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A Kinetic Characterization of (Na^+, K^+)-ATPase Activity in the Gills of the Pelagic Seabob Shrimp *Xiphopenaeus kroyeri* (Decapoda, Penaeidae)

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Abstract We characterize the kinetic properties of a gill (Na^+, K^+)-ATPase from the pelagic marine seabob *Xiphopenaeus kroyeri*. Sucrose density gradient centrifugation revealed membrane fractions distributed mainly into a heavy fraction showing considerable (Na^+, K^+)-ATPase activity, but also containing mitochondrial F_0F_1- and Na^+- and V-ATPases. Western blot analysis identified a single immunoreactive band against the (Na^+, K^+)-ATPase α-subunit with an M_r of ≈110 kDa. The α-subunit was immunolocalized to the intralamellar septum of the gill lamellae. The (Na^+, K^+)-ATPase hydrolyzed ATP obeying Michaelis–Menten kinetics with $V_M = 109.5 \pm 3.2$ nmol Pi min^{-1} mg^{-1} and $K_M = 0.03 \pm 0.003$ mmol L^{-1}. Mg^{2+} ($V_M = 109.8 \pm 2.1$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 0.60 \pm 0.03$ mmol L^{-1}), Na^+ ($V_M = 117.6 \pm 3.5$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 5.36 \pm 0.14$ mmol L^{-1}), K^+ ($V_M = 112.9 \pm 1.4$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 1.32 \pm 0.08$ mmol L^{-1}), and NH_4^+ ($V_M = 200.8 \pm 7.1$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 2.70 \pm 0.04$ mmol L^{-1}) stimulated (Na^+, K^+)-ATPase activity following site–site interactions. K^+ plus NH_4^+ does not synergistically stimulate (Na^+, K^+)-ATPase activity, although each ion modulates affinity of the other. The enzyme exhibits a single site for K^+ binding that can be occupied by NH_4^+, stimulating the enzyme. Ouabain ($K_I = 84.0 \pm 2.1$ μmol L^{-1}) and orthovanadate ($K_I = 0.157 \pm 0.001$ μmol L^{-1}) inhibited total ATPase activity by ≈50 and ≈44 %, respectively. Ouabain inhibition increases ≈80 % in the presence of NH_4^+ with a threefold lower K_I, suggesting that NH_4^+ is likely transported as a K^+ congener.

Keywords Ammonium excretion · Immunolocalization · Kinetic properties · Microsomal fraction · (Na^+, K^+)-ATPase · Penaeid shrimp gill

Introduction

Osmotic and ionic homeostasis in the Crustacea is accomplished by the multi-functional gills, together with excretory organs like the antennal glands (Péqueux 1995; Freire et al. 2008). The gill epithelial cells also play a central role in hemolymph acid–base regulation and in the excretion of nitrogenous metabolic end products (Péqueux 1995; Lucu and Towle 2003; Weihrauch et al. 2004). Most marine crustaceans are isosmotic with their surrounding medium, using Na^+ and Cl^- ions as their primary hemolymph osmolytes (Péqueux 1995; Lucu and Towle 2003; Kirschner 2004; Freire et al. 2008; McNamara and Faria 2012). However, the selection of a stable extracellular osmolyte concentration relative to that found in fluctuating salinities may have sustained the invasion of estuaries and low salinity biotopes by the Crustacea (Morris 2001; Lee et al. 2011). A species independence of fluctuations in environmental salinity relies on efficient mechanisms of salt uptake and excretion, finely regulated by the activities

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of specific ion transporters in its osmoregulatory organs, such as the gills (Lucu and Towle 2003; Freire et al. 2008).

Although gill-based osmoregulatory mechanisms have been intensively studied in decapods from distinct osmotic niches (Péqueux 1995; Morris 2001; Freire et al. 2008; McNamara and Faria 2012), the role of molecular components in such transport mechanisms is still under active discussion. One suggestion for coupled Na^+ and Cl^- transport across the gill epithelium of weakly hyper-osmoregulating crabs proposes that the gill epithelium uses the (Na^+, K^+)-ATPase located in the basal membrane invaginations to drive active Na^+ uptake, maintaining hemolymph Na^+ concentration fairly constant (Towle and Kays 1986; Freire et al. 2008; McNamara and Faria 2012). Na^+ movement across the apical membrane can be provided by an apical $Na^+/K^+/2Cl^-$ symporter (Towle and Weihrauch 2001) and/or an apical Na^+/H^+ antiporter (Weihrauch and Towle 2000). This Na^+ gradient is supplemented by apical Cs^+-sensitive K^+ channels (Onken et al. 2003) that hyperpolarize the apical membrane, driving Cl^- efflux to the hemolymph through Cl^- channels in the basal invaginations (Morris 2001; Freire et al. 2008). Sodium influx may also follow a paracellular route (Onken et al. 2003; Freire et al. 2008).

Most investigations of crustacean osmoregulation have focused on reduced salinity and its effects on gill and excretory organ functions, evaluating compensatory ion uptake mechanisms (Freire et al. 2008) and alterations in urine volume and concentration (Robinson 1982; Péqueux 1995; Sáez et al. 2009). The gill (Na^+, K^+)-ATPase also appears to play a major role in ammonia excretion (Weihrauch et al. 2004). NH_4^+ can replace K^+ in sustaining ATP hydrolysis (Furriel et al. 2000; Masui et al. 2002, 2005; Garçon et al. 2009; Lucena et al. 2012; Leone et al. 2014) as also seen in the vertebrate enzyme (Skou and Esmann 1992). The synergistic stimulation by K^+ and NH_4^+ of gill (Na^+, K^+)-ATPase activity in various crustaceans suggests a significant physiological role for the (Na^+, K^+)-ATPase in active nitrogen excretion by the gill epithelium (Masui et al. 2002; Gonçalves et al. 2006; Garçon et al. 2007; Santos et al. 2007; Lucena et al. 2012; França et al. 2013; Leone et al. 2014).

The (Na^+, K^+)-ATPase (E.C.3.6.1.37) or sodium pump belongs to the P_2-type ATPase enzyme family, and is phosphorylated by an ATP-derived γ-phosphate group at an aspartate residue in the highly conserved DKTGS/T sequence during the ion transport cycle (Palmgren and Nissen 2011). The (Na^+, K^+)-ATPase is an oligomeric enzyme consisting of a catalytic α-subunit and a β-subunit required for the correct folding, stabilization and expression of the active α-protomer in the plasma membrane (Kaplan 2002; Morth et al. 2007; Poulsen et al. 2010). Recently, a short, single-span membrane protein belonging to the FXYD2 peptide family has been shown to interact with transmembrane helix αM9, fine-tuning the kinetic behavior of the (Na^+, K^+)-ATPase to the specific needs of a given cell type, tissue or physiological state (Garty and Karlish 2006; Geering 2008; Shindo et al. 2011). This FXYD peptide is also present in the crustacean (Na^+, K^+)-ATPase (Silva et al. 2012).

All P-type ATPases function similarly, hydrolyzing ATP and occluding ions during the translocation process within the membrane-embedded protein moiety. As a consequence, the specific ion binding sites on the enzyme are accessible from one side of the membrane only. The overall reaction mechanism of the (Na^+, K^+)-ATPase involves at least two different conformations: E_1, exhibiting high affinity for intracellular Na^+, and E_2, showing high affinity for extracellular K^+, each existing in a phosphorylated or dephosphorylated form (Horisberger 2004). Cycling between the E_1 and E_2 forms results in the counter transport of three Na^+ ions from the cytosol and two K^+ ions into the cytosol across the cell membrane, at the expense of ATP hydrolysis (Kaplan 2002; Jorgensen et al. 2003).

The pelagic marine shrimp *Xiphopenaeus kroyeri* (Heller 1862) is widely distributed along the coast of the Western Atlantic ocean from Virginia (USA), throughout the Gulf of Mexico and the Caribbean Sea to Southern Brazil (Rio Grande do Sul) (Williams 1984; Costa et al. 2003). This species, popularly known as the "camarão sete-barbas" in Brazilian waters, or the "seabob" around the world, is one of the most important commercial shallow water crustaceans exploited along the northern coast of São Paulo State. It is subject to intensive trawling and accounts for approximately 80 % of the penaeid shrimp catch (Mantelatto et al. 1999). The species' plays an important ecological role through its trophic relationships, maintaining the stability of benthic communities (Pires 1992).

Adult *X. kroyeri* migrates to deep offshore areas during their reproductive period, and various physiological and biological processes establish the recruitment pattern of the juveniles (Dall et al. 1990). While juvenile *Xiphopenaeus* spp. inhabit estuarine areas, their migration patterns between the estuarine and fully marine biotopes are poorly known, and it is uncertain to what extent they depend on estuaries as recruitment sites. The penaeid shrimps possess bi-lobate, dendrobranchiate gills (Taylor and Taylor 1992). However, there is no information available as to gill morphology and epithelial ultrastructure in *X. kroyeri*. While knowledge of the biology and ecology of *X. kroyeri* has slowly accumulated (for review see Heckler et al. 2014), there is a dearth of information on the species' physiology and biochemistry.

