340.37 ± 14.1 µmol L^{-1}, similar to 21 ‰-acclimated *M. olfersi* [56], *Macrobrachium rosenbergii* (J. França, unpublished data) and 33 ‰-acclimated *Callinectes ornatus* [77]. However, the *C. vittatus* K_I is half those of *M. olfersi* [18] and fresh-caught *C. danae* [19] and 21 ‰-acclimated *C. ornatus* [77], and freshwater- and 21 ‰-acclimated *M. amazonicum* [57]. Except for *X. kroyeri* [34] and 21 ‰-acclimated *M. amazonicum* [57], these K_I values are considerably greater than that for ATP hydrolysis, and are similar to vertebrate enzymes [17]. Ouabain or orthovanadate inhibited 72% of the K^+-phosphatase activity. These data thus show unequivocally that *p*-NPP hydrolysis results from the K^+-phosphatase activity of the (Na^+, K^+)-ATPase.

Stimulation by K^+ and NH_4^+ of gill K^+-phosphatase activity

In the absence of one another, K^+ or NH_4^+ ions stimulate *p*-NPP hydrolysis by the *C. vittatus* gill enzyme to comparable rates (48.9 ± 2.4 U mg^{-1} and 50.1 ± 1.7 U mg^{-1}, for K^+ and NH_4^+, respectively) as seen in 21 ‰-acclimated *M. olfersii* [56], fresh-caught and 15 ‰-acclimated *C. danae* [19,60], 33 ‰-acclimated *C. ornatus* [77] and *C. pagurus* [71]. Stimulation of *p*-NPP hydrolysis by NH_4^+ ions may reflect substitution of K^+ by NH_4^+ at the same binding sites [78]. Although $K_{0.5}$ values were greater for NH_4^+ (19.3 ± 0.7 mmol L^{-1}) compared to K^+ (3.7 ± 0.1 mmol L^{-1}) they are similar to those for various crustaceans [18, 19, 34, 56, 57, 71].

Synergistic stimulation of ATP hydrolysis by K^+ and NH_4^+ is a well-known characteristic of the crustacean (Na^+, K^+)-ATPase [51, 59, 63, 79, 80]. However, except for 33‰-acclimated *C. ornatus* [77], *p*-NPP hydrolysis by the crustacean enzyme is not synergistically stimulated apparently due to the fact that the sites responsible for stimulation of (Na^+, K^+)-ATPase activity by NH_4^+ (or K^+) under saturating K^+ (or NH_4^+) concentrations are unavailable for cation binding to the E_2 enzyme form. Further, the differences observed in the calculated kinetic parameters V, n_H and K_M for the different species we have studied, may reflect either the expression of different isoenzymes during salinity adaptation or the activation of species-specific regulatory mechanisms. Two α-subunit (Na^+, K^+)-ATPase isoforms have been identified in some crustaceans [32, 81, 82] and different isoenzymes also may be expressed in the crab gill [60, 83, 84].

Concluding, it is difficult to formulate a simple kinetic model to explain how crustacean enzymes exert K^+-phosphatase activity [14]. Findings showing a threshold K^+ concentration for the appearance of high-affinity ATP binding sites, and a low-affinity phosphorylation site on the enzyme [85], suggest differences in the catalytic mechanism of the crustacean compared to vertebrate enzyme. For the mammalian (Na^+, K^+)-ATPase, the biphasic curve for ATP phosphorylation seen only when *p*-NPP is used as an exogenous, non-physiological inhibitor has been attributed to the formation of a tetramer bearing two distinct, high- and low-affinity, ATP-binding catalytic α-subunits that may act cooperatively [86]. Thus, it seems likely that, as seen in mammalian tissues, the *C. vittatus* gill enzyme undergoes multiple E2 subconformations during *p*-NPP hydrolysis. However, the interactions at the K^+ sites as well as the response to NH_4^+ are quite different from those seen in other animal models.

ACKNOWLEDGEMENTS

This work constitutes part of a Ph.D. thesis by RRG and was supported by research grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho de Desenvolvimento Científico e Tecnológico (CNPq), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Instituto Nacional de Ciência e Tecnologia (INCT) Adapta/Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM, 573976/2008-2). RRG and MNL received scholarships from FAPESP. DPG and MRP received post-doctoral scholarships from FAPESP and CNPq, respectively. FAL and JCM received research scholarships from CNPq. We thank Nilton Rosa Alves for technical assistance. This laboratory (FAL) is integrated with the Amazon Shrimp Network (Rede de Camarão da

Amazônia) and with ADAPTA (Centro de Estudos de Adaptações da Biota Aquática da Amazônia).

