

Partial characterization and response under hyperregulating conditions of Na⁺-K⁺ ATPase and levamisole-sensitive alkaline phosphatase activities in chela muscle of the euryhaline crab *Cyrtograpsus angulatus*

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SUMMARY: The occurrence, characteristics and response to changes in environmental salinity of Na⁺-K⁺ ATPase and levamisole-sensitive alkaline phosphatase (AP) activities were studied in chela muscle of the euryhaline crab *Cyrtograpsus angulatus*. Chela muscle exhibited an Na⁺-K⁺ ATPase activity which was strongly dependent on ATP concentration, pH and temperature of the reaction mixture. Maximal activity was found at 1 mM ATP, 30-37°C and pH 7.4. Levamisole-sensitive AP activity was characterised at physiological pH 7.4 and at pH 8.0. I₅₀ for levamisole-sensitive AP activity was 8.8 mM and 8.0 mM at pH 7.4 and 8.0, respectively. At both pH levels, levamisole-sensitive AP activity exhibited Michaelis-Menten kinetics (K_m=3.451 mM and 6.906 mM at pH 7.4 and 8.0, respectively). Levamisole-sensitive AP activities were strongly affected by temperature, exhibiting a peak at 37°C. In crabs acclimated to low salinity (10; hyperregulating conditions), Na⁺-K⁺ ATPase activity and levamisole-sensitive AP activity at the physiological pH were higher than in 35 psu (osmoconforming conditions). The response to low salinity suggests that both activities could be components of muscle regulatory mechanisms at the biochemical level secondary to hyperregulation of *C. angulatus*. The study of these activities under hyperregulating conditions contributes to a better understanding of the complexity of biochemical mechanisms underlying the adaptive process of euryhaline crabs.

Keywords: alkaline phosphatase, Na⁺-K⁺ ATPase, crabs, *Cyrtograpsus angulatus*, levamisole, muscle.

RESUMEN: CARACTERIZACIÓN PARCIAL Y RESPUESTA BAJO CONDICIONES DE HIPERREGULACIÓN DE LAS ACTIVIDADES DE Na⁺-K⁺ ATPASA Y FOSFATASA ALCALINA LEVAMISOL-SENSIBLE EN MÚSCULO DE LA QUELA DEL CANGREJO EURIHALINO *CYRTOGRAPSUS ANGULATUS*. – En el presente trabajo se estudió la presencia, características y respuesta a la salinidad ambiental de las actividades de Na⁺-K⁺ ATPasa y fosfatasa alcalina (AP) levamisol-sensible en el músculo de la quela del cangrejo eurialino *Cyrtograpsus angulatus*. El músculo de la quela exhibió una actividad de Na⁺-K⁺ ATPasa marcadamente dependiente de la concentración de ATP, pH y temperatura del medio de reacción. La actividad fue máxima a 1 mM de ATP, 30-37°C y a pH 7.4. La actividad de AP levamisol-sensible se caracterizó parcialmente a pH fisiológico (pH 7.4) y a pH 8.0. La I₅₀ fue de 8.8 y 8.0 mM de levamisol a pH 7.4 y 8.0 respectivamente. A ambos pH la actividad de AP levamisol-sensible exhibió una cinética michaeliana (K_m=3.451 y 6.606 mM de pNPP, respectivamente). Las actividades de AP levamisol-sensibles fueron fuertemente afectadas por la temperatura, exhibiendo un pico a 37°C. En cangrejos aclimatados a baja salinidad (10) (condiciones de hiperregulación), las actividades de Na⁺-K⁺ ATPasa y de AP levamisol-sensible a pH fisiológico fueron mayores que a 35 de salinidad (condiciones de osmoionconformación). La respuesta a baja salinidad sugiere que ambas actividades serían componentes de los mecanismos regulatorios a nivel bioquímico secundarios a la hiperregulación en *C. angulatus*. El estudio de estas actividades contribuye a un mejor conocimiento de los complejos mecanismos bioquímicos en el proceso adaptativo bajo condiciones de hiperregulación de cangrejos eurialinos.

Palabras clave: fosfatasa alcalina, Na⁺-K⁺ ATPasa, cangrejos, *Cyrtograpsus angulatus*, levamisol, músculo.

INTRODUCTION

Estuarine crabs have to cope with a variety of challenges, including frequent and abrupt changes in environmental salinity. Environmental fluctuations in salinity can trigger adjustments at different levels (biochemical, physiological, morphological and/or behavioural) to control movements of water and ions between animals and their medium (Kirschner, 1991). In low salinities, hyperregulating crabs maintain the hemolymph osmotic concentration above that of the external medium by absorbing both sodium and chloride from the environment. Posterior gills are considered to be the main site of the biochemical adaptations of hyperregulation (reviewed by Lucu and Towle, 2003; Kirschner, 2004). In the euryhaline crab *Cyrtograpsus angulatus*, a hyperregulatory role for the anterior gills has also been suggested (López Mañanes *et al.*, 2002). Branchial Na⁺-K⁺ ATPase activity appears to be a central component of the ionoregulatory process at the biochemical level (reviewed by Towle, 1997; Lucu and Towle, 2003). Little is known about the occurrence and characteristics of Na⁺-K⁺ ATPase in other tissues of hyperregulating crabs.

The biochemical adaptations in other organs or tissues (i.e. muscle) of crabs to different environmental salinities, have received little attention. Hypoosmotic stress led to adjustments associated with acid-base regulation in leg muscle of *Eriocheir sinensis*, (Whiteley *et al.*, 2001) and to an increase of arginine kinase flux in muscle of *Callinectes sapidus* (Holt and Kinsey, 2002). In muscle of *Chasmagnathus granulatus*, an increase in the mobilization of lipids occurred upon acclimation to low salinity (Luvizotto-Santos *et al.*, 2003) and in phosphoenolpyruvate carboxykinase and gluconeogenic activities under hyperosmotic stress (Schein *et al.*, 2004). Muscle cells of the freshwater red crab *Dilocarcinus pagei* display a certain degree of regulatory volume increase upon volume loss in hyperosmotic medium (Amado *et al.*, 2006).