One of the novelties of this work is the species examined (*X. kroyeri*), a penaeid shrimp with a complex life cycle that unfolds in different habitats. Such a life history is

underpinned by a complex physiology and biochemistry, as demonstrated by our findings for the gill (Na^+, K^+)-ATPase. This is the first study to evaluate these aspects in a very intriguing species. Our findings lay useful groundwork for future studies and aid in comprehending the evolution of biochemical processes related to osmoregulation in decapod crustaceans.

We provide a full kinetic characterization of the (Na^+, K^+)-ATPase present in a microsomal fraction from the gill epithelium of *X. kroyeri* held in seawater in an effort to better understand the role of this enzyme in the active excretion of NH_4^+ by the gills in different decapod crustacean groups. We also examine the distribution of (Na^+, K^+)-ATPase activity in a sucrose density gradient and immunolocalize the enzyme to the intralamellar septum of the bi-lobate gill lamellae.

Materials and Methods

Material

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water. Tris, ATP ditris salt, pyruvate kinase (PK), phosphoenolpyruvate (PEP), NAD^+, NADH, imidazole, N-(2-hydroxyethyl) piperazine-N19-ethanesulfonic acid (HEPES), lactate dehydrogenase (LDH), ouabain, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indole phosphate (BCIP), 4′,6-diamidino-2-phenylindole (DAPI), alamethicin, imidazole, sodium orthovanadate, 3-phosphoglyceraldehyde diethyl acetal, ethacrynic acid, oligomycin, thapsigargin, bafilomycin A_1 were purchased from the Sigma Chemical Company (Saint Louis, USA). Dimethyl sulfoxide (DMSO) and triethanolamine (TEA) were from Merck (Darmstadt, Germany). The protease inhibitor cocktail (1 mmol L^{-1} benzamidine, 5 μmol L^{-1} antipain, 5 μmol L^{-1} leupeptin 1 μmol L^{-1}, pepstatin A and 5 μmol L^{-1} phenyl-methanesulfonyl-fluoride) was from Calbiochem (Darmstadt, Germany). Mouse monoclonal α-5 IgG antibody raised against the α-subunit of chicken (Na^+, K^+)-ATPase was from the Development Studies Hybridoma Bank, maintained by the University of Iowa (Iowa, USA). Antimouse IgG, alkaline phosphatase conjugate was purchased from the Promega Corporation (Madison, USA). Optimal Cutting Temperature Compound was from Sakura Tissue-Tek (Torrance, USA). Alexa-fluor 488-conjugated goat anti-mouse IgG, was from Invitrogen (Carlsbad, USA); fluoromount-G and paraformaldehyde were from Electron Microscopy Sciences (Hatfield, USA). All other reagents were of the highest purity commercially available.

Crystalline suspensions of LDH and PK in 2.9 mol L^{-1} ammonium sulfate (200 μL) were centrifuged at 14,000 rpm for 15 min at 4 °C in an Eppendorf Model 5810 refrigerated centrifuge (Hamburg, Germany). The pellet was resuspended in 500 μL of 50 mmol L^{-1} HEPES buffer, pH 7.5, transferred to a YM-10 Microcon filter (Millipore Corporation, Billerica, USA) and washed five times at 10,000 rpm for 15 min at 4 °C in the same buffer until complete removal of ammonium ions (tested with the Nessler reagent). Finally, the pellet was resuspended to the original volume. For PGK and GAPDH, the suspension was treated as above with 50 mmol L^{-1} triethanolamine buffer, pH 7.5, containing 1 mmol L^{-1} dithiothreitol. Ammonium sulfate-depleted PK, LDH, PGK and GAPDH suspensions were used within 2 days. Glyceraldehyde-3-phosphate (G3P) was prepared by hydrolysis of 3-phosphoglyceraldehyde diethyl acetal, barium salt, with 150 μL HCl ($d = 1.18$ g mL^{-1}) in a boiling water bath for 2 min, after removal of the barium salt with Dowex $50H^+$ resin, as recommended by the manufacturer (see Sigma Chem. Co. Product Information for Product Number G5376). Final pH was adjusted to 7.0 with 50 μL triethanolamine just before use. When necessary, enzyme solutions were concentrated on Amicon Ultra 10 K centrifugal filters (Millipore Corporation, Billerica, USA). The stock solution of ATP was prepared by dissolving ATP di-Tris salt in water and adjusting the pH to 7.0 with triethanolamine ($d = 1.12$ g mL^{-1}). The exact concentration was established from the extinction coefficient ($\varepsilon_{260\ nm,pH\ 7.0} = 15{,}400$ mol L^{-1} cm^{-1}) and adjusted to 100 mmol L^{-1}. Concentrated bafilomycin A1 (200 μmol L^{-1}) and thapsigargin (28 μmol L^{-1}) solutions were prepared in DMSO; oligomycin (100 μg mL^{-1}) and aurovertin (5 mmol L^{-1}) were prepared in ethanol. Sodium orthovanadate solution was prepared according to Gordon (1991).

Shrimps

Adult *X. kroyeri* were caught from Ubatuba Bay (23°26′ S, 45°02′ W), São Paulo State (Brazil) using double rig trawl nets. The shrimps were transported to the laboratory and maintained in tanks containing aerated seawater (33 ‰ salinity, 25 °C) overnight. For each homogenate prepared, 15–30 intermolt specimens of about 10 cm total length were anesthetized by chilling in a freezer (−20 °C) and killed by quickly removing the carapace and appendages and destroying the thoracic ganglion. All gills were rapidly excised and placed in 25 mL ice-cold 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose, 6 mmol L^{-1} EDTA and the protease inhibitor cocktail (homogenization buffer).

Preparation of Microsomal Fractions

The gills were rapidly diced and homogenized in a Potter homogenizer in 20 mmol L^{-1} imidazole homogenization buffer (20 mL buffer/g wet tissue). 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 the homogenization buffer. After further centrifugation as above, the two supernatants were gently pooled and centrifuged at 100,000×g for 90 min at 4 °C. The resulting pellet containing the microsomal fraction was homogenized in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose (15 mL buffer/g wet tissue). Finally, 0.5-mL aliquots were rapidly frozen in liquid nitrogen and stored at −20 °C. No appreciable loss of (Na^+, K^+)-ATPase activity was seen after two-months' storage of the microsomal enzyme prepared as above. When required, the aliquots were thawed, placed on crushed ice and used immediately.

Continuous-Density Sucrose Gradient Centrifugation

An aliquot (1.9 mg) of the ATPase-rich gill microsomal fraction was layered into a 10–50 % (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 a PV50T2 Hitachi vertical rotor. Fractions (0.5 mL) collected from the bottom of the gradient were then assayed for total ATPase activity, ouabain-insensitive ATPase activity (assayed in the presence of 3 mmol L^{-1} ouabain), protein and refractive index.

Measurement of ATP Hydrolysis

Total ATPase activity was assayed at 25 °C using a PK/LDH coupling system (Rudolph et al. 1979) in which ATP hydrolysis was coupled to NADH oxidation according to Leone et al. (2014). The oxidation of NADH was monitored at 340 nm ($\varepsilon_{340\ nm,\ pH\ 7.5} = 6{,}200\ mol^{-1}\ L\ cm^{-1}$) in a Hitachi U-3000 spectrophotometer equipped with thermostatted cell holders. Standard conditions were: 50 mmol L^{-1} HEPES buffer, pH 7.5, 1 mmol L^{-1} ATP, containing 2 mmol L^{-1} $MgCl_2$, 30 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 0.14 mmol L^{-1} NADH, 2 mmol L^{-1} PEP, 82 μg PK (49 U), and 110 μg LDH (94 U) in a final volume of 1 mL.

ATP hydrolysis was also estimated using 3 mmol L^{-1} ouabain to assess ouabain-insensitive activity. The difference in activity measured in the absence (total ATPase activity) or presence of ouabain (ouabain-insensitive activity) represents the (Na^+, K^+)-ATPase activity. Alternatively, for K^+ and NH_4^+, ATPase activity was estimated using a GAPDH/PGK-linked system coupled to the reduction of NAD^+ at 340 nm (Leone et al. 2014). Standard conditions were: 50 mmol L^{-1} TEA buffer, pH 7.5, 1 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1 mmol L^{-1} G3P, 150 μg GAPDH (12 U) and 20 μg PGK (9 U) in a final volume of 1 mL. The two coupling systems gave equivalent results with a difference of less than 10 %.

ATP hydrolysis was also estimated at 25 °C 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. Initial velocities were constant for at least 15 min provided that less than 5 % of the total NADH (or NAD^+) was oxidized (or reduced). The reaction rate for each modulator was estimated in duplicate using identical aliquots from the same preparation. For each microsomal preparation, assay linearity was checked using samples containing 5–50 μg protein; total microsomal protein added to the cuvette always fell well within the linear range of the assay. Neither NADH, PEP, LDH, PK, NAD^+, G3P, PGK nor GAPDH was rate-limiting over the initial course of the assay, and no activity could be measured in the absence of NADH or NAD^+. Mean values were used to fit each corresponding saturation curve, which was repeated three times utilizing different microsomal homogenates ($N = 3$). One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1.0 nmol of ATP per minute, at 25 °C, and (Na^+, K^+)-ATPase specific activity is given as nmol Pi min^{-1} mg^{-1} total protein.

