The Journal of Experimental Biology 205, 1775–1785 (2002)
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JEB4027

Osmotic effects on arginine kinase function in living muscle of the blue crab *Callinectes sapidus*

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Accepted 8 April 2002

Summary

Flux was examined through the reaction catalyzed by arginine kinase in intact blue crab (*Callinectes sapidus*) muscle during simulated changes in salinity. Isolated dark levator muscles from the swimming leg were superfused with a saline solution that had an osmolarity equivalent to that of the hemolymph under different salinity regimes. Animals were acclimated for 7 days to a salinity of 5, 17 or 35‰, which corresponds to a hemolymph osmolarity of 640, 720 or 960 mosmol l^{-1}, respectively. Experiments were conducted under control conditions, in which the osmolarity of the superfusion medium matched that of the acclimated hemolymph, as well as under hypo- and hyperosmotic conditions. These latter treatments were meant to simulate a rapid change in environmental salinity. Pseudo-first-order unidirectional rate constants and flux rates were measured for arginine kinase in the forward and reverse directions using a ^{31}P-nuclear magnetic resonance saturation transfer method. There were no differences in the rate constants or flux rates among the controls, indicating that arginine kinase function is not modulated by salinity if the animal has had sufficient acclimation time. However, the rate constants and flux rates of arginine kinase varied over a modest 1.7-fold range across the three types of osmotic treatments, although the range for the flux data was reduced when cell volume changes were taken into account. The hyperosmotic treatments led to a reduction in arginine kinase flux, while the hypo-osmotic treatments led to an enhanced arginine kinase flux. We propose that this effect is mediated by an increase in the concentration of perturbing inorganic ions under hyperosmotic conditions and a decrease in the concentration of such ions during the hypo-osmotic treatments.

Key words: arginine kinase, osmoregulation, haemolymph, muscle, blue crab, *Callinectes sapidus*, crustacean, nuclear magnetic resonance, salinity.

Introduction

The blue crab *Callinectes sapidus* inhabits estuarine environments that range in salinity from full-strength sea water to fresh water (Mangum and Amende, 1972; Lynch et al., 1973; Cameron, 1978). Like many euryhaline organisms, blue crabs have evolved compensatory mechanisms to minimize perturbations to the intracellular environment during osmotic stress. While the hemolymph of blue crabs fluctuates iso-osmotically with the environment at salinities above 25‰, below this threshold the hemolymph is maintained hyperosmotic relative to the surrounding water (Ballard and Abbott, 1969; Mangum et al., 1985; Lynch et al., 1973; Robinson, 1994). The major site of active osmoregulation in blue crabs is the posterior gills, where a number of membrane-bound ion pumps and other transport-related enzymes associated with osmoregulation are located (Mantel and Farmer, 1983; Lucu, 1990; Towle, 1997; Towle and Weihrauch, 2001). In addition, reductions in the permeability of membranes to inorganic ions and water can play a role in hyperosmoregulation in the blue crab (Robinson, 1982, 1994).

Although the blue crab is a capable osmoregulator at low salinities, hemolymph osmolarity may still fluctuate from approximately 600 to 1000 mosmol l^{-1} as the environmental salinity changes from 0 to 35‰, respectively (Lynch et al., 1973; Mantel and Farmer, 1983; Piller et al., 1995). Since the tissues remain iso-osmotic with the hemolymph, the intracellular osmolyte composition and concentrations may change dramatically when the animal experiences a change in environmental salinity (Gérard and Gilles, 1972; Gilles, 1979). One way in which the tissues of blue crabs, like those of other euryhaline organisms, compensate for salinity challenges is by altering the concentrations of free amino acids and of other compatible or counteracting solutes to maintain cells in an iso-osmotic condition (Gérard and Gilles, 1972). These compounds help to maintain proper cell volume without the destabilizing effects on protein structure that are typically induced by inorganic ions (Brown and Simpson, 1972; Clark and Zounes, 1977; Bowlus and Somero, 1979; Yancey et al., 1982; Pierce, 1982). A large body of *in vitro* studies conducted

by these and other authors have detailed the nature of the perturbing effects of inorganic ions on enzymes and some of the mechanisms by which compatible and counteracting solutes preserve enzyme function (for reviews, see Yancey, 1994, 2001). More recently, it has been demonstrated that cells made hypertonic with elevated levels of NaCl experience double-strand breaks in DNA (Kültz and Chakravarty, 2001) and induce heat-shock proteins (Petronini et al., 1993; Sheikh-Hamad et al., 1994). These studies underscore the protective effects of organic osmolytes, since cells made hypertonic with the stabilizing solute urea did not undergo DNA degradation (Kültz and Chakravarty, 2001), and heat-shock protein induction was inhibited by the presence of the compatible solute betaine (Petronini et al., 1993; Sheikh-Hamad et al., 1994).

Despite a sizable literature describing the effects of solutes on proteins, the consequences of salinity challenges and the associated alterations in the intracellular environment on enzyme function in intact organisms or tissues are largely unknown. Osmotic stress may be particularly disruptive to cell function during rapid changes in salinity since the compensatory adjustments of intracellular organic solute concentrations often occur over a period of hours or days, lagging behind the onset of changes in hemolymph osmolarity (Dall, 1975; Bartberger and Pierce, 1976; Gilles, 1979; Pierce, 1982). During this transient period of acclimation, inorganic ions that perturb enzyme function may be the principal intracellular osmolytes involved in cell volume regulation (Warren and Pierce, 1982).