Alkaline phosphatases (AP) (EC 3.1.3.1) are ubiquitous non-specific metalloenzymes which hydrolyse many types of phosphate esters at a range of optimal pH above 7.0. In mammals, AP plays a role in several essential functions (Hessle *et al.*, 2002; Ali *et al.*, 2006a,b; Nakano *et al.*, 2006). In amphibians, AP has been suggested to be involved in adaptive osmoregulation (Dore *et al.*, 2000). Although AP has been identified and characterised in several tissues of invertebrates (Itoh *et al.*, 1999; Chen *et al.*, 2000;

2005; Park *et al.*, 2001; Mazorra *et al.*, 2002; Xiao *et al.*, 2002), its exact physiological role has not always been clearly established.

The role of AP in euryhaline crab adaptation to environmental salinity has been scarcely studied. An AP activity sensitive to low salinity occurs in the posterior gills of the euryhaline crab *C. sapidus*, which would modulate the osmoregulatory response, probably being an effector for the increases of branchial Na⁺-K⁺ ATPase activity (Lovett *et al.*, 1994). We have recently demonstrated the occurrence of a levamisole-insensitive AP activity with optimal pH 7.7, which decreased upon acclimation to low salinity in chela muscle of *C. granulatus*, whereas levamisole-sensitive AP activity appeared not to be affected. The differential response of both muscle AP activities to low salinity suggests a different participation in mechanisms of adjustments to varying environmental conditions (Pinoni *et al.*, 2005). *C. angulatus* is a euryhaline crab which is found from Rio de Janeiro (Brazil) to Patagonia (Argentina) in habitats with varying salinities (Boschi, 1964). In Mar Chiquita coastal lagoon (Buenos Aires Province, Argentina), it is exposed to highly and abruptly variable environmental salinity (Anger *et al.*, 1994; Spivak *et al.*, 1994). The regulatory mechanisms at the biochemical level in other organs or tissues of *C. angulatus* under low salinity conditions have been poorly investigated. As part of our integrative studies on the identification of enzyme activities involved in biochemical adaptations to environmental salinity in estuarine crabs, the aim of this work was to determine the occurrence, characteristics and response to low salinity at physiological pH of AP activity and Na⁺-K⁺ ATPase activity in muscle of *C. angulatus* from Mar Chiquita coastal lagoon. The study of the responses of muscle total AP, levamisole-sensitive AP and Na⁺-K⁺ ATPase activities under hyperregulating conditions contributes to a better understanding of the complexity of biochemical mechanisms underlying adaptive process of euryhaline crabs.

MATERIALS AND METHODS

Chemicals

Na₂ATP (adenosine 5' triphosphate, vanadium-free), Tris-(hydroxymethylamino-methane) (Tris), ethylenglicol *N, N', N'*-tetraacetic acid (EGTA), imidazole, G-Strophanthin (ouabain), pNPP (p-nitrophenylphosphate), levamisole (L [-]-2, 3, 5, 6-

Tetrahydro-6-phenylimidazol [2, 1-*b*] thiazole) and bovine serum albumin were from Sigma (St. Louis, MO, USA); sucrose and sodium chloride were from Merck (Darmstadt, Germany); magnesium chloride was from ICN (Ohio, USA); potassium chloride, magnesium sulphate and Coomassie Blue G250 were from Fluka (Germany). All solutions were prepared in glass-distilled water.

Animal collection and maintenance

Crabs were caught from a single area from Mar Chiquita lagoon which exhibited high and abrupt variations in salinity ranging from 4 to 35 psu. For all the experiments salinity was measured in practical salinity units (psu). Only adult male crabs with a carapace width greater than 2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. Crabs were maintained in natural seawater (35 psu) or dilute seawater (10 psu) for at least 10 days prior to use. The aquaria contained 36 l of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at $22 \pm 2^\circ\text{C}$. Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Cichlid T.E.N., Wardley, USA) (about 0.07 g per individual) but they were starved 48 h prior to experiments. Dilute seawater was obtained by dilution of natural seawater with distilled water.

Preparation of enzyme muscle extract

The crabs were cryoanaesthetised by putting them on ice for about 15 min. After removal of the chelae, the muscle was immediately excised, mixed with homogenising medium (0.25 M sucrose/0.5 mM EGTA-Tris, pH 7.4; 8 ml g^{-1} of muscle tissue) and homogenised (CAT homogeniser x120, tool T10) on ice. The muscles from both chelae of one individual were pooled and used for each preparation of enzyme extract. The homogenate was fractionated into 200 μl aliquots and used immediately (for assay of $\text{Na}^+\text{-K}^+\text{ATPase}$ activity) or stored at -20°C until use (for assay of AP activities). Glycerol (1.3% v/v) was added to samples before freezing.