Western Blot Analysis

SDS-PAGE of the gill microsomes from shrimps held in sea water was performed as described by Laemmli (1970) using 4 and 160 μg protein/slot for protein staining and blotting analysis, respectively. After electrophoresis, the gel was split, one half being stained with silver nitrate and the other electro-blotted using a Gibco BRL Mini-V 8–10 system (Gaithersburg, USA) employing a nitrocellulose membrane according to Towbin et al. (1979). The nitrocellulose membrane was blocked for 10 h with 5 % nonfat dry milk powder freshly prepared in 50 mmol L^{-1} Tris. HCl buffer, pH 8.0, containing 150 mmol L^{-1} NaCl and 0.1 % Tween 20, with constant agitation. The membrane was incubated for 30 min at 25 °C in a 1: 10 dilution (2.1 μg mL^{-1}) of the α-5 monoclonal antibody. After washing three times in 50 mmol L^{-1} Tris. HCl buffer, pH 8.0, containing 150 mmol L^{-1} NaCl and 0.1 % Tween 20, the membrane was incubated for 30 min at 25 °C with an anti-mouse IgG, alkaline phosphatase conjugate, diluted 1: 7,500. The membrane was washed three times in 50 mmol L^{-1} Tris. HCl buffer, pH 8.0, containing 150 mmol L^{-1} NaCl and 0.1 % Tween 20, and specific

antibody binding was developed in 100 mmol L^{-1} Tris. HCl buffer, pH 9.5, containing 100 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} NBT and 0.8 mmol L^{-1} BCIP. Controls consisting of membranes incubated with the secondary antibody without previous incubation with the α-5 antibody were included in each experiment. Western blot analysis for each experiment was repeated three times using different gill preparations from separate pools of 15–30 shrimps each. Immunoblots were scanned and imported as JPG files into a commercial software package (Kodak 1D 3.6) where immuno-reaction densities were compared.

Immunofluorescence Microscopy

Fourth, left side gills were dissected and incubated in a fixative solution containing 2.5 % *p*-formaldehyde in a phosphate buffered saline PBS, (10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 290 mOsm kg^{-1} H_2O), pH 7.4, for 1 h, and then embedded in Optimal Cutting Temperature Compound. Thick cryosections (15 µm) were taken transversely to the gill lamellae long-axes using a Micron model HM 505E Cryostatic Microtome (Walldorf, Germany) at −20 °C and collected on gelatin-coated slides (Bloom 225).

Cryosectioning was performed according to França et al. (2013), using a primary α-5 IgG antibody raised against chicken (Na^+, K^+)-ATPase α-subunit, diluted to 21 mg mL^{-1} in PBS (Takeyasu et al. 1988). A goat anti-mouse IgG secondary antibody conjugated with Alexa-fluor 488 was used to provide the fluorescence signal. The sections were observed and photographed using an Olympus BX-50 fluorescence microscope (Olympus America Inc., Melville, USA) equipped with a SPOT RT3 25.4 2 Mb Slider camera (SPOT Imaging Solutions Inc., Sterling Heights, USA) employing phase contrast microscopy and excitation/emission wavelength of 495/519 nm (Alexa-fluor 488) and 358/461 nm (DAPI).

Protein Measurement

Protein concentration was estimated using the Coomassie Blue G dye-binding assay (Read and Northcote 1981) employing bovine serum albumin as the standard.

Estimation of Kinetic Parameters

The kinetic parameters V_M (maximum velocity), $K_{0.5}$ (apparent dissociation constant) and K_M (Michaelis–Menten constant), and the n_H (Hill coefficient) values for ATP hydrolysis under the different assay conditions were calculated using SigrafW software (Leone et al. 2005). The apparent dissociation constant, K_I, of the enzyme-inhibitor complex was estimated as described by Marks and Seeds (1978). The kinetic parameters furnished in the tables are calculated values and represent the mean (±SEM) derived from three different microsomal preparations ($N = 3$). SigrafW software can be freely obtained from http://portal.ffclrp.usp.br/sites/fdaleone/downlods.

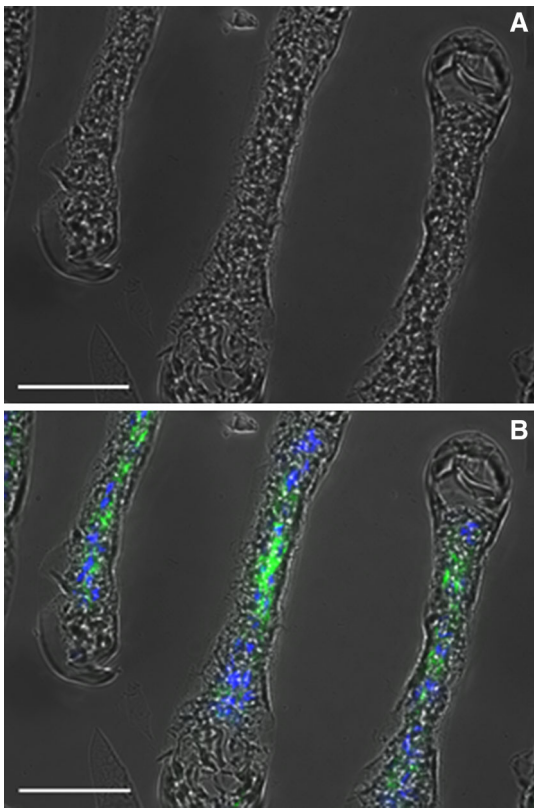

Fig. 1 Immunolocalization of the (Na^+, K^+)-ATPase α-subunit in cryosections of gill lamellae from *Xiphopenaeus kroyeri*. **a** Phase contrast image revealing typical structure of the dendrobranchiate gills with an intralamellar septum extending between the two marginal channels that delimit each gill tip. **b** Immunofluorescence labeling (Alexa-fluor 488; 495/519 nm) showing distribution of the (Na^+, K^+)-ATPase α-subunit (*green*) located predominantly in the intralamellar septum. Nuclei were stained with DAPI (*blue*). *Scale bars* = 50 µm

Results

Transverse sections near the tips of gill lamellae from *X. kroyeri* (Fig. 1a) reveal the lamellar epithelium to consist of an intralamellar septum extending from one marginal channel to the other. A fine epithelium of pillar cell flanges underlies the cuticle. Immunofluorescence labeling reveals the (Na^+, K^+)-ATPase α-subunit to be located predominantly in the intralamellar septum of the gill lamellae (Fig. 1b). Although irregular in distribution, fluorescent signal is concentrated in the central region of

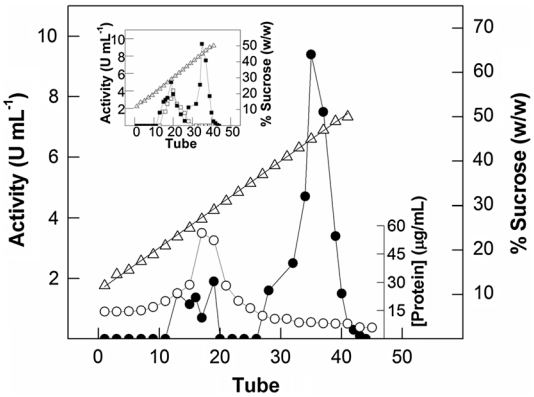

Fig. 2 Sucrose density gradient centrifugation of a microsomal fraction from the gill epithelium of *X. kroyeri*. An aliquot containing 1.9 mg protein was layered into a 10–50 % (w/w) continuous sucrose density gradient. Fractions (0.5 mL) were collected from the bottom of the gradient and analyzed for total ATPase activity (*filled square*); ouabain-insensitive ATPase activity (*open square*); (Na^+, K^+)-ATPase activity (*filled circle*); protein concentration (*open circle*); sucrose concentration (*open triangle*)

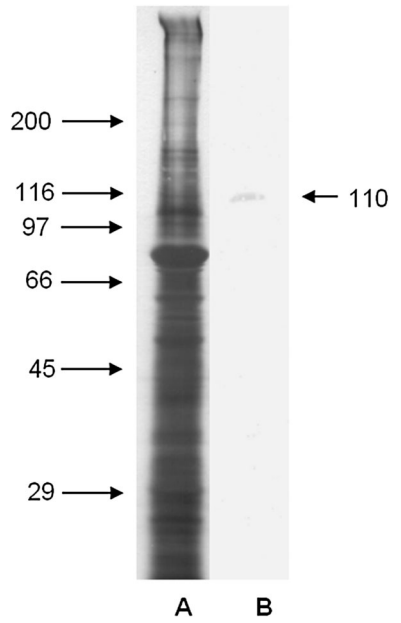

Fig. 3 SDS-PAGE and western blot analysis of the microsomal fraction from the gill epithelium of *X. kroyeri*. Electrophoresis was performed in a 5–20 % polyacrylamide gel using 4 and 160 µg protein/slot for protein staining and blotting analysis, respectively. *Lane A* silver nitrate-stained SDS-PAGE. *Lane B* Western blotting against the α-subunit of the (Na^+, K^+)-ATPase as revealed by an anti-mouse IgG, alkaline phosphatase conjugate

the lamellar transect, declining toward the peripheral marginal channels whose epithelia show very little or no signal.

