Comparative Biochemistry and Physiology, Part A 153 (2009) 141–148

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Na^+, K^+-ATPase activity in gill microsomes from the blue crab, *Callinectes danae*, acclimated to low salinity: Novel perspectives on ammonia excretion

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ARTICLE INFO

Article history:
Received 29 October 2008
Received in revised form 28 January 2009
Accepted 28 January 2009
Available online 8 February 2009

Keywords:
Acclimation
Ammonium excretion
Callinectes danae
Euryhaline crab
(Na^+,K^+)-ATPase
Potassium ion

ABSTRACT

This investigation provides an extensive characterization of the modulation by ATP, Mg^{2+}, Na^+, K^+ and NH_4^+ of a gill microsomal (Na^+,K^+)-ATPase from *Callinectes danae* acclimated to 15‰ salinity. Novel findings are the lack of high-affinity ATP-binding sites and a 10-fold increase in enzyme affinity for K^+ modulated by NH_4^+, discussed regarding NH_4^+ excretion in benthic marine crabs. The (Na^+,K^+)-ATPase hydrolyzed ATP at a maximum rate of 298.7 ± 16.7 nmol Pi min^{-1} mg^{-1} and $K_{0.5} = 174.2 \pm 9.8$ mmol L^{-1}, obeying cooperative kinetics ($n_H = 1.2$). Stimulation by sodium ($V = 308.9 \pm 15.7$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 7.8 \pm 0.4$ mmol L^{-1}), magnesium (299.2 ± 14.1 nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 767.3 \pm 36.1$ mmol L^{-1}), potassium (300.6 ± 15.3 nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 1.6 \pm 0.08$ mmol L^{-1}) and ammonium ($V = 345.1 \pm 19.0$ nmol Pi min^{-1} mg^{-1}, $K_{0.5} = 6.0 \pm 0.3$ mmol L^{-1}) ions showed site–site interactions. Ouabain inhibited (Na^+,K^+)-ATPase activity with $K_I = 45.1 \pm 2.5$ μmol L^{-1}, although affinity for the inhibitor increased ($K_I = 22.7 \pm 1.1$ μmol L^{-1}) in 50 mmol L^{-1} NH_4^+. Inhibition assays using ouabain plus oligomycin or ethacrynic acid suggest mitochondrial F_0F_1- and K^+-ATPase activities, respectively. Ammonium and potassium ions synergistically stimulated specific activity up to 72%, inferring that these ions bind to different sites on the enzyme molecule, each modulating stimulation by the other.

1. Introduction

The (Na^+, K^+)-ATPase (E.C.3.6.1.37), or sodium pump, is a member of the P_{2C} ATPase family. This ion-transporting enzyme couples ATP hydrolysis to the uphill transfer of two K^+ ions into, and three Na^+ ions out of cells, generating an electrochemical gradient that establishes their resting membrane potential, determines their excitable properties and underlies their osmotic equilibrium. Such ion transport across the plasma membrane involves repeated cycling between at least two phosphorylated or dephosphorylated enzyme conformations: E_1, which exhibits high affinity for intracellular Na^+, and E_2 in which extracellular K^+ ions bind with high affinity (for reviews see Kaplan, 2002; Horisberger, 2004; Morth et al., 2007; Pedersen, 2007).

Most marine crustaceans are osmoconformers, essentially isosmotic with their surrounding medium. Usually stenohaline, they show little tolerance of external salinity changes, and exposure to dilute or fluctuating media may be lethal (Péqueux, 1995; Lucu and Towle, 2003; Kirschner, 2004; Tsoi et al., 2005). In contrast, euryhaline crustaceans inhabit media of variable salinity and hyper-regulate their hemolymph osmolality when in dilute media, employing ion uptake mechanisms that involve considerable energy expenditure (Péqueux, 1995; Guerin and Stickle, 1997; Henry et al., 2002; Genovese et al., 2004). In such species, coordinated ion transport across the gills maintains hemolymph osmotic and ionic equilibria and compensates for diffusive and urinary ion losses (Péqueux, 1995; Onken and Riestenpatt, 1998, 2002; Lucu and Towle, 2003; Kirschner, 2004).