Assay of $\text{Na}^+\text{-K}^+\text{ATPase}$ activity

Total ($\text{Mg}^{2+}\text{-Na}^+\text{-K}^+$) ATPase activity was determined by measuring ATP hydrolysis in a reaction

medium containing 100 mM NaCl, 30 mM KCl, 10 mM MgCl_2 and 0.5 mM EGTA in 20 mM imidazole buffer (pH 7.4). Residual ($\text{Mg}^{2+}\text{-Na}^+$) ATPase activity was assayed in the same medium but without KCl and in the presence of 1 mM ouabain (specific inhibitor of $\text{Na}^+\text{-K}^+\text{ATPase}$). $\text{Na}^+\text{-K}^+\text{ATPase}$ activity was determined as the difference between the two assays. An aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 30°C . The reaction was initiated by the addition of ATP (final concentration 1 mM). Incubation was carried out at 30°C for 15 min. The reaction was stopped by addition of 2 ml of cooled Bonting's reagent (560 mM sulphuric acid, 8.1 mM ammonium molybdate and 176 mM ferrous sulphate). After 20 min at room temperature, the amount of released Pi was determined by reading the absorbance at 700 nm of the reduced phosphomolybdate complex (Bonting, 1970). To study the effect of ATP concentrations, pH and temperature on $\text{Na}^+\text{-K}^+\text{ATPase}$ activity, the procedure was the same as that described above, except that the activities were determined in the presence of varying ATP concentrations or at different pH levels of the reaction mixture, respectively. Individuals acclimated to 10 psu salinity were used in these experiments. Since a residual ($\text{Mg}^{2+}\text{-Na}^+$) ouabain-insensitive ATPase activity is usually determined as part of the assay of $\text{Na}^+\text{-K}^+\text{ATPase}$ activity, we also determined the characteristics and responses of this activity in chela muscle of *C. angulatus*. The determination of enzyme activity was always performed with samples, without previous freezing.

Assay of alkaline phosphatase activity

In the standard assay, AP activity was determined by measuring pNPP hydrolysis in a reaction medium containing 1 mM MgSO_4 in 100 mM Tris-HCl buffer (pH 7.4 or 8.0) in the absence (total AP activity) and presence of 16 mM levamisole (levamisole-insensitive AP activity). Levamisole-sensitive AP activity was estimated as the difference between the two assays. An aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) (200-350 μg of proteins) was added to the reaction mixture and pre-incubated for 5 min at 37°C . The reaction was initiated by the addition of pNPP (final concentration 9.5 mM). Incubation was carried out at 37°C for 30 min. The reaction was stopped by addition of 2 ml of 0.1 M KOH. The amount of released

pNP was determined by reading the absorbance at 410 nm. To study the effect of pH on AP activity, the procedure was the same as that described above, except that the activity was determined in the presence of varying pH levels of the reaction mixture. To study the effect of pNPP concentration on AP activity, the procedure was the same as that described above, except that the activity was determined in the presence of varying pNPP concentrations in the reaction mixture. The effect of temperature on AP activity was determined as described above, but the incubation temperature was varied. Individuals acclimated to 35 psu salinity were used in these experiments. The determination of enzyme activity was always performed with samples which had been stored at -20°C , without any previous thawing. This freezing procedure did not alter the activity values.

Measurement of hemolymph ionic concentration

Hemolymph (about 500 μl) was sampled from the infrabranchial sinus by mean of a syringe at the base of the cheliped, and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10000 Xg (Beckman, Microfuge, B) for 30 s. Na^+ and K^+ were determined by flame photometry (Radiometer Copenhagen, FLM3). Cl^- was determined by a colorimetric method (Randox Commercial Kit) based on the formation of a blue Fe-2,4,6-tri-(2-pyridyl)-1,3,5-triazine-ferrous sulphate complex.

Protein analysis

Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard.

Statistical analysis

Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A parametric (one-way ANOVA or repeated measures ANOVA) or non-parametric (Kruskal-Wallis) analysis of variance was used. A posteriori ANOVA test using the Holm-Sidak method was used to identify differences and $p < 0.05$ was considered significant. Results of the effect of varying concentrations of pNPP on AP activity were analysed by means of non-linear regression analysis (GraphPad Prism 2.01 software). The corresponding curves shown are those which

best fit the experimental data. K_m values (Michaelis-Menten constant) were estimated by analysis of data using a Lineweaver-Burk plot (GraphPad Prism 2.01 software). I_{50} (levamisole concentration at which levamisole-sensitive AP activity was 50% inhibited) was calculated from the inhibition curve (GraphPad Prism 2.01 software).

RESULTS

$\text{Na}^+\text{-K}^+$ ATPase activity of muscle of *Cyrtograpsus angulatus*

The effect of ATP concentration on $\text{Na}^+\text{-K}^+$ ATPase activity of muscle of *Cyrtograpsus angulatus* is shown in Figure 1. Maximal activity occurred at 1 mM ATP. At 5.0-10.0 mM ATP, $\text{Na}^+\text{-K}^+$ ATPase activity was about 40% lower than maximal activity (Fig. 1). The response to ATP of muscle $\text{Na}^+\text{-K}^+$ ATPase activity did not fit to any of the equations tested (one-site binding, two-site binding, sigmoidal, sigmoidal variable slope) (non-linear regression analysis GraphPad Prism software version 2.01). Residual ($\text{Mg}^{2+}\text{-Na}^+$) ATPase activity was similar within the range of ATP concentrations used (Fig. 1, Inset). $\text{Na}^+\text{-K}^+$ ATPase activity in chela muscle was affected by pH and temperature of the reaction mixture. Maximal