Sucrose density gradient centrifugation of the gill microsomal preparation (Fig. 2) revealed a single protein peak in the light fractions (15–20 % sucrose), and a second component spread evenly over the heavy fractions (30–40 % sucrose). There are two well-defined peaks showing (Na^+, K^+)-ATPase activity (Fig. 2). The light fraction shows low (Na^+, K^+)-ATPase activity and is highly contaminated by other ATP hydrolyzing enzymes. The heavy fraction exhibits significant (Na^+, K^+)-ATPase activity and very low levels of other ATP hydrolyzing enzymes (inset to Fig. 2). Protein recovery from the gradient was greater than 90 %, and no significant loss of (Na^+, K^+)-ATPase activity was seen when the microsomal fraction was maintained at 4 °C for up to 6 h (data not shown).

Western blot analysis identified a single immunoreactive band of the α-5 monoclonal antibody against the α-subunit of the (Na^+, K^+)-ATPase with an Mr of ≈110 kDa, suggesting the presence of a single α-subunit isoform (Fig. 3). The data also show that the (Na^+, K^+)-ATPase represents just a small proportion of the total protein content in the gill microsomal fraction from *X. kroyeri*.

The effect of increasing ATP concentrations on (Na^+, K^+)-ATPase activity of the gill microsomal preparation is shown in Fig. 4. Under saturating K^+ (10 mmol L^{-1}), Na^+ (30 mmol L^{-1}) and Mg^{2+} (2 mmol L^{-1}) concentrations, ATP hydrolysis follows a well-defined saturation curve in the range of 10^{-7}–10^{-3} mol L^{-1} ATP, obeying Michaelis–Menten kinetics, with $V_M = 109.5 \pm 3.2$ nmol Pi min^{-1} mg^{-1} and $K_M = 0.03 \pm 0.003$ mmol L^{-1} (Table 1). At ATP concentrations as low as 10^{-7} mol L^{-1}, an ATPase

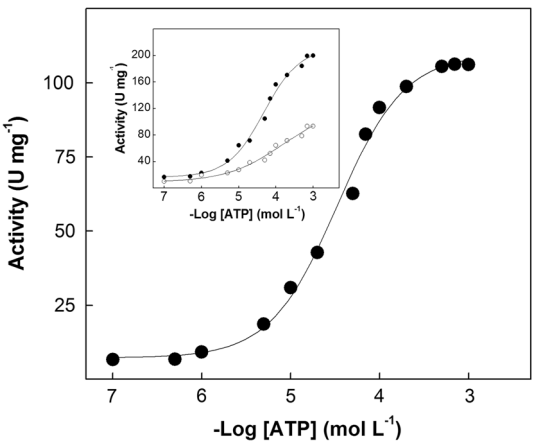

Fig. 4 Effect of ATP concentration on (Na^+, K^+)-ATPase activity in the microsomal fraction from the gill epithelium of *X kroyeri*. Activity was assayed continuously at 25 °C using 7.4 µg protein in 50 mmol L^{-1} HEPES buffer, pH 7.5, as described in the "Materials and Methods" section. A representative curve obtained from one homogenate is given. The experiment was performed using duplicate enzyme aliquots from three ($N = 3$) different gill microsomal fractions. *Inset* Ouabain-insensitive ATPase activity (*open circle*) Total ATPase activity (*filled circle*)

activity of ≈10 nmol Pi min^{-1} mg^{-1} was measured independently of the presence of ouabain. Ouabain-insensitive ATPase activity is stimulated up to 95 nmol Pi min^{-1} mg^{-1}

Table 1 Kinetic parameters for the stimulation by ATP, Mg^{2+}, Na^+, K^+, and NH_4^+ and inhibition by ouabain of (Na^+, K^+)-ATPase activity in a gill microsomal fraction from *Xiphopenaeus kroyeri*

Modulator	V_M (nmol Pi min^{-1} mg^{-1})	K_M (or $K_{0.5}$) (mmol L^{-1})	n_H	K_I (μmol L^{-1})	V_M/K_M ($\times 10^{-6}$)
ATP	109.5 ± 3.2	0.03 ± 0.003	1.0	–	3,650
Mg^{2+}	109.8 ± 2.1	0.60 ± 0.03	2.2	–	183
Na^+	117.6 ± 3.5	5.36 ± 0.14	1.2	–	22
K^+	112.9 ± 1.4	1.32 ± 0.08	0.6	–	85
NH_4^+	200.8 ± 7.1	2.70 ± 0.04	1.3	–	74
Ouabain	–	–	–	84.0 ± 2.1	–
				28.4 ± 0.7 (NH_4^+)	
Orthovanadate	–	–	–	0.157 ± 0.001	–

Assays were performed in 50 mmol L^{-1} HEPES buffer, pH 7.5, using 7.4 μg protein in a final volume of 1 mL, as described in "Materials and Methods" section. Data are the mean ± SD ($N = 3$)

over the same ATP concentration range, which represents around 48 % of total ATPase activity (inset to Fig. 4), suggesting the presence of ATPases other than (Na^+, K^+)-ATPase. Above 1 mmol L^{-1}, (Na^+, K^+)-ATPase activity is significantly inhibited by excess free ATP (not shown). Alamethicin has no effect on (Na^+, K^+)-ATPase activity, suggesting that ATP has free access to the enzyme.

The modulation by Mg^{2+} and Na^+ of (Na^+, K^+)-ATPase activity is shown in Fig. 5. Magnesium ions are essential for (Na^+, K^+)-ATPase activity of the gill microsomal fraction of *X. kroyeri* (Fig. 5a). Under saturating ATP (1 mmol L^{-1}), Na^+ (30 mmol L^{-1}) and K^+ (10 mmol L^{-1}) concentrations, increasing Mg^{2+} concentrations from 10^{-5} to 3×10^{-3} mol L^{-1} stimulated (Na^+, K^+)-ATPase activity up to 109.8 ± 2.1 nmol Pi min^{-1} mg^{-1} with a $K_{0.5}$ = 0.60 ± 0.03 mmol L^{-1} (Table 1). Cooperative effects ($n_H = 2.2$) resulting from Mg^{2+} interaction with the enzyme suggest multiple Mg^{2+} binding sites. No significant (Na^+, K^+)-ATPase activity was seen at Mg^{2+} concentrations as low as 10^{-5} mol L^{-1}. However, ouabain-insensitive ATPase activity, corresponding to ≈48 % of total ATPase activity (≈98 nmol Pi min^{-1} mg^{-1}), was estimated over the same Mg^{2+} concentration range (inset to Fig. 5a) and corroborates the presence of ATPases other than (Na^+, K^+)-ATPase. Above 10 mmol L^{-1} (Na^+, K^+)-ATPase activity was significantly inhibited by excess free Mg^{2+} (not shown).

Under saturating ATP (1 mmol L^{-1}), K^+ (10 mmol L^{-1}) and Mg^{2+} (2 mmol L^{-1}) concentrations, the Na^+-dependence of (Na^+, K^+)-ATPase activity (Fig. 5b) was characterized by positive cooperativity ($n = 1.2$) with V = 117.6 ± 3.5 nmol Pi min^{-1} mg^{-1} and $K_{0.5} = 5.36 \pm 0.14$ mmol L^{-1} over the range from 10^{-5} to 5×10^{-2} mol L^{-1} (Table 1). Ouabain-insensitive activity was stimulated from ≈80 to ≈95 nmol Pi min^{-1} mg^{-1} over the same Na^+ concentration range (inset to Fig. 5b), suggesting the presence of a Na^+-stimulated ATPase in the microsomal preparation.

