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A kinetic study of the gill (Na^+, K^+)-ATPase, and its role in ammonia excretion in the intertidal hermit crab, *Clibanarius vittatus*

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Abstract

To better comprehend the role of gill ion regulatory mechanisms, the modulation by Na^+, K^+, NH_4^+ and ATP of (Na^+, K^+)-ATPase activity was examined in a posterior gill microsomal fraction from the hermit crab, *Clibanarius vittatus*. Under saturating Mg^{2+}, Na^+ and K^+ concentrations, two well-defined ATP hydrolyzing sites were revealed. ATP was hydrolyzed at the high-affinity sites at a maximum rate of $V=19.1\pm0.8$ U mg^{-1} and $K_{0.5}=63.8\pm2.9$ nmol L^{-1}, obeying cooperative kinetics ($n_H=1.9$); at the low-affinity sites, hydrolysis obeyed Michaelis–Menten kinetics with $K_M=44.1\pm2.6$ μmol L^{-1} and $V=123.5\pm6.1$ U mg^{-1}. Stimulation by Na^+ ($V=149.0\pm7.4$ U mg^{-1}; $K_M=7.4\pm0.4$ mmol L^{-1}), Mg^{2+} ($V=132.0\pm5.3$ U mg^{-1}; $K_{0.5}=0.36\pm0.02$ mmol L^{-1}), NH_4^+ ($V=245.6\pm9.8$ U mg^{-1}; $K_M=4.5\pm0.2$ mmol L^{-1}) and K^+ ($V=140.0\pm4.9$ U mg^{-1}; $K_M=1.5\pm0.1$ mmol L^{-1}) followed a single saturation curve and, except for Mg^{2+}, obeyed Michaelis–Menten kinetics. Under optimal ionic conditions, but in the absence of NH_4^+, ouabain ($K_I=117.3\pm3.5$ μmol L^{-1}) and orthovanadate inhibited up to 67% of the ATPase activity. The inhibition studies performed suggest the presence of F_0F_1, V- and P-ATPases, but not Na^+-, K^+- or Ca^{2+}-ATPases as contaminants in the gill microsomal preparation. (Na^+, K^+)-ATPase activity was synergistically modulated by NH_4^+ and K^+. At 20 mmol L^{-1} K^+, a maximum rate of $V=290.8\pm14.5$ U mg^{-1} was seen as NH_4^+ concentration was increased up to 50 mmol L^{-1}. However, at fixed NH_4^+ concentrations, no additional stimulation was found for increasing K^+ concentrations ($V=135.2\pm4.1$ U mg^{-1} and $V=236.6\pm9.5$ U mg^{-1} and for 10 and 30 mmol L^{-1} NH_4^+, respectively). This is the first report to detail ionic modulation of gill (Na^+, K^+)-ATPase in *C. vittatus*, revealing an asymmetrical, synergistic stimulation of the enzyme by K^+ and NH_4^+, as yet undescribed for other (Na^+, K^+)-ATPases, and should provide a better understanding of NH_4^+ excretion in pagurid crabs.

Keywords: (Na^+, K^+)-ATPase; Ammonium excretion; Gill microsomes; Ionic regulation; *Clibanarius vittatus*; Hermit crab; Asymmetrical stimulation; Ouabain inhibition

1. Introduction

The (Na^+, K^+)-ATPase (E.C.3.6.1.37) is an ion-transporting protein, located in the plasma-membrane, that exports three Na^+ from the cell cytoplasm against two K^+ from the extracellular fluid per ATP molecule hydrolyzed. This enzyme is a heterodimer comprising a catalytic, 110-kDa α-subunit and a glycosylated, 55-kDa β-subunit. The energy of ATP hydrolysis induces conformational changes in the enzyme, which lead to the vectorial, electrogenic, ion transport process. The β-subunit appears to be involved in the proper folding and trafficking of the enzyme and may participate in ion transport. The electrochemical Na^+ and K^+ gradients generated by the (Na^+, K^+)-ATPase activity underlie cell osmotic equilibria, in addition to many other important functions (for review see Jorgensen et al., 2003; Horisberger, 2004).

Crustacean gills play a central role in active osmotic and ionic regulatory processes (Péqueux, 1995), respiratory gas exchange (Böttcher and Siebers, 1993), hemolymph acid-base regulation (Henry and Wheatly, 1992) and the excretion of nitrogenous metabolic end products (Cameron and Batterton, 1978; Weihrauch et al., 1998, 1999, 2004). Aquatic crustaceans are ammoniotelic, excreting nitrogenous waste products mainly

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as ammonia ($NH_3+NH_4^+$), principally through the gill epithelium; the gill (Na^+, K^+)-ATPase appears to play a major role in such excretion (Weihrauch et al., 2004; Masui et al., 2002, 2005; Furriel et al., 2004). The similar ionic radii of hydrated NH_4^+ and K^+ ions suggest that NH_4^+ may interact with K^+ binding sites (Knepper et al., 1989) and, indeed, NH_4^+ substitutes for K^+ in stimulating (Na^+, K^+)-ATPase hydrolytic activity in vertebrate (Skou and Esmann, 1992; Kurtz and Balaban, 1986; Robinson, 1970) and crustacean gill tissues (Holliday, 1985; Towle et al., 1976; Towle and Holleland, 1987; Lucu et al., 1989). Further, NH_4^+ can synergistically stimulate *Callinectes danae* and *Macrobrachium olfersii* gill (Na^+, K^+)-ATPase (Masui et al., 2002; Furriel et al., 2004).