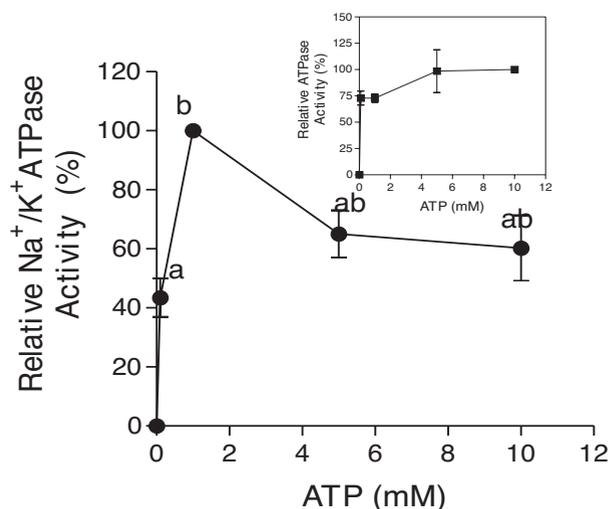


FIG. 1. – Effect of ATP (0.1-10.0 mM) on $\text{Na}^+\text{-K}^+$ ATPase activity in chela muscle of *C. angulatus*. Inset: residual ($\text{Mg}^{2+}\text{-Na}^+$) ATPase activity. The values of $\text{Na}^+\text{-K}^+$ ATPase and residual ($\text{Mg}^{2+}\text{-Na}^+$) ATPase activity are expressed as a relation to the maximal activity (100%, $\text{Na}^+\text{-K}^+$ ATPase activity: 61.8 ± 7.7 and ($\text{Mg}^{2+}\text{-Na}^+$) ATPase activity: 63.6 ± 5.1 nmoles $\text{Pi} \times \text{min}^{-1} \times \text{mg prot}^{-1}$). In some cases, deviation bars were smaller than the symbols used. Data are the mean \pm S.E. for three to five crabs. Different letters indicate significant differences ($p < 0.05$).

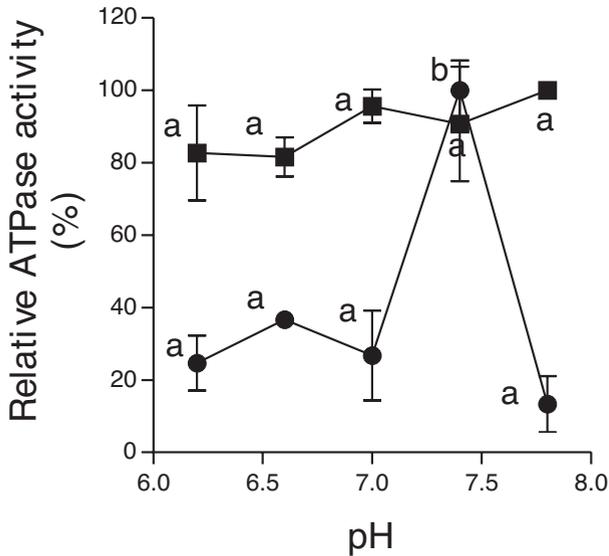


FIG. 2. – Effect of pH on Na⁺-K⁺ ATPase activity in chela muscle of *C. angulatus*. The values of Na⁺-K⁺ ATPase activity and residual (Mg²⁺-Na⁺) ATPase activity are expressed as a relation to the activity at pH 7.4 (100%, Na⁺-K⁺ ATPase activity: 66.3±5.5 and (Mg²⁺-Na⁺) ATPase activity: 110±28.6 nmoles Pi x min⁻¹ x mg prot⁻¹). In some cases, deviation bars were smaller than the symbols used. Data are the mean ± S.E. for three individuals. Circles: Na⁺-K⁺ ATPase activity. Squares: Residual (Mg²⁺-Na⁺) ATPase activity. Different letters indicate significant differences (p<0.05).

At pH between 6.2 and 7.0 and at pH 7.8 the activity was only about 20% of the maximal activity. Muscle residual (Mg²⁺-Na⁺) ATPase activity was not affected

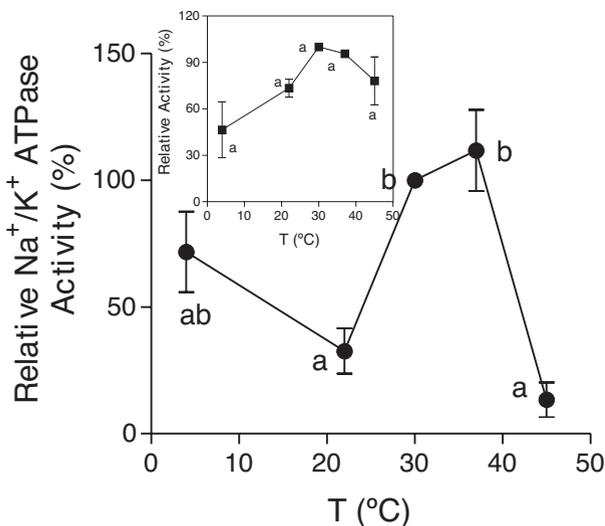


FIG. 3. – Effect of temperature on Na⁺-K⁺ ATPase activity at pH 7.4 in chela muscle of *C. angulatus*. Inset: Residual (Mg²⁺-Na⁺) ATPase activity. The values of Na⁺-K⁺ ATPase and residual (Mg²⁺-Na⁺) ATPase activities are expressed as a relation to the corresponding activity at 30°C (100%, Na⁺-K⁺ ATPase activity: 135.9±70.2 and (Mg²⁺-Na⁺) ATPase activity: 105.3±23.1 nmoles Pi x min⁻¹ x mg prot⁻¹). In some cases, deviation bars were smaller than the symbols used. Data are the mean ± S.E. for three individuals. Different letters indicate significant differences (p<0.05).

by pH within the range used (Fig. 2). Na⁺-K⁺ ATPase activity increased upon enhancement of temperature from 22 to 30-37°C. At higher temperature (45°C) Na⁺-K⁺ ATPase activity decreased strongly to about 13% of the activity at 30°C (Fig. 3). Residual (Mg²⁺-Na⁺) ATPase activity was similar within the same range of temperature (Fig. 3, Inset).