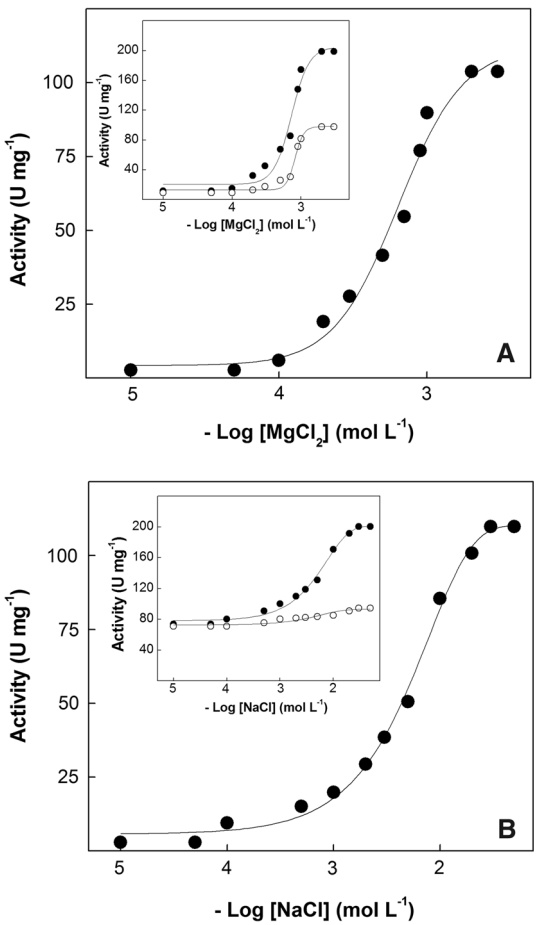

Fig. 5 Effect of Mg^{2+} and Na^+ concentration on (Na^+, K^+)-ATPase activity in the microsomal fraction from the gill epithelium of *X. kroyeri*. Activity was assayed continuously at 25 °C using 7.4 μg protein in 50 mmol L^{-1} HEPES buffer, pH 7.5, as described in the "Materials and Methods" section. A representative curve obtained from one homogenate is given. The experiment was performed using duplicate enzyme aliquots from three ($N = 3$) different gill microsomal fractions. **a** Mg^{2+}. **b** Na^+. *Insets* Ouabain-insensitive ATPase activity (*open circle*) Total ATPase activity (*filled circle*)

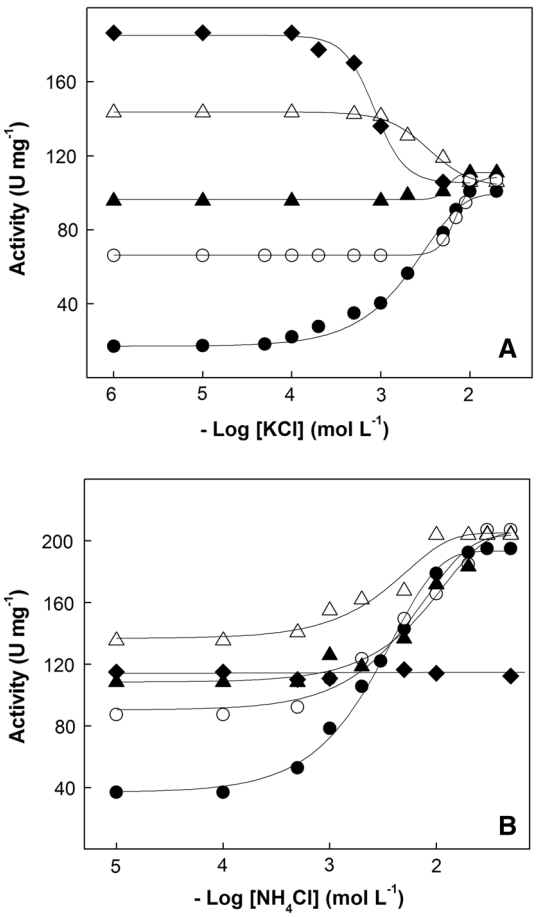

Fig. 6 Effect of potassium plus ammonium ions on (Na^+, K^+)-ATPase activity in the microsomal fraction from the gill epithelium of *X. kroyeri*. Activity was assayed continuously at 25 °C using 7.4 µg protein in 50 mmol L^{-1} TEA buffer, pH 7.5, 1 mmol L^{-1} NAD^+, 0.5 mmol L^{-1} sodium phosphate, 1 mmol L^{-1} G3P, 150 µg GAPDH (12 U) and 20 µg PGK (9 U) in a final volume of 1 mL. Representative curves obtained from one homogenate are given. The experiment was performed using duplicate enzyme aliquots from three ($N = 3$) different gill microsomal fractions **a** (*filled circle*) No NH_4^+; (*open circle*) 5 mmol L^{-1} NH_4^+; (*filled triangle*) 10 mmol L^{-1} NH_4^+; (*open triangle*) 20 mmol L^{-1} NH_4^+; (*closed diamond*) 50 mmol L^{-1} NH_4^+. **b** (*filled circle*) No K^+; (*open circle*) 0.5 mmol L^{-1} K^+; (*filled triangle*) 1 mmol L^{-1} K^+; (*open triangle*) 5 mmol L^{-1} K^+; (*filled diamond*) 10 mmol L^{-1} K^+

The effect of NH_4^+ on K^+-stimulated (Na^+, K^+)-ATPase activity of *X. kroyeri* is shown in Fig. 6a. Under saturating ATP (1 mmol L^{-1}), Na^+ (30 mmol L^{-1}) and Mg^{2+} (2 mmol L^{-1}) concentrations, and without NH_4^+, stimulation of (Na^+, K^+)-ATPase activity by K^+ (from 10^{-6} to 2×10^{-2} mol L^{-1}) reached a maximum rate of 112.9 ± 1.4 nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 1.32 \pm 0.08$ mmol L^{-1}, obeying cooperative kinetics (Table 1). (Na^+, K^+)-ATPase activity of ≈20 nmol Pi min^{-1} mg^{-1} was seen at K^+ concentrations as low as 10^{-6} mol L^{-1}, and ouabain-insensitive ATPase activity was not stimulated over the same concentration range, suggesting the absence of a K^+-stimulated ATPase in the microsomal fraction (not shown). Notable responses were revealed when ATPase activity was assayed in the presence of both K^+ plus NH_4^+. Modulation by K^+ in the presence of low fixed NH_4^+ concentrations (5 and 10 mmol L^{-1}) stimulated (Na^+, K^+)-ATPase activity ≈fivefold (from ≈20 to ≈110 nmol Pi min^{-1} mg^{-1}) as K^+ concentration increased from 10^{-6} to 5×10^{-2} mol L^{-1}. No synergistic effect was seen under such ionic conditions. However, for the higher NH_4^+ concentrations (20 and 50 mmol L^{-1}), further addition of K^+ (10^{-6} to 5×10^{-2} mol L^{-1}) inhibited (Na^+, K^+)-ATPase activity. Activity decreased from ≈200 nmol Pi min^{-1} mg^{-1} (with 50 mmol L^{-1} NH_4^+ and 10^{-6} mol L^{-1} K^+) and ≈140 nmol Pi min^{-1} mg^{-1} (with 20 mmol L^{-1} NH_4^+ and 10^{-6} mol L^{-1} K^+) to ≈110 nmol Pi min^{-1} mg^{-1} (Table 2). This inhibitory effect suggests that even when fully saturated by NH_4^+, increasing K^+ concentrations displace NH_4^+ from the K^+ binding sites.

The effect of K^+ on NH_4^+-stimulated (Na^+, K^+)-ATPase activity is shown in Fig. 6b. Under saturating ATP (1 mmol L^{-1}), Na^+ (30 mmol L^{-1}) and Mg^{2+} (2 mmol L^{-1}) concentrations, and without K^+, (Na^+, K^+)-ATPase activity was stimulated by NH_4^+ (from 10^{-6} to 7×10^{-3} mol L^{-1}) to maximum values of 200.8 ± 7.1 nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 2.70 \pm 0.04$ mmol L^{-1}, obeying cooperative kinetics (Table 1). A residual (Na^+, K^+)-ATPase activity of ≈40 nmol Pi min^{-1} mg^{-1} was observed for NH_4^+ concentrations as low as 10^{-6} mol L^{-1}, but ouabain-insensitive ATPase activity was not stimulated over the same concentration range, suggesting the absence of an NH_4^+-stimulated ATPase in the microsomal fraction (not shown). (Na^+, K^+)-ATPase activity assayed in the presence of fixed K^+ concentrations (0.5–5 mmol L^{-1}) also attained maximum values of around 200 nmol Pi min^{-1} mg^{-1}. Cooperative kinetics was seen in the presence of both ions, and there were no relevant changes in $K_{0.5}$ values when the enzyme is fully saturated by both ions (Table 2). No synergistic effects were found in the presence of NH_4^+ plus K^+. However, the kinetic response to 10 mmol L^{-1} NH_4^+ is remarkable. (Na^+, K^+)-ATPase activity remaining unchanged at ≈110 nmol Pi min^{-1} mg^{-1} over the same K^+ concentration range. Likely, as enzyme becomes fully saturated by K^+ (10 mmol L^{-1}), NH_4^+ does not displace K^+ from its binding site. Table 2 summarizes the values of the kinetic parameters estimated for the stimulation by K^+ plus NH_4^+ of (Na^+, K^+)-ATPase activity from the gill epithelium of *X. kroyeri*.