REFERENCES

1. Kaplan, J. H. 2002, Ann. Rev. Biochem., 71, 511.
2. Jorgensen, P. L., Hakansson, K. O. and Karlish, S. J. D. 2003, Ann. Rev. Physiol., 65, 817.
3. Martin, D. W. 2005, Seminars Nephrol., 198, 282.
4. Sáez, A. G., Lozano, E. and Zaldívar-Riverón, A. 2009, Genetica, 136, 479.
5. Jorgensen, P. L. and Pedersen, P. A. 2001, Biochim. Biophys. Acta, 1505, 57.
6. Axelsen, K. B. and Palmgren, M. G. 1998, J. Mol. Evol., 46, 84.
7. Poulsen, H., Morth, P., Egebjerg, J. and Nissen, P. 2010, FEBS Lett., 584, 2589.
8. Morth, J. P., Pedersen, B. P., Toustrup-Jensen, M. S., Sorensen, T. L. M., Petersen, J., Eersen, J. P., Vilsen, B. and Nissen, P. 2007, Nature, 450, 1043.
9. Geering, K. 2008, Curr. Opin. Nephrol. Hypertension, 17, 526.
10. Robinson, J. D. 1981, Biochim. Biophys. Acta, 642, 405.
11. Glynn, I. M. 1985, The Enzymes of Biological Membranes, Martonosi, A. (Ed.), Plenum Press, New York, 3, 35.
12. Robinson, J. D. 1982, Neurochem. Res., 7, 1403.
13. Drapeau, P. and Blostein, R. 1980, J. Biol. Chem., 255, 7827.
14. Gatto, C., Arnett, K. L. and Milanick, M. A. 2007, J. Memb. Biol., 216, 49.
15. Huang, W. and Askari, A. 1975, Analyt. Biochem., 66, 265.
16. Swann, A. C. and Albers, R. W. 1980, Arch. Biochem. Biophys., 203, 422.
17. Esmann, M. 1988, Methods Enzymol., 156, 105.
18. Furriel, R. P. M., McNamara, J. C. and Leone, F. A. 2001, Comp. Biochem. Physiol., 130A, 665.
19. Masui, D. C., Furriel, R. P. M., McNamara, J. C., Mantelatto, F. L. M. and Leone, F. A. 2003, Comp. Biochem. Physiol., 134B, 631.
20. Gache, C., Rossi, B. and Lazdunski, M. 1976, Eur. J. Biochem., 6, 293.
21. Krumscheid, R., Ettrich, R., Sovová, Z., Susankova, K., Lansky, Z., Hofbauerova, K., Linnertz, H., Teisinger, J., Amler, E. and Schoner, W. 2004, Eur. J. Biochem., 271, 3923.
22. Ratheal, I. M., Virgin, G. K., Yu, H., Roux, B., Gatto, C. and Artigas, P. 2010, PNAS, 107, 18718.
23. Homareda, H. and Ushimaru, M. 2005, FEBS J., 272, 673.
24. Robinson, J. D. and Pratap, P. R. 1991, Biochim. Biophys. Acta, 1061, 267.
25. Berberian, G. and Beaugé, L. 1992, Biochim. Biophys. Acta, 1103, 85
26. Jorgensen, P. L., Nielsen, J. M., Rasmussen, J. H. and Pedersen, P. A. 1998, Biochim. Biophys. Acta, 1365, 65.
27. Blostein, R. and Chu, L. 1977, J. Biol. Chem., 252, 3035.
28. Taylor, H. H. and Taylor, E. W. 1992, Microscopic anatomy of invertebrates. Decapod Crustacea, Harrison, F. W. and Humas, A. G. (Eds.), Wiley-Liss, New York, 10, 203.
29. Péqueux , A. 1995, J. Crustacean Biol., 15, 1.
30. Freire, C. A., Onken, H. and McNamara, J. C. 2008, Comp. Biochem. Physiol., 151A, 272.
31. McNamara, J. C. and Faria, S. C. 2012, J. Comp. Physiol. B, Biochem. Syst. Env. Physiol. in press.
32. Lucu, C. and Towle, D. W. 2003, Comp. Biochem. Physiol., 135A, 195.
33. Kirschner, L. B. 2004, J. Exp. Biol., 207, 1439.
34. Leone, F. A., Furriel, R. P. M., McNamara, J. C., Mantelatto, F. L. M. Masui, D. C., Rezende, L. A., Gonçalves, R. R. and Garçon, D. P. 2005, Trends Comp. Biochem. Physiol., 11, 1.
35. Bracken, H. D., Toon., A., Felder, D. L., Martin, J. W., Finley, M., Rasmussen, J., Palero, F. and Creall, K. A. 2009, Arthropod Syst. Phylogeny, 67, 99.
36. Hazlett, B. A. 1981, Ann. Rev. Ecol. Syst. 12, 1.
37. Williams, J. D. and McDermott, J. J. 2004, J. Exp. Mar. Biol. Ecol., 305, 1.
38. Coelho, P. A. and Ramos, R. A. 1972, Trabls. Oceanogr. Univ. Fed. PE, 13, 133.
39. Williams, A. B. 1984, Shrimps, lobsters, and crabs of the Atlantic coast of the eastern United States, Maine to Florida. Smithsonian Institution Press. Washington, D.C.