Levamisole-sensitive AP activity of muscle of *Cyrtograpsus angulatus*

Initially, AP activity of chela muscle was determined within the range of pH 7.4-10.0 in the absence and presence of the AP inhibitor levamisole (Fig. 4). The inhibition by levamisole revealed the presence in chela muscle of *C. angulatus* of a levamisole-sensitive AP activity which was high between the range of pH 7.4-8.4, and maximal at pH 8.0 (Fig. 4). Levamisole-sensitive AP activity was not inhibited by ouabain or activated by K⁺ (not shown). Further characterisation of AP activity in chela muscle of *C. angulatus* was made at physiological pH 7.4 and comparatively at pH 8.0. I₅₀ (the concentration that produced 50% levamisole-sensitive AP activity inhibition) was similar at pH 7.4 and 8.0 (8.8 and 8.0 mM, respectively) (Fig. 5). Levamisole-sensitive AP activity exhibited Michaelis-Menten kinetics; Km of p-nitrophenylphosphate being lower at the physiological pH (K_m= 3.451 mM and 6.906 mM at pH 7.4 and 8.0, respectively; Fig. 6). Levamisole-sensitive AP activity was notably affected by temperature. At 4°C, no activity at pH 7.4 was detected, whereas at pH 8.0 it was very low. At higher temperatures, levamisole-sensitive AP activity at both pH levels increased, being maximal at 37°C and decreasing by about 60% at 45°C (Fig. 7).

Effect of acclimation to low salinity on Na⁺-K⁺

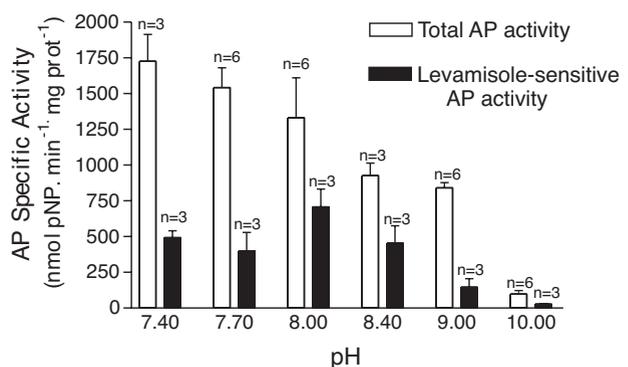


FIG. 4. – AP activity in chela muscle of *C. angulatus* in the absence and presence of 16 mM levamisole. Data are the mean ± S.E.

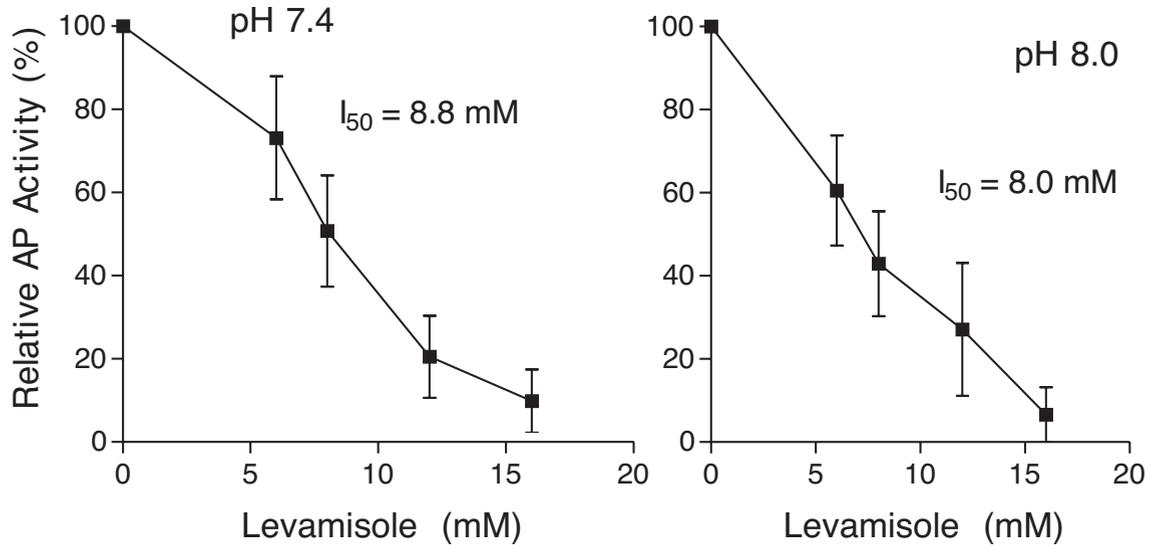


FIG. 5. – Dose-dependent inhibition of AP activity by levamisole in chela muscle of *C. angulatus* at pH 7.4. Maximal levamisole-sensitive AP activity was taken as 100% (1955±524.1 and 1689.5±278.1 nmoles pNP x min⁻¹ x mg prot⁻¹ at pH 7.4 and 8.0, respectively). I₅₀: levamisole concentration that produced 50% of inhibition, calculated by GraphPad Prism 2.01. Data are the mean ± S.E. for three individuals.