The effect of a wide range of ouabain and orthovanadate concentrations on total ATPase activity in the microsomal fraction from *X. kroyeri* gills is shown in Fig. 7. Using 1 mmol L^{-1} ATP, 2 mmol L^{-1} Mg^{2+}, 30 mmol L^{-1} Na^+, and 10 mmol L^{-1} K^+, increasing ouabain concentrations up to 7×10^{-3} mol L^{-1} inhibited total ATPase activity by

Table 2 Kinetic parameters for the stimulation by both K^+ and NH_4^+ of (Na^+, K^+)-ATPase activity in a gill microsomal fraction from *Xiphopenaeus kroyeri*

[K^+] (mmol L^{-1})	[NH_4^+] (mmol L^{-1})	V_M (nmol Pi min^{-1} mg^{-1})	$K_{0.5}$ (mmol L^{-1})	n_H	$V_M/K_{0.5}$ ($\times 10^{-6}$)
Variable	0	112.9 ± 1.4	1.32 ± 0.08	0.6	85
Variable	5	108.3 ± 2.2	7.00 ± 0.14	4.8	15
Variable	10	110.8 ± 1.2	–	–	–
Variable	20	109.9 ± 1.5	–	–	–
Variable	50	112.7 ± 1.7	–	–	–
0	Variable	200.8 ± 6.1	2.70 ± 0.04	1.3	74
0.5	Variable	203.2 ± 5.4	6.20 ± 0.08	1.0	33
1	Variable	210.7 ± 6.8	7.11 ± 0.15	1.3	30
5	Variable	206.9 ± 5.3	2.45 ± 0.06	1.5	84
10	Variable	111.3 ± 3.1	–	–	–

Assays were performed in 50 mmol L^{-1} TEA buffer, pH 7.5, using 7.4 µg protein in a final volume of 1 mL, as described in "Materials and Methods" section. Data are the mean ± SD ($N = 3$)

≈50 % obeying a single ouabain-binding site model (Fig. 7a), with $K_I = 84.0 \pm 2.1$ µmol L^{-1} (inset to Fig. 7a and Table 1). With 50 mol L^{-1} NH_4^+, inhibition increased to ≈80 %, and $K_I = 28.4 \pm 0.7$ µmol L^{-1} was threefold lower compared to that estimated in the absence of NH_4^+ (Table 1). Low orthovanadate concentrations (≈10^{-7} mol L^{-1}) did not affect microsomal ATPase activity, but increasing concentrations up to 10^{-4} mol L^{-1} resulted in ≈40 % inhibition (79.6 ± 2.4 nmol Pi min^{-1} mg^{-1}) ATPase activity (Fig. 7b). The calculated K_I for orthovanadate inhibition was 0.157 ± 0.001 µmol L^{-1} (inset to Fig. 7b and Table 1).

(Na^+, K^+)-ATPase activity represents ≈50 % of the total ATP hydrolyzing activity present in the gill epithelium. The relative proportions of putative ATPases other than (Na^+, K^+)-ATPase accounting for the ouabain-insensitive activity in *X. kroyeri* gill microsomes are given in Table 3. The lack of an additional inhibitory effect by ouabain plus theophylline suggests a minor contribution by neutral phosphatases to the ouabain-insensitive ATPase activity. In contrast, the additional inhibition estimated with ouabain plus aurovertin B and ouabain plus Bafilomycin A_1 suggests the presence of ≈22 % mitochondrial F_0F_1-ATPase and ≈14 % $V(H^+)$-ATPase activity, respectively. The substantial inhibition by ouabain plus ethacrynic acid, corroborated by the stimulation by Na^+ of the ouabain-insensitive ATPase activity (see inset to Fig. 5b) is a strong indication of Na^+-stimulated ATPase activity (≈10 %). Finally, inhibition by ouabain plus thapsigargin of ouabain-insensitive ATPase activity suggests some Ca^{2+}-ATPase activity (≈6 %).

Discussion

This systematic characterization of the gill (Na^+, K^+)-ATPase from *X. kroyeri* discloses two important findings. Firstly, K^+ (or NH_4^+) modulates stimulation of the (Na^+, K^+)-ATPase by NH_4^+ (or K^+) without synergistic stimulation of enzyme activity. Secondly, modulation of the enzyme by NH_4^+ and K^+ together shows that both ions bind to the K^+ binding site, NH_4^+ being displaced at increasing K^+ concentrations.

Characterization of the Gill Microsomal Fraction

The two peaks of (Na^+, K^+)-ATPase activity appearing at different sucrose densities suggest that the corresponding light and heavy membrane fractions originate from different regions of the gill epithelium (Furriel et al. 2010; Lucena et al. 2012), possibly the intralamellar septum and pillar cells (Freire and McNamara 1995). Two such peaks occur in gill microsomal fractions from the freshwater crab *Dilocarcinus pagei* (Furriel et al. 2010), salinity-acclimated blue crab *Callinectes ornatus* (Garçon et al. 2009), high salinity (45 ‰ S)-acclimated hermit crab *Clibanarius vittatus* (Lucena et al. 2012), and the freshwater shrimp *Macrobrachium rosenbergii* (França et al. 2013). In contrast, only a single peak appears in the fractions from *M. olfersi* and *M. amazonicum* (Furriel et al. 2000; Santos et al. 2007; Leone et al. 2014) and fresh-caught *C. vittatus* (Gonçalves et al. 2006) and *C. ornatus* (Garçon et al. 2007).

The Mr of ≈110 kDa estimated for the *X. kroyeri* gill (Na^+, K^+)-ATPase is similar to other decapods (Furriel et al. 2000; Donnet et al. 2001; Masui et al. 2002, 2005; Lucu and Towle 2003; Belli et al. 2009; Garçon et al. 2007, 2009; Lucena et al. 2012; França et al. 2013; Leone et al. 2014). The sole immunoreactive band revealed by Western blotting suggests the presence of a single α-subunit isoform typical of various crustacean species (Furriel et al. 2000; Masui et al. 2002, 2005; Lucu and Towle 2003; Belli et al. 2009; Garçon et al. 2007, 2009; Lucena et al. 2012; França et al. 2013; Leone et al. 2014). This is corroborated by the single titration curve for ouabain inhibition, but differs from the biphasic inhibition curve for *D. pagei*, for

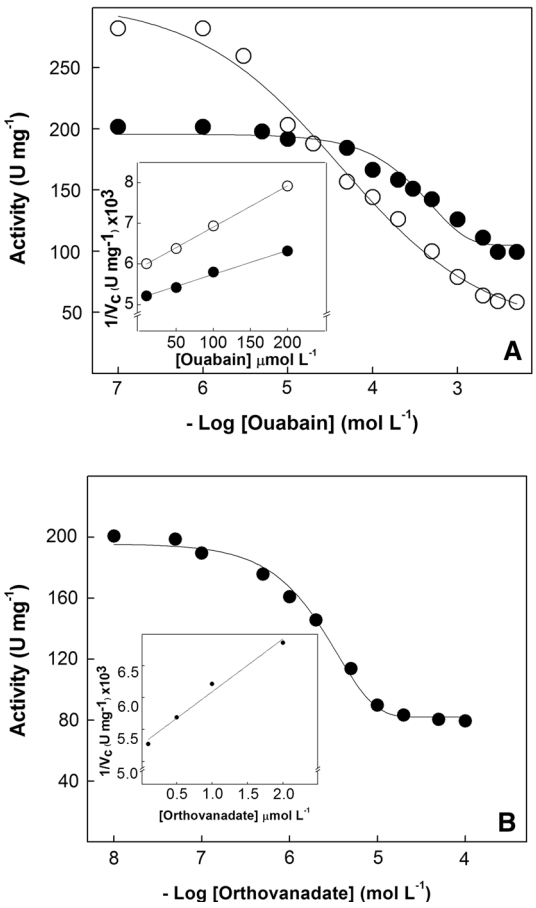

Fig. 7 Effect of ouabain and orthovanadate on (Na^+, K^+)-ATPase activity in the microsomal fraction from the gill epithelium of *X. Kroyeri*. Activity was assayed continuously at 25 °C using 7.4 µg protein in 50 mmol L^{-1} HEPES buffer, pH 7.5, containing 1 mmol L^{-1} ATP, 2 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl, 30 mmol L^{-1} NaCl, 0.14 mmol L^{-1} NADH, 2 mmol L^{-1} PEP, 82 µg PK (49 U) and 110 µg LDH (94 U). A representative curve obtained from one homogenate is given. The experiment was performed using duplicate enzyme aliquots from three ($N = 3$) different gill microsomal fractions. **a** ouabain: (*open cirlce*) without NH_4^+; (*filled circle*) with 50 mol L^{-1} NH_4^+. **b** Orthovanadate. *Insets* Dixon plot for estimation of K_I in which v_c is the reaction rate corresponding to (Na^+, K^+)-ATPase activity

example (Furriel et al. 2010). Immunolocalization reveals the enzyme to be distributed predominantly throughout the intralamellar septum of the gill lamellae as seen in *M. rosenbergii* (França et al. 2013) and *M. amazonicum* (Boudour-Boucheker et al. 2014).