Crustacean gills are multifunctional organs, performing respiratory gas exchange, hemolymph acid–base and osmo-ionic regulation, as well as the excretion of nitrogenous metabolites (for reviews see Lucu and Towle, 2003; Weihrauch et al., 2004; Tresguerres et al., 2008; Freire et al., 2008). Covered by a thin cuticle, the gill epithelium constitutes a selective interface between the animal's extracellular space and the environment, and across which Na^+ and Cl^- are actively absorbed from dilute media. Both weak and strong hyper-osmoregulating crustaceans possess a common set of ion transporters, including the (Na^+, K^+)-ATPase and K^+ and Cl^- channels located in the basolateral epithelial cell membranes. However, while Na^+ channels, and a V-type proton pump and Cl^-/HCO_3^- antiporter furnished with H^+ and HCO_3^- *via* the cytoplasmic carbonic anhydrase, characterize the apical membranes of strong hyperosmoregulators, weak hyperosmoregulators possess an apical $Na^+/K^+/2Cl^-$ symporter, Na^+/H^+ and Cl^-/HCO_3^- antiporters and K^+ channels (see reviews by Kirschner, 2004; Freire et al., 2008). The basolateral (Na^+, K^+)-ATPase constitutes a key enzyme in ion uptake in both cases (Péqueux, 1995; Furriel et al., 2000; Lucu and Towle, 2003; Kirschner, 2004; Lovett et al., 2006; Jayasundara et al., 2007) and there is an apparent relationship between enzyme activity, hyperosmoregulation and ammonia excretion in many species (Péqueux, 1995; Lucu and Towle 2003; Kirschner, 2004; Weihrauch et al., 2004; Leone et al.,

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1095-6433/$ – see front matter
doi:10.1016/j.cbpa.2009.01.020

2005b, Masui et al., 2005; Freire et al., 2008). However, while ammonia excretion rates correlate with Na^+ absorption in *C. sapidus* (Pressley et al., 1981), *C. maenas* (Lucu et al., 1989) and *E. sinensis* (Péqueux, 1995), a direct relationship between active ion uptake and ammonia excretion remains tenuous.

Aquatic crustaceans are ammoniotelic, excreting their nitrogenous metabolites across the gill epithelium largely as ammonia ($NH_3 + NH_4^+$), which at physiological pH, is mostly in the ionic form (Weihrauch et al., 1998, 1999, 2004). The demonstration that NH_4^+ can substitute for K^+ in the gill (Na^+,K^+)-ATPase initially suggested a role in active NH_4^+ transport into the gill cell cytoplasm from the hemolymph (Weihrauch et al., 2004; Weiner and Hamm, 2007). However, NH_4^+ and K^+ synergistically stimulate the *Callinectes danae* gill (Na^+, K^+)-ATPase in a manner regulated by Mg^{2+}, suggesting additional NH_4^+-binding sites on the enzyme, which may guarantee the outwardly directed, active transport of ammonia even at usual hemolymph K^+ concentrations (Masui et al., 2002, 2005). These findings corroborate current models of ammonia transport across the crustacean gill epithelium (reviewed by Weihrauch et al., 2004; Freire et al., 2008) and open new perspectives on ammonia excretion.

Callinectes danae Smith 1869, is a euryhaline, benthic brachyuran crab widely distributed in the Western Atlantic from Florida to southern Brazil (Melo, 1996; Chacur and Negreiros-Fransozo, 2001). In Ubatuba Bay, Brazil, *C. danae* is often found in seawater affected by freshwater runoff (Mantelatto and Fransozo, 1999, 2000) and is exposed to variable salinities; efficient mechanisms of hyperosmoregulation are vital for survival. Since *C. danae* characteristically buries itself in bottom sediments, local ammonia concentration may also increase, reducing passive efflux and even leading to ammonia influx across the gill epithelium (Weihrauch et al., 1999). Since both osmoregulation and ammonia excretion in aquatic brachyurans occur mainly across the gill epithelium (Péqueux, 1995; Weihrauch et al., 1999), we have employed *C. danae* as a model to characterize the crab gill (Na^+,K^+)-ATPase (Masui et al., 2002). In fresh-caught, unacclimated *C. danae*, total gill microsomal ATPase activity is predominantly (Na^+,K^+)-ATPase activity. The enzyme exhibits two distinct sites for ATP hydrolysis and is synergistically stimulated by K^+ and NH_4^+ (Masui et al., 2002).

The present study provides a kinetic characterization of the (Na^+, K^+)-ATPase expressed in the gill tissue of *C. danae* acclimated to dilute seawater of 15‰ salinity. Our data confirm the synergistic stimulation of the enzyme by K^+ and NH_4^+ but, in contrast to fresh-caught, unacclimated crabs (Masui et al., 2002), modulation of enzyme activity by NH_4^+ increases K^+ affinity 10-fold. These findings provide a better understanding of the biochemical mechanisms underlying ammonia excretion in crustaceans.