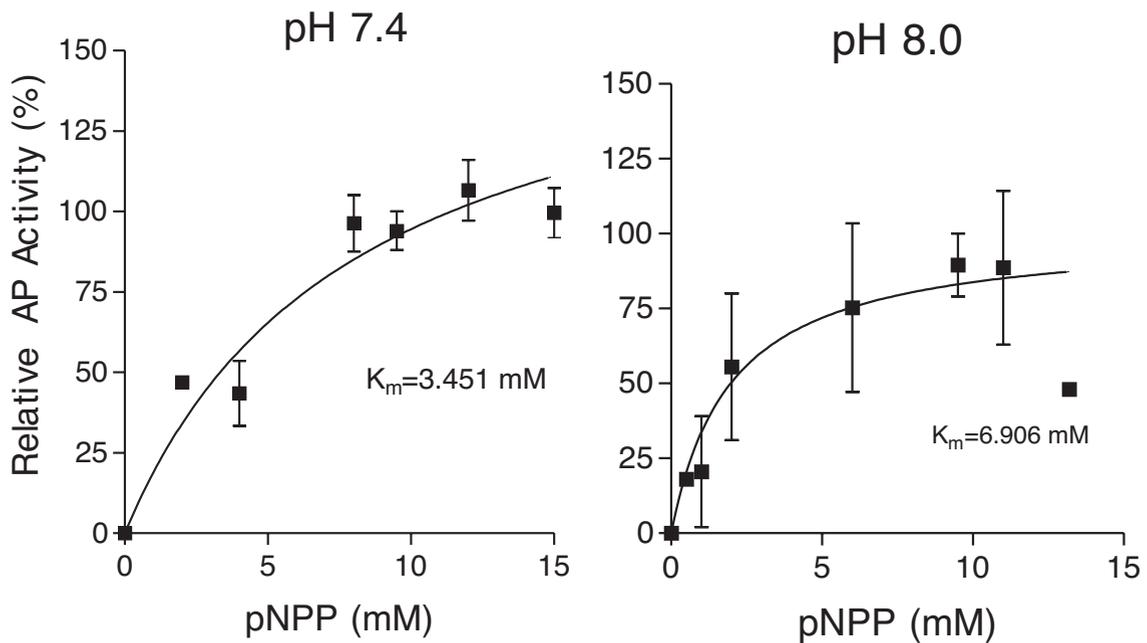


FIG. 6. – Effect of pNPP on levamisole-sensitive AP activity at pH 7.4 in chela muscle of *C. angulatus*. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The values of AP activity are expressed as a relation to the activity at 9.5 mM pNPP (100%). In some cases, deviation bars were smaller than the symbols used. Data are the mean ± S.E. for three individuals.

TABLE 1. – Concentration of ions (mEq l⁻¹) in external medium and in *C. angulatus* hemolymph.

	Medium	35 psu ^a Hemolymph	Medium	10 psu ^a Hemolymph
Na ⁺	420.8±18.8 (4)	406.7±18.8 (9)	179±3.2 (4)	332.8±6.6 (6)*
K ⁺	10.2±2.7 (4)	7.8±1.9 (9)	3.8±0.3 (4)	8.6±0.4 (6)*
Cl ⁻	497.6±34.2 (4)	440±12.1 (9)	152±3.3 (4)	363.3±16.1 (6)*

^a Hemolymph of crabs acclimated to either 35 or 10 psu. *Denotes significantly different from the corresponding concentration of the external medium (ANOVA, p<0.05). Data are the mean ± S.E. (): Number of specimens.

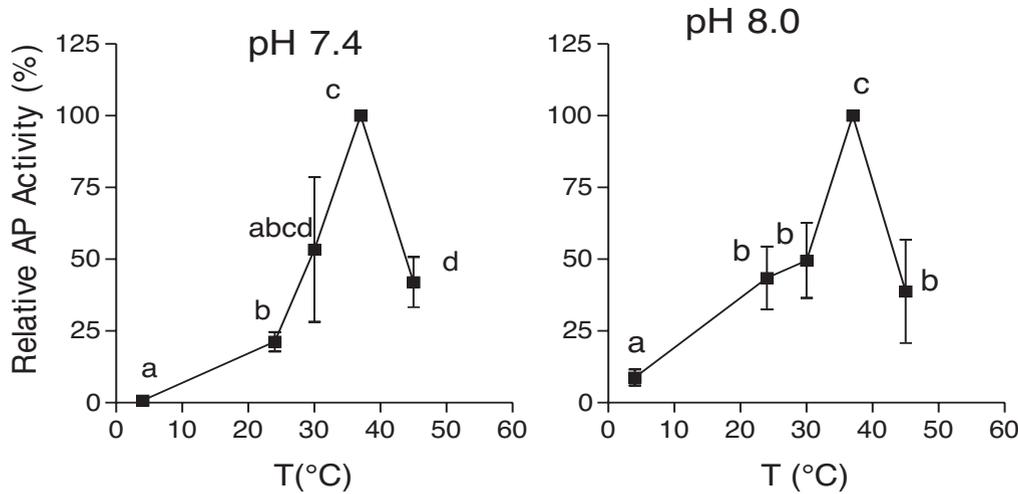


FIG. 7. – Effect of temperature (4–45°C) on levamisole-sensitive AP activity at pH 7.4 in chela muscle of *C. angulatus*. The values of AP activity are expressed as a relation to the corresponding activity at 37°C (100%, 464.3±61.4 and 1186.6±199.7 nmoles pNP x min⁻¹ x mg prot⁻¹ at pH 7.4 and 8.0 respectively). In some cases, deviation bars were smaller than the symbols used. Data are the mean ± S.E. for three individuals. Different letters indicate significant differences (p<0.05).

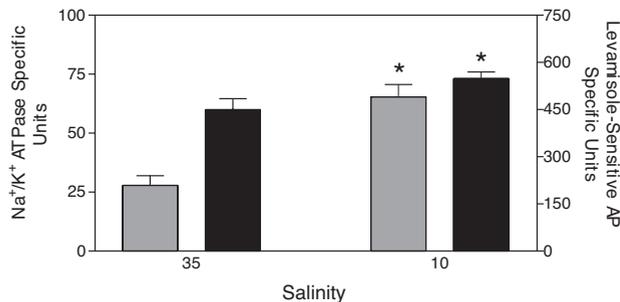


FIG. 8. – Effect of acclimation to low salinity on Na⁺-K⁺ ATPase activity (grey bars) and levamisole-sensitive AP activity at pH 7.4 (black bars) in chela muscle of *C. angulatus*. Data are the mean ± S.E. Number of specimens given in Table 2. * Significantly different from the corresponding activity of individuals acclimated to 35 psu (p<0.05). Units: Na⁺-K⁺ ATPase: nmol Pi x min⁻¹ x mg prot⁻¹, AP: nmol pNP x min⁻¹ x mg prot⁻¹.