Modulation by ATP of the Gill (Na^+, K^+)-ATPase

ATP hydrolysis by *X. kroyeri* (Na^+, K^+)-ATPase (109.5 ± 3.2 nmol Pi min^{-1} mg^{-1}) is similar to the gill enzyme from various brachyuran crabs acclimated to seawater (Lucu et al. 2000; Corotto and Holliday 1996; Holliday 1985; Lucu and Flik 1999; Masui et al. 2002; Lovett and Watts 1995; Piller et al. 1995; Lucu et al. 2000) but is considerably lower than for freshwater crustaceans (Furriel et al. 2000; Santos et al. 2007; Leone et al. 2012) and in blue crab *C. danae* (Masui et al. 2002). This may be a characteristic of marine species in general, owing to their elevated ion and water permeabilities and lesser osmoregulatory capacity (Péqueux 1995; Corotto and Holliday 1996) or may be a species-specific characteristic of the *X. kroyeri* (Na^+, K^+)-ATPase in particular. While two families of ATP binding sites are present in *C. danae* (Masui et al. 2002), *C. vittatus* (Gonçalves et al. 2006), and *M. amazonicum* (Santos et al. 2007), hydrolysis of ATP by the *X. kroyeri* (Na^+, K^+)-ATPase revealed only a single family of sites ($K_M = 0.03 \pm 0.003$ mmol L^{-1}) as seen in *C. ornatus* (Garçon et al. 2007; 2009), *Cancer pagurus* (Gache et al. 1976), *M. olfersi* (Furriel et al. 2000), *M. rosenbergii* (Wilder et al. 2000; França et al. 2013), *C. sapidus* (Wheatly and Henry 1987) and some euryhaline brachyuran crabs (Holliday 1985; D'Orazio and Holliday 1985; Corotto and Holliday 1996). However, this K_M value is ≈tenfold lower than in euryhaline brachyurans (Holliday 1985; D'Orazio and Holliday 1985; Corotto and Holliday 1996). The affinity of the (Na^+, K^+)-ATPase for ATP seems to be independent of both species' biotope and tissue. These K_M values are similar to those for the low-affinity sites of the vertebrate enzyme (Glynn 1985; Ward and Cavieres 1998).

Modulation by Mg^{2+} and Na^+ of the Gill (Na^+, K^+)-ATPase

The interaction between the (Na^+, K^+)-ATPase and Mg^{2+} is difficult to interpret under physiological conditions since Mg^{2+} is both a ligand and substrate (Mg-ATP), and the enzyme is inhibited by excess Mg^{2+} (Glynn 1985; Karlish 2003). Such inhibition may result from binding to a second inhibitory site on the enzyme (Pedemonte and Beaugé 1983). The (Na^+, K^+)-ATPase affinity for Mg^{2+} is not dependent on habitat salinity: the affinity of *X. kroyeri* enzyme for Mg^{2+} is similar to that of both marine and freshwater crustaceans like *C. vittatus* (Gonçalves et al. 2006; Lucena et al. 2012), *C. danae* (Masui et al. 2002; 2009), *C. ornatus* (Garçon et al. 2007; 2009), *M. amazonicum* (Santos et al. 2007; Leone et al. 2012), *M. rosenbergii* (França et al. 2013), *M. olfersi* (Furriel et al. 2000), and *D. pagei* (Furriel et al. 2010). Further, as seen in gill enzymes from *C. danae* (Masui et al. 2002), *C. ornatus* (Garçon et al. 2007; 2009) and *C. vittatus* (Gonçalves et al. 2006; Lucena et al. 2012) stimulation of hydrolysis is cooperative, suggesting multiple Mg^{2+} binding sites.

Each (Na^+, K^+)-ATPase α-subunit isoform may have a different apparent affinity for Na^+ and K^+ when using ATP as a substrate (Blanco and Mercer 1998; Crambert et al.

Table 3 Effect of various inhibitors on ATPase activity in a gill microsomal fraction from *Xiphopenaeus kroyeri*

Inhibitor	V_M (nmol Pi min^{-1} mg^{-1})	% V_M	Specific ATPase activity (%)	ATPase type likely present
Control	197.9 ± 9.5	100	–	
Ouabain (3 mmol L^{-1})	98.7 ± 2.5	49.8	50.1	Na^+, K^+
Orthovanadate (50 µmol L^{-1})	87.6 ± 1.9	44.3	5.6	–
Ouabain (3 mmol L^{-1}) + Orthovanadate (50 µmol L^{-1})	86.0 ± 1.2	43.4	6.4	P
Ouabain (3 mmol L^{-1}) + Ethanol (20 µL mL^{-1})	99.8 ± 1.9	50.4	–	–
Ouabain (3 mmol L^{-1}) + DMSO (20 µg mL^{-1})	98.2 ± 1.6	49.6	–	–
Ouabain (3 mmol L^{-1}) + Aurovertin B (10 µmol L^{-1})	55.8 ± 0.3	28.2	21.7	F_0F_1
Ouabain (3 mmol L^{-1}) + Bafilomycin A_1 (0.4 µmol L^{-1})	70.2 ± 1.1	35.5	14.4	$V(H^+)$
Ouabain (3 mmol L^{-1}) + Ethacrynic acid (2 mmol L^{-1})	80.3 ± 1.3	40.6	9.3	Na^+ or K^+
Ouabain (3 mmol L^{-1}) + Theophylline (5 mmol L^{-1})	97.1 ± 2.3	49.1	≈0	Neutral phosphatases
Ouabain (3 mmol L^{-1}) + Thapsigargin (0.5 µmol L^{-1})	86.9 ± 1.6	43.9	6.0	Ca^{2+}

Assays were performed in 50 mmol L^{-1} HEPES buffer, pH 7.5, using 7.4 µg protein in a final volume of 1 mL, as described in "Materials and Methods" section. Data are the mean ± SD ($N = 3$)

2000; Blanco et al. 2005). Although a striking correlation exists between apparent affinity for Na^+ and habitat (Péqueux 1995), affinities are unaffected by acclimation in fresh-caught, 21 ‰S- and 33 ‰S-acclimated *C. ornatus* (Garçon et al. 2007, 2009) and fresh-caught and 15 ‰ S-acclimated *C. danae* (Masui et al. 2002, 2009). In fact, specific activity rather than apparent affinity may be the most reliable parameter for predicting the osmoregulatory ability of a given species in a particular medium (Péqueux 1995). The apparent affinity for Na^+ ($K_{0.5} = 5.36 \pm 0.014$ mmol L^{-1}) of the *X. kroyeri* (Na^+, K^+)-ATPase is similar to *C. danae* (Masui et al. 2002), *C. ornatus* (Garçon et al. 2007; 2009), *C. vittatus* (Gonçalves et al. 2006), *M. amazonicum* (Santos et al. 2007; Leone et al. 2012), and *D. pagei* (Furriel et al. 2010), but is about threefold less than for *M. rosenbergii* (França et al. 2013) and 45 ‰ S-acclimated *C. vittatus* (Lucena et al. 2012). Further, the cooperative kinetics seen for the *X. kroyeri* enzyme are similar to *C. danae* (Masui et al. 2002), *C. ornatus* (Garçon et al. 2007, 2009), *D. pagei* (Furriel et al. 2010), *M. amazonicum* (Leone et al. 2012), *M. rosenbergii* (França et al. 2013) and *M. olfersi* (Furriel et al. 2000) but contrast with the Michaelis–Menten behavior seen for *C. vittatus* (Gonçalves et al. 2006; Lucena et al. 2012).

Modulation by K^+ and NH_4^+ of the Gill (Na^+, K^+)-ATPase

The apparent affinity of the *X. kroyeri* (Na^+, K^+)-ATPase for K^+ ($K_{0.5} = 1.32 \pm 0.082$ mmol L^{-1}) is similar to marine species like *C. vittatus* (Gonçalves et al. 2006), *C. danae* (Masui et al. 2002) and *C. ornatus* (Garçon et al. 2007; Garçon et al. 2009) but is about twofold less than in 45 ‰S-acclimated *C. vittatus* (Lucena et al. 2012) and freshwater crab *D. pagei* (Furriel et al. 2010), and roughly twofold greater than in the freshwater shrimps *M. amazonicum* (Leone et al. 2012), *M. rosenbergii* (França et al. 2013), and *M. olfersi* (Furriel et al. 2000). The negative cooperativity ($n_H = 0.6$) seen for stimulation by K^+ without NH_4^+ contrasts with the Michaelis–Menten behavior of the enzyme from *M. olfersi* (Furriel et al. 2000), *M. amazonicum* (Leone et al. 2012), *M. rosenbergii* (França et al. 2013), and *C. vittatus* (Gonçalves et al. 2006), and with the positive cooperativity for *C. danae* (Masui et al. 2002; Masui et al. 2009), *C. ornatus* (Garçon et al. 2007; 2009) and *D. pagei* (Furriel et al. 2010) gill enzymes.