2. Materials and methods

2.1. Material

All solutions were prepared using Millipore MilliQ ultrapure, apyrogenic water and all reagents were of the highest purity commercially available. Imidazole, pyruvate kinase (PK), phospho*enol*pyruvate (PEP), NAD^+, NADH, N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (Hepes), lactate dehydrogenase (LDH), ouabain, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate (G3P), alamethicin, 3-phosphoglyceraldehyde diethyl acetal, ATP ditris salt, ouabain, ethacrynic acid, oligomycin, thapsigargin, bafilomycin A_1, sodium orthovanadate and alamethicin were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Dimethyl sulfoxide and triethanolamine were from Merck. The protease inhibitor cocktail was from Calbiochem. The alpha-5 monoclonal antibody against the α-subunit of the (Na^+, K^+)-ATPase (all isoforms) was purchased from Developmental Studies Hybridoma Bank (IA, USA). Antimouse IgG, alkaline phosphatase conjugate was purchased from Promega Corporation (Madison, WI, USA).

2.2. Reagents

Glyceraldehyde-3-phosphate was prepared by hydrolysis of 3-phospho-glyceraldehyde diethyl acetal with 150 μL HCl ($d = 1.18$ g mL^{-1}) in a boiling-water bath for 2 min and neutralized with 50 μL triethanolamine. Sodium orthovanadate solution was prepared according to Furriel et al. (2000).

2.3. Depletion of ammonium ions from enzyme suspensions

Crystalline suspensions of LDH and PK were centrifuged at 20,000 *g* for 15 min, at 4 °C, in an Eppendorf 5810 refrigerated centrifuge. The pellet was ressuspended in 300 μL of 50 mmol L^{-1} Hepes buffer, pH 7.5, transferred to a YM-10 Microcon filter and centrifuged 5 times with the same buffer as above until complete depletion of ammonium ions (tested with the Nessler reagent). Finally, the pellet was ressuspended to the original volume. For PGK and GAPDH, the suspension was treated as above with 50 mmol L^{-1} triethanolamine buffer, pH 7.5, and containing 1 mmol L^{-1} dithiothreitol. When necessary, enzyme solutions were concentrated using YM-10 Amicon cones or Microcon filters.

2.4. Gill excision

Specimens of *C. danae* were collected using double rig trawl nets from Ubatuba Bay (23° 26′ S; 45° 02′ W), São Paulo State, Brazil and were transported to the laboratory and held in tanks containing aerated seawater (33‰ salinity). One lot of crabs was killed within two days and constitutes the fresh-caught (unacclimated) group. The other group was acclimated to 15‰ salinity for 10 days at 25 °C, and fed on alternate days with shrimp tails. For each homogenate prepared, 8 adult intermolt crabs were anesthetized by chilling at −20 °C and the entire carapace was quickly removed. Posterior gill pairs 6, 7, and 8 were rapidly excised and placed in 10 mL of ice-cold homogenization buffer consisting of 20 mmol L^{-1} imidazole buffer, pH 6.8, 250 mmol L^{-1} sucrose, 6 mmol L^{-1} EDTA and the protease inhibitor cocktail (5 μmol L^{-1} leupeptin, 5 μmol L^{-1} antipain, 1 mmol L^{-1} benzamidine and 1 μmol L^{-1} pepstatin A).

2.5. Preparation of the gill microsomal fraction

The gills were rapidly diced and homogenized in the homogenization buffer (20 mL/g wet tissue) using a Potter homogenizer. After centrifugation of the crude extract at 20,000× g for 30 min at 4 °C, the supernatant was placed on crushed ice and the pellet was resuspended in an equal volume of homogenization buffer. After further centrifugation as above the two supernatants were gently pooled and centrifuged at 100,000× g for 2 h at 4 °C. The resulting pellet was ressuspended in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose (10 mL buffer/g wet tissue mass). Finally, 0.5-mL aliquots were rapidly frozen in liquid nitrogen and stored at −20 °C. Under these conditions, no appreciable changes in (Na^+, K^+)-ATPase activity were seen after two-month's storage (fresh preparation, $V = 298.8 \pm 16.7$ nmol Pi min^{-1} mg^{-1}; after two month's freezing $V = 286.8 \pm 8.6$ nmol Pi min^{-1} mg^{-1}). When required, aliquots were thawed, placed on crushed ice and used immediately.

2.6. Continuous-density sucrose gradient centrifugation

An aliquot (900 μg protein) of the (Na^+, K^+)-ATPase-rich microsomal fraction was layered into a 10 to 50% (w/w) continuous-density sucrose gradient in 20 mM imidazole buffer, pH 6.8, and centrifuged at 180,000× g and 4 °C for 2 h using an Hitachi PV50T2 vertical rotor. Fractions (0.5 mL) were collected from the bottom of the tube and were assayed for protein, total ATPase and ouabain-insensitive activities and refractive index.