ATPase and levamisole-sensitive AP activity of muscle of *Cyrtograpsus angulatus*

To determine the effect of environmental salinity on Na⁺-K⁺ ATPase and on levamisole-sensitive AP activity at the physiological pH in chela muscle of *C. angulatus*, specimens were acclimated to 35 and 10 psu, salinities at which this crab ionoconforms

and hyperregulates, respectively (Table 1). In crabs acclimated to 35 psu, chela muscle exhibited a low Na⁺-K⁺ ATPase activity (27.9±3 nmoles Pi x min⁻¹ x mg prot⁻¹). In individuals acclimated to low salinity Na⁺-K⁺, ATPase activity was about 2.3 times higher (65±5 nmoles Pi x min⁻¹ x mg prot⁻¹) than at 35 psu (Fig. 8; Table 2). No differences in residual (Mg²⁺-Na⁺) ATPase activity were found in muscle of individuals acclimated to either 35 or 10 psu (Table 2).

In individuals acclimated to 10 psu, levamisole-sensitive AP activity at pH 7.4 (548.5 ± 21.5 nmol pNP min⁻¹ mg protein⁻¹) was higher than the activity in crabs acclimated to 35 psu (449.7 ± 34.8 nmol pNP min⁻¹ mg protein⁻¹; Fig. 8; Table 2). Levamisole-insensitive AP activity was not affected by acclimation of crabs to 10 psu (Table 2).

DISCUSSION

Our results show the occurrence of a Na⁺-K⁺ ATPase activity and a levamisole-sensitive AP activity in chela muscle of the euryhaline crab *C. angulatus*, which increased upon acclimation to low

TABLE 2. – Na⁺-K⁺ ATPase, (Mg²⁺-Na⁺) ATPase and AP specific activities in chela muscle of *C. angulatus*.

Salinity	Na ⁺ -K ⁺ ATPase (nmoles Pi x min ⁻¹ x mg prot ⁻¹)	(Mg ²⁺ -Na ⁺) ATPase	Levamisole-Sensitive AP (nmoles pNP x min ⁻¹ x mg prot ⁻¹)	Total AP
35	27.9 ± 3.1 (5)	61.1 ± 3.1 (5)	449.7 ± 34.8 (7)	2137.7 ± 426.6 (6)
10	65.4 ± 5.3 (5)*	99.8 ± 20.3 (5)	548.5 ± 21.5 (5)*	2119.0 ± 401.6 (5)

*Denotes significantly different from the corresponding activity at 35 psu (ANOVA, p<0.05). Data are the mean ± S.E. (): Number of specimens.

salinity. Na⁺-K⁺ ATPase in chela muscle of *C. angulatus* appeared to be quite sensitive to ATP (Fig. 1). In crustaceans, the pattern of response of Na⁺-K⁺ ATPase activity to varying ATP concentrations varies according to species. Na⁺-K⁺ ATPase activity in gills of various crabs exhibits Michaelis-Menten kinetics (Neufeld *et al.*, 1980; D'Orazio and Holliday, 1985; Corotto and Holliday, 1996). However, Na⁺-K⁺ ATPase from posterior gills of the swimming crab *Callinectes danae* follows a biphasic curve (Masui *et al.*, 2002). In gill homogenates from the air-breathing crab *Leptograpsus variegates*, Na⁺-K⁺ ATPase activity exhibited a markedly low optimum ATP concentration (0.87 mM), being inhibited at higher ATP concentrations (Cooper and Morris, 1997).

The strong dependency on pH of Na⁺-K⁺ ATPase activity of chela muscle of *C. angulatus* (Fig. 2) is in agreement with that found for this activity from crustaceans (reviewed by Lucu and Towle, 2003). Branchial Na⁺-K⁺ ATPase activity shows optimum pH values of between 7.0 and 7.7 in species so far studied (reviewed by Lucu and Towle, 2003). In homogenates from anterior and posterior gills of *C. granulatus* the optimum pH of Na⁺-K⁺ ATPase activity was found in a sharp maximal at about 7.4-7.6, a dramatic decrease in activity occurring at lower and higher pH values (Castilho *et al.*, 2001). The strong dependency on temperature and the low Na⁺-K⁺ ATPase activity in chela muscle of *C. angulatus* at high temperature, 45°C (Fig. 3), is similar to that described for this activity in homogenates from posterior gills of *C. granulatus* (Castilho *et al.*, 2001; Genovese *et al.*, 2004). The highest Na⁺-K⁺ ATPase activity in chela muscle of *C. angulatus* at 30-37°C is in agreement with the optimum temperature for this activity in gills from several crustaceans (reviewed by Lucu and Towle, 2003).

Levamisole, a well-known AP inhibitor, is commonly used to discriminate between mammal AP isoforms and in clinical studies (Van Belle, 1976; Calhau *et al.*, 2000; Ali *et al.*, 2006a). In euryhaline crabs, the occurrence of a levamisole-sensitive AP activity has been shown in gill homogenates of *C. sapidus* (Lovett *et al.*, 1994). *C. angulatus* from Mar Chiquita coastal lagoon exhibited a levamisole-sensitive AP activity which is high within the broad range of pH 7.4-8.4 (Fig. 4). AP from different invertebrate tissues exhibits an optimum pH ranging between 7.1 and 10.9 (Lovett *et al.*, 1994; Itoh *et al.*, 1999; Funk, 2001; Mazon *et al.*, 2002; Xiao *et al.*, 2002). The response to pH of levamisole-sensitive AP activity in chela muscle of *C. angulatus* was also similar to

that described for this activity in chela muscle of *C. granulatus* (Pinoni *et al.*, 2005). The Michaelis-Menten kinetics of levamisole-sensitive AP activity of chela muscle of *C. angulatus* (Fig. 6) is in agreement with that described for this activity in gills of *C. sapidus* (Lovett *et al.*, 1994) and in chela muscle of *C. granulatus* (Pinoni *et al.*, 2005).