40. Hazlett, B. A., Rittschof, D. and Bach, C. E. 2005, J. Exp. Mar. Biol. Ecol., 323, 93.
41. Young, A. M. 1978, Estuar. Coast. Marin. Sci., 6, 117.
42. Rhodes-Ondi, S. E. and Turner, R. L. 2010, Estuar. Coast. Shelf. Sci., 86, 189.
43. Fotheringham, N. 1975, J. Exp. Mar. Biol. Ecol., 18, 47.
44. Melo, G. A. S. 1999, Manual de Identificação dos Crustacea Decapoda do Litoral Brasileiro: Anomura, Thalassinidea, Palinuridea, Astacidea, Editora Plêiade, São Paulo.
45. Mantel, L. H. and Farmer, L. L. 1983, The biology of Crustacea, internal anatomy and physiological regulation, Mantel, L. H. and Bliss, D. E. (Eds.), Academic Press, New York, 5, 53.
46. Young, A. M. 1979, Comp. Biochem. Physiol., 63A, 377.
47. Sharp, M. S. and Neff, J. M. 1980, Comp. Biochem. Physiol., 66A, 455.
48. Sabourin, T. D. and Stickle, W. B. 1980, J. Exp. Mar. Biol. Ecol., 46, 241.
49. Young, A. M. and Hazlett, T. L. 1978, J. Exp. Mar. Biol. Ecol., 34, 131.
50. Antunes, C. D. 2011, Relação da arquitetura branquial e ultraestrutura de células íon-transportadoras nos mecanismos fisiológicos que permitiram a invasão de meio diluído pelos Anomura (Crustacea, Decapoda): Aegla franca (Schmitt) e Clibanarius vittatus (Bosc). M.S. thesis, University of São Paulo.
51. Gonçalves, R. R., Masui, D. C., McNamara, J. C., Mantelatto, F. L. M., Garçon, D. P., Furriel, R. P. M. and Leone, F. A. 2006, Comp. Biochem. Physiol., 145A, 346.
52. Read, S. M. and Northcote, D. H. 1981, Analyt. Biochem., 116, 53.
53. Leone, F. A., Baranauskas, J. A., Furriel, R. P. M. and Borin, I. A. 2005, Biochem. Mol. Biol. Ed., 33, 399.
54. Marks, M. J. and Seeds, N. W. 1978, Life Sc., 23, 2735.
55. Gache, C., Rossi, B. and Lazdunski, M. 1977, Biochemistry, 16, 2957.
56. Mendonça, N. N., Masui, D. C., McNamara, J. C., Leone, F. A. and Furriel, R. P. M. 2007, Comp. Biochem. Physiol., 146A, 534.
57. Belli, N. M., Faleiros, R. O., Firmino, K. C. S., Masui, D. C., Leone, F. A., McNamara, J. C. and Furriel, R. P. M. 2009, Comp. Biochem. Physiol., 152A, 431.
58. Lucena, M. N., Garçon, D. P., Mantelatto, F. L. M., Pinto, M. R., McNamara, J. C. and Leone, F. A. 2012, Comp. Biochem. Physiol., 161B, 380.
59. Santos, L. C. F., Belli, N. M. , Augusto, A., Masui, D. C., Leone, F. A. , McNamara, J. and Furriel, R. P. M. 2007, Comp. Biochem. Physiol., 148A, 178.
60. Masui, D. C., Furriel, R. P. M., Mantelatto, F. L. M., McNamara, J. C. and Leone, F. A. 2005, J. Exp. Zool., 303A, 294.
61. Beaugé, L. and Berberian, G. 1983, Biochim. Biophys. Acta, 727, 336.
62. Davis, R. L. and Robinson, J. D. 1988, Biochim. Biophys. Acta, 953, 26.
63. Masui, D. C., Furriel, R. P. M., McNamara, J. C., Mantelatto, F. L. M. and Leone, F. A. 2002, Comp. Biochem. Physiol., 132C, 471.
64. Furriel, R. P. M., Firmino, K. C. S., Masui, D. C., Faleiros, R. O., Torres, A. H. and McNamara, J. C. 2010, J. Exp. Zool., 313A, 508.
65. Beaugé, L. A., Gadsby, D. C. and Garrahan, P. J. 1997, Ann. N.Y. Acad. Sci., 834, 1.
66. Martin, D. W. and Sachs, J. R. 2000, J. Biol. Chem., 275, 24512.
67. Ward, D. G. and Cavieres, J. D. 1998, J. Biol. Chem., 273, 33759.
68. Lowery, W. A. and Nelson, W. G. 1988, J. Crust. Biol., 8, 548.
69. Reese, E. S. 1969, Amer. Zool., 9, 343.
70. Wernick, A. M., McNamara, J. C. and Moreira, G. S. 1984, Braz. J. Med. Biol. Res., 17, 492.
71. Rossi, B., Gache, C. and Lazdunski, M. 1978, Eur. J. Biochem., 85, 561.
72. Karlish, S. J. 2003, Ann. N.Y. Acad. Sci., 986, 39.
73. Albers, R. W. and Koval, G. J. 1966, J. Biol. Chem., 241, 1896.
74. Lovett, D. L., Towle, D. W. and Faris, J. E., 1994, Comp. Biochem. Physiol., 109B, 163.
75. Reddy, M. S. and Rao, K. V. 1990, Biochem. Int., 22, 1033.
76. Flik, G. and Haond, C. 2000, J. Exp. Biol., 203, 213.