NH_4^+ can substitute for K^+ in sustaining ATP hydrolysis by the crustacean gill (Na^+, K^+)-ATPase (Holliday 1985; Weihrauch et al. 2004; Masui et al. 2002; Furriel et al. 2004; Lucena et al. 2012; Leone et al. 2014). NH_4^+ stimulated activity up to values ≈50 % greater than did K^+, as also seen in vertebrate (Robinson 1970; Wall 1996) and other crustacean gill enzymes, although with a lower affinity (Holliday 1985; Masui et al. 2002; Furriel et al. 2004; Gonçalves et al. 2006; Garçon et al. 2007; Santos et al. 2007; Furriel et al. 2010; Lucena et al. 2012; França et al. 2013; Leone et al. 2014). Except for the freshwater crab *D. pagei* (Furriel et al. 2010), the synergistic stimulation by K^+ and NH_4^+ of the crustacean gill (Na^+, K^+)-ATPase, first described in *C. danae* (Masui et al. 2002, 2005), appears to be a phenomenon widespread among the decapod Crustacea (Furriel et al. 2004; Gonçalves et al. 2006; Garçon et al. 2007, 2009; Santos et al. 2007; Lucena et al. 2012; França et al. 2013; Leone et al. 2014). The absence of a synergistic effect induced by K^+ plus NH_4^+ in *X. kroyeri* suggests that both ions compete for the same site on the enzyme molecule.

Like K^+, NH_4^+ can be actively transported by the vertebrate (Na^+, K^+)-ATPase (Wall 1996). Organic cations

such as acetamidinium and formamidinium can act as K^+ surrogates in the (Na^+, K^+)-ATPase cycle and are transported in exchange for Na^+ (Ratheal et al. 2010). However, active ammonia excretion in the crab *Cancer pagurus* is completely inhibited by ouabain, a specific inhibitor of the (Na^+, K^+)-ATPase; this suggests that NH_4^+ is likely transported as a K^+ congener (Weihrauch et al. 1999). The 80 % increase in ouabain inhibition of the (Na^+, K^+)-ATPase in the presence of NH_4^+, reflected in a threefold lower K_I, also suggests that NH_4^+ is likely transported as a K^+ congener. The significantly higher affinity for ouabain in the presence of NH_4^+ compared to K^+ also suggests that NH_4^+ facilitates E_1–E_2 conversion toward to E_2. Discrepantly, K^+ binding, which facilitates dephosphorylation to E_2P, is antagonistic to ouabain binding that prevents ion transport (Crambert al. 2004; Nesher et al. 2007). Likely, the significantly lower rate of E_2P dephosphorylation in the presence of NH_4^+ compared to K^+ is sufficient maintain the enzyme cycle.

With few exceptions, crustaceans are ammoniotelic (Weihrauch et al. 1998, 1999). Most ammonia excretion occurs via the gills (Weihrauch et al. 1999); less than 2 % is eliminated via the urine in *C. sapidus* (Cameron and Batterton 1978). Much of the ammonia content is excreted as NH_4^+ (Weihrauch et al. 1998, 1999), and the sensitivity of active transepithelial NH_4^+ ion fluxes to basolateral ouabain strongly suggests involvement of the (Na^+, K^+)-ATPase (Weihrauch et al. 1998, 1999; Lucu et al. 1989). According to Weihrauch et al. (1998), the basolateral (Na^+, K^+)-ATPase participates directly in NH_4^+ translocation from the hemolymph into the gill epithelial cells where it can be counter exchanged for Na^+ via an apical Na^+/NH_4^+ antiporter, contributing to Na^+ uptake (Mangum and Towle 1977; Armstrong et al. 1981; Weihrauch et al. 1999).

Ambient ammonia in the aquatic environment is usually low as a consequence of bacterial nitrification of ammonia to nitrite and nitrate followed by absorption by autotrophs (Weihrauch et al. 1999). In unpolluted, oxygenated sea water, NH_4^+ concentrations rarely exceed 5 μmol L^{-1} (Koroleff 1983) in contrast to hemolymph concentrations of approximately 100 μmol L^{-1} in various brachyuran species adapted to different salinities (Weihrauch et al. 1999). Body surface permeabilities can play a key role in cell NH_4^+ titers. In seawater, *C. pagurus* gills exhibit high permeabilities and constitute little or no barrier to NH_4^+ influx (Weihrauch et al. 1999). Active ammonia excretion by *C. pagurus* is twofold greater than in the diadromous crab *Eriocher sinensis*, reflecting its leaky epithelium, suggesting that an efficient mechanism of active ammonia excretion compensates for ammonia influx in marine crustaceans (Weihrauch et al. 1999). In *X. kroyeri*, the increased V_M in the presence of NH_4^+ may guarantee appropriate cytosolic NH_4^+ concentrations, constituting a rapid response underlying the excretion of accumulated NH_4^+.

Effect of Inhibitors on Gill (Na^+, K^+)-ATPase Activity

K_I values for ouabain inhibition of the *X. kroyeri* (Na^+, K^+)-ATPase (28.4 ± 2.1 and 84 ± 0.7 μmol L^{-1} with and without NH_4^+, respectively) lie within the range known for various crustaceans (D'Orazio and Holliday 1985; Holliday 1985; Lucu 1990; Corotto and Holliday 1996; Masui et al. 2002; Gonçalves et al. 2006; Garçon et al. 2007, 2009; Furriel et al. 2010; Lucena et al. 2012; Leone et al. 2012; França et al. 2013). The threefold lower K_I estimated with NH_4^+ may derive from the fact that this ion apparently shifts the conformational E_1–E_2 equilibrium to the E_2 conformation. Thus, the stable orthovanadate trigonal bipiramidal structure might compete with the stable transitional phosphate analog apparently producing a stable intermediate with the E_2 conformation causing inhibition (Pick 1982). Yoda and Yoda (1982) suggest that different phosphorylated intermediates occurring between E_1P and E_2P and the E_2P phosphorylated enzyme subconformations (Fedosova et al. 1998) might explain such alterations in K_I values.

The residual ≈50 % ATPase activity found with 3 mM ouabain suggests that the (Na^+, K^+)-ATPase represents only half the total ATPase activity of the microsomal preparation from the *X. kroyeri* gill epithelium (see Table 3). Further, the additional 6 % inhibition seen with 3 mmol L^{-1} ouabain plus 50 μmol L^{-1} orthovanadate likely reveals neutral phosphatases or P-ATPases other than (Na^+, K^+)-ATPase. A Ca^{2+}-ATPase in the crustacean gill epithelium is well known (Gonçalves et al. 2006; Santos et al. 2007; Lucena et al. 2012; Leone et al. 2014).

The inhibition by 10 μmol L^{-1} aurovertin strongly suggests an F_0F_1-ATPase in the gill microsomal fraction, as also seen in *M. olfersi* (Furriel et al. 2000; Gonçalves et al. 2006; Santos et al. 2007; Garçon et al. 2009; França et al. 2013; Leone et al. 2014) owing to the elevated mitochondrial density in the intralamellar septum (Freire and McNamara 1995; McNamara and Lima 1997). The significant fraction of ouabain-insensitive ATPase activity revealed by bafilomycin A_1, indicates the presence of a $V(H^+)$ATPase (Bowman et al. 1988) in *X. kroyeri*. A gill $V(H^+)$-ATPase has been characterized kinetically in microsomal gill preparations from *M. amazonicum* (Faleiros et al. 2010; Lucena et al. 2015) and *D. pagei* (Firmino et al. 2011). In contrast, $V(H^+)$-ATPase activity is very reduced in the blue crabs *C. danae* (Masui et al. 2002) and *C. ornatus* (Garçon et al. 2009), and in *M. amazonicum* after high salinity acclimation (Faleiros et al. 2010).

The presence of a Na^+-ATPase in the gill epithelium of *X. kroyeri* revealed by ouabain plus ethacrynic acid is corroborated by the Na^+-stimulated ouabain-insensitive ATPase activity ($\approx$15 nmol Pi min^{-1} mg^{-1}, Fig. 5b). While Na^+-ATPase activity is found in the gill epithelia of various crustaceans (Proverbio et al. 1990; Moretti et al. 1991; Santos et al. 2007; Garçon et al. 2009; Lucena et al. 2012; França et al. 2013; Leone et al. 2014), it is absent from *M. olfersi* (Furriel et al. 2000) and *C. danae* gill preparations (Masui et al. 2002).

Concluding, the pivotal role of the (Na^+, K^+)-ATPase in osmoregulation and in the excretion of nitrogen end products in aquatic crustaceans is well known (Péqueux 1995; Weihrauch et al. 1999). Since both processes occur mainly in the gill epithelia (Péqueux 1995; Weihrauch et al. 1999), our kinetic characterization of the (Na^+, K^+)-ATPase from the gill epithelium of *X. kroyeri*, a pelagic marine shrimp, together with findings from other crustaceans from various habitats constitutes a valuable tool to better comprehend the biochemical adjustments of crustaceans to biotopes of different salinity. Many of the kinetic characteristics exhibited by the *X. kroyeri* enzyme are similar to those of marine crabs. However, some kinetic properties are more closely allied with those of estuarine and freshwater decapods. Considering that *X. kroyeri* inhabits estuarine regions during its juvenile phase (Dall et al. 1990), it seems likely that the adult shrimps conserve this kinetic profile after migration to marine coastal waters.

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