Animal APs from different tissues exhibit a variable sensitivity to temperature (Olsen *et al.*, 1991; Åsgeirsson *et al.*, 1995; Funk, 2001). In invertebrates, the optimum temperature of AP activity appeared to be species- and tissue-dependent. The response of levamisole-sensitive AP activity of chela muscle of *C. angulatus* to temperature (Fig. 7) is in agreement with that found for this activity in chela muscle of *C. granulatus* (Pinoni *et al.*, 2005). An inhibition of AP activity at high temperatures has also been described for AP purified from viscera of *P. fucata* (Xiao *et al.*, 2002) and from the digestive tract of *S. serrata* (Chen *et al.*, 1997).

The ability of hyperregulating crabs to adapt to varying environmental salinity may imply responses ranging from the molecular level to the organism. In dilute media, hyperregulating crabs absorb both sodium and chloride from the external medium via the gills, thus regulating their concentrations in the hemolymph and compensating for salt losses. In posterior gills of several hyperregulating crabs, adaptive increases of Na⁺-K⁺ ATPase activity occurs both upon acclimation and after transfer to reduced salinity. Thus, this enzyme plays a central role in the biochemical adaptation to low salinity (reviewed by Lucu and Towle, 2003; Towle, 1997; Kirshner, 2004). We have previously shown the occurrence of differential changes in Na⁺-K⁺ ATPase activity of anterior and posterior gills of *C. angulatus* from the Mar Chiquita coastal lagoon suggesting that this enzyme is a component of the biochemical adaptations of this crab to low salinity (López Mañanes *et al.*, 2002). The participation of Na⁺-K⁺ ATPase activity as a component of the biochemical adaptation of *C. angulatus* to environmental salinity in other tissues is still unknown. The higher Na⁺-K⁺ ATPase activity of chela muscle of *C. angulatus* at 10 psu (Fig. 8), a salinity at which this crab exhibits a strong hyperregulatory capacity (Table 1), along with the fact that under osmoionconforming conditions (35) Na⁺-K⁺ ATPase activity in chela muscle was low (Fig. 8), suggest the role of this enzyme and of the muscle in regulatory mechanisms at the biochemical level secondary to hyperregulation. In mammal skeletal

muscle, Na⁺-K⁺ ATPase activity has been involved in the response to various stressful conditions and the maintenance of resting membrane potential and osmotic balance. The Na⁺ gradient created by the enzyme also appears to be the driving force for the maintenance of Na⁺-dependent secondary processes such as transport of nutrients and Ca²⁺ (Clausen, 1996; McCarter *et al.*, 2001). In frog muscle, Na⁺-K⁺ ATPase has been shown to have a role in mechanisms of adjustment to hypo-osmotic stress (Venosa, 1991, 2003). Muscle of euryhaline crabs has been involved in cellular volume regulation (Lang and Gaener, 1969) and acid-base balance (Whiteley *et al.*, 2001), and provides an energy source through mobilisation of lipids (Luvizotto-Santos *et al.*, 2003) under hypo-osmotic stress. The enhanced Na⁺-K⁺ ATPase activity in chela muscle of individuals acclimated to low salinity (Fig. 8; Table 2) could support the ion concentrations and electrochemical gradients necessary for functioning of the transport systems that are probably involved in these physiological processes secondary to osmo-ionoregulation.

The role of AP as a component of euryhaline crabs' responses to environmental salinity has received little attention (Pinoni and López Mañanes, 2004). Levamisole-sensitive and levamisole-insensitive AP activities in the gills of *C. sapidus* have been shown to decrease upon acclimation of crabs to low salinity (Lovett *et al.*, 1994). AP has been suggested to be an effector for the adaptive changes of this crab to low salinity in branchial Na⁺-K⁺ ATPase activity. AP regulates the synthesis or delivery of polyamines which, in turn, modulate Na⁺-K⁺ ATPase activity (Lovett *et al.*, 1994). The higher levamisole-sensitive AP activity at physiological pH in chela muscle of *C. angulatus* (Fig. 8) acclimated to low salinity suggests that this activity could also be a component of the biochemical adaptation to low salinity in this crab. Several physiological processes in animals are regulated via dephosphorylation of key components (i.e. several enzymes) mediated by different phosphatase activities. In chela muscle of *C. angulatus*, it remains to be established whether levamisole-sensitive AP activity is involved in phosphorylation/dephosphorylation processes regulating key components involved in mechanisms underlying biochemical adaptation to low salinity (i.e. cell volume and acid-base regulation, and mobilisation of energy substrates). Whether the increase of levamisole-sensitive AP activity could be related to a physiological link with Na⁺/K⁺ ATPase activity in chela muscle of *C. angulatus*, as was suggested in the gills of *C. sapidus*

(Lovett *et al.*, 1994), requires further investigation. In muscle of the squirrel, Na⁺/K⁺ ATPase has been described to be regulated via dephosphorylation by AP (Mac Donald and Storey, 1999).

In summary, our results show the existence of Na⁺-K⁺ ATPase and levamisole-sensitive AP activities in muscle of *C. angulatus*. The response of Na⁺-K⁺ ATPase and of levamisole-sensitive AP activities to low salinity under hyperregulating conditions suggests the participation of these enzymes in responses at the biochemical level to varying environmental salinity. Whether or not these activities in chela muscle of *C. angulatus* are involved in physiological processes secondary to osmo-ionoregulation (i.e. cell volume regulation, acid-base equilibrium, mobilisation of substrates) remains to be investigated. Future studies should focus on establishing the exact physiological roles of these muscle activities in the integrative adaptive responses of euryhaline crabs to varying environmental conditions.

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