77. Garçon, D. P., Lucena, M. N., Pinto, M. R., Fontes, C. F. L., McNamara, J. C and Leone, F. A. 2013, Arc. Biochem. Biophys., 530, 55.
78. Robinson, J. D. 1970, Arch. Biochem. Biophys., 139, 17.
79. Garçon, D. P., Masui, D. C., Mantelatto, F. L. M., McNamara, J. C., Furriel, R. P. M. and Leone, F. A. 2007, Comp. Biochem. Physiol., 147A, 145.
80. Garçon, D. P., Masui, D. C., Mantelatto, F. L. M., McNamara, J. C., Furriel, R. P. M. and Leone, F. A. 2009, Comp. Biochem. Physiol., 154A, 44.
81. Lovett, D. L., Colella, T., Cannon, A. C., Lee, H., Evangelisto, A., Muller, E. M. and Towle, D. W. 2006, Biol. Bull., 210, 132.
82. Jayasundara, N., Towle, D. W., Weihrauch, D. and Spanings-Pierrot, C. 2007, J. Exp. Biol., 210, 2070.
83. Harris, R. R. and Bayliss, D. 1988, Comp. Biochem. Physiol., 90A, 303.
84. Genovese, G., Luchetti, C. G. and Luquet, C. M. 2004, Mar. Biol., 144, 111.
85. Masui, D. C., Silva, E. C. C., Mantelatto, F. L. M., McNamara, J. C., Barrabin, H., Scofano, H., Fontes, C., Furriel, R. P. M. and Leone, F. A. 2008, Arch. Biochem. Biophys., 479, 139.
86. Tanoue, K., Kaya, S., Hayashi, Y., Abe, K., Imagawa, R., Taniguchi, K. and Sakaguchi, K. 2006, J. Biochem., 140, 599.