The Anomura are amongst the most morphologically and ecologically diverse groups of decapod crustaceans, and include many species that inhabit a wide variety of biotopes. While the Coenobitidae include well adapted semi-terrestrial and terrestrial species, and the Aeglidae are restricted to freshwater, other families and genera are almost exclusively marine, inhabiting the intertidal to abyssal zones (Forest et al., 2000; Scelzo et al., 2004). This diversity renders the Anomura a promising group to study since their establishment in such varied environments derives from the evolution of suitable adaptive strategies (Mantelatto and Sousa, 2000; Greenaway, 2003).

Clibanarius vittatus (Bosc, 1802) is an abundant hermit crab distributed from the West Atlantic coast of the United States to southern Brazil (Coelho and Ramos, 1972; Williams, 1984). It occurs in the intertidal zone, and is common on beaches and in mangroves (Coelho and Ramos, 1972; Williams, 1984), frequently inhabiting regions associated with the deposition of organic matter and susceptible to freshwater influence (Lowery and Nelson, 1988). As a consequence of exposure by tides, *C. vittatus* confronts ample seasonal and diurnal salinity changes (Fotheringham, 1975). Many studies have described the distribution and basic biology of *C. vittatus* (see Melo, 1999 for review); however, few physiological and biochemical data are available. *C. vittatus* hyperosmoregulates well between 5 and 25‰ salinity, but only slightly at higher salinities (Young, 1979a; Sharp and Neff, 1980; Sabourin and Stickle, 1980). Seawater is necessary for embryonic and larval development, with metamorphosis to the juvenile phase occurring at 25–30‰ salinity (Young and Hazlett, 1978).

C. vittatus possesses fourteen pairs of gills disposed laterally in the cephalothorax, and arranged as pairs of ten arthrobranchiae and four pleurobranchiae (Forest and Saint Laurent, 1968). Little is known of the histological and ultrastructural characteristics of *C. vittatus* gills. However, in brachyuran crab gills, the basolateral membrane of the epithelial cells houses the (Na^+, K^+)-ATPase (Towle and Kays, 1986). There appears to be no information available concerning the gill (Na^+, K^+)-ATPase for this species.

The adaptive physiological and biochemical strategies employed by the Crustacea to confront the osmotic challenges of life in freshwater and estuarine habitats may be better comprehended through comparative, systematic studies of the biochemical characteristics of the gill (Na^+, K^+)-ATPase in species from different habitats. Here, we provide a kinetic characterization of a gill microsomal (Na^+, K^+)-ATPase from *C. vittatus*, an intertidal species, particularly well adapted to wide salinity fluctuations. We also focus on the effect of NH_4^+ on ATP hydrolysis, to further comprehend the role of the (Na^+, K^+)-ATPase in active ammonia excretion. This is the first study providing a kinetic characterization of the (Na^+, K^+)-ATPase from anomuran crab gills.

2. Materials and methods

2.1. Materials

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-*N*′-ethanesulfonic acid (HEPES), lactate dehydrogenase (LDH), ouabain, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate (G3P), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indole phosphate (BCIP), alamethicin, imidazole, sodium orthovanadate, 3-phosphoglyceraldehyde diethyl acetal, ethacrynic acid, oligomycin, thapsigargin, bafilomycin A_1 were purchased from Sigma Chemical Co (Saint Louis, MO, USA). Dimethyl sulfoxide and triethanolamine were from Merck (Darmstadt, Germany). The protease inhibitor cocktail (1 mmol L^{-1} benzamidine, 5 μmol L^{-1} antipain, 5 μmol L^{-1} leupeptin and 1 μmol L^{-1} pepstatin A) was from Calbiochem (San Diego, CA, USA). All other reagents were of the highest purity commercially available. Crystalline suspensions of LDH and PK were centrifuged at 16,000 g for 15 min, at 4 °C in a refrigerated centrifuge. The pellet was re-suspended in 500 μL of 50 mmol L^{-1} Hepes buffer, pH 7.5, transferred to an YM-10 Microcon filter and centrifuged 5 times with the same buffer as above to complete depletion of ammonium ions (tested with Nessler reagent). Finally, the pellet was re-suspended 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. G3P was prepared by hydrolysis of 3-phospho-glyceraldehyde diethyl acetal with 150 μL HCl (d=1.18 g/mL) 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). When necessary, enzyme solutions were concentrated on YM-10 Amicon Microcon filters. The alpha-5 monoclonal antibody against the α-subunit of the (Na^+, K^+)-ATPase (all isoforms) was purchased from Developmental Studies Hybridoma Bank (Iowa, USA). Antimouse IgG, alkaline phosphatase conjugate was purchased from Promega Corporation (USA).

2.2. Gill dissection

Adult *C. vittatus* were collected at low tide along rocky shore areas of the Araçá Mangrove near São Sebastião, São Paulo