Arginine kinase (AK) is a member of the phosphagen kinase family that catalyzes the reversible transfer of a high-energy phosphate from the phosphagen arginine phosphate to ADP to form ATP:

$$\text{Arginine phosphate} + \text{MgADP} + \alpha\text{H}^+ \leftrightarrow \text{Arginine} + \text{MgATP}. \quad (1)$$

Here, α represents a partial proton. In muscle, AK functions as a temporal ATP buffer during rest-to-work transitions and it may also serve to buffer ATP concentration spatially during sustained contractile activity (for a review, see Ellington, 2001). AK is also one of a handful of enzymes whose function can be examined in living tissue using nuclear magnetic resonance (NMR) magnetization transfer methods. ^{31}P-NMR has been applied extensively in comparative physiology for the non-invasive assessment of the relative levels of phosphagen, ATP and inorganic phosphate (P_i) in living cells, tissues and whole organisms (for reviews, see Wasser et al., 1996; van den Thillart and van Waarde, 1996). To date, NMR magnetization transfer is the only method suitable for the non-invasive measurement of the forward and reverse flux rates of enzymatic reactions.

AK has been the subject of more *in vivo* kinetic analyses than any other phosphagen kinase except creatine kinase, which is the functional analog to AK found in vertebrates and some invertebrates. Magnetization transfer NMR has been employed to measure steady-state reaction flux in the phasic adductor muscle of the scallop *Argopecten irradians* (Graham et al., 1986) and the abdominal muscle of the crayfish *Orconectes virilis* (Butler et al., 1985). The *in vivo* temperature-dependence of AK has been examined in leg muscle of the crab *Carcinus maenas* (Briggs et al., 1985) and in abdominal muscle of the shrimp *Sycionia ingentis* (Fan et al., 1992). More recently, magnetization transfer was used to assess the effects of pentachlorophenol and hypoxia on the rates of AK flux in red abalone *Haliotis rufrescens* (Shofer et al., 1996). However, to our knowledge, there is no information regarding intracellular AK function related to physiologically relevant variations in environmental salinity.

The present study used ^{31}P-NMR saturation transfer to examine AK flux in isolated blue crab muscle in osmotic steady state and under hyperosmotic and hypo-osmotic conditions. AK flux varied by nearly twofold across the entire range of osmotic conditions examined, although the enzyme appeared to be largely unaffected by moderate osmotic challenges.

Materials and methods

Animal maintenance

Specimens of *Callinectes sapidus* Rathbun were bought at a local seafood store in Wilmington, NC, USA. The animals were maintained at room temperature in air-equilibrated 55-gallon (200 l) tanks and were fed shrimp daily. Crabs were held for 7 days at a salinity of 35, 17 or 5 ‰. This salinity is referred to throughout as the acclimation salinity. Crabs were packed in ice for 15 min prior to dissection.

Arginine phosphate metabolite assays

Arginine phosphate concentrations in muscle were measured spectrophotometrically for animals that had been exposed to each of the acclimation salinities. The concentrations of ATP and P_i could then be determined from their NMR peak area relative to the peak area of arginine phosphate (see below). Between 200 and 500 mg of dark levator muscle was homogenized using a Fisher Powergen 125 homogenizer in 2.5 ml of ice-cold 7 % $HClO_4$ containing 1 mmol l^{-1} EDTA. The sample was centrifuged for 10 min at 9880 ***g*** in an Eppendorf 5415C microcentrifuge at 4 °C. The supernatant was titrated to pH 6.5 using 3 mol l^{-1} KOH containing 50 mmol l^{-1} Pipes and allowed to sit on ice for 10 min. The sample was centrifuged again at 9880 ***g*** for 15 min at 4 °C. The concentrations were measured *via* an enzymatically linked assay. The AK reaction was coupled to the production of NADPH, which was monitored at a wavelength of 340 nm on a Pharmacia Ultrospec 4000 spectrophotometer. The assay medium contained 30 mmol l^{-1} Tris/HCl, 2.5 mmol l^{-1} $MgCl_2$, 2.5 mmol l^{-1} D-glucose, 0.63 mmol l^{-1} NADP, 150 units of glucose-6-phosphate dehydrogenase and the muscle extract. Hexokinase (3 units) was then added, and the absorbance was allowed to stabilize following the consumption of endogenous ATP. This was followed by the addition of 0.5 mmol l^{-1} ADP and 5 units of arginine kinase; the observed change in

absorbance was proportional to the concentration of arginine phosphate.

Measurement of arginine kinase flux

Swimming leg dark levator muscle was excised and immediately transferred to a Petri dish containing blue crab saline solution gassed with a mixture of 99.5 % O_2/0.5 % CO_2. The white muscle was mechanically stripped to isolate the dark levator from surrounding muscle. The superfusion medium meant to mimic that of animals exposed to a salinity of 35 ‰ had an osmolarity of 960 mosmol l^{-1} and consisted of 470 mmol l^{-1} NaCl, 8 mmol l^{-1} KCl, 15 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} $MgSO_4$ and 10 mmol l^{-1} Hepes at pH 7.4 (Tse et al., 1983). The solutions for animals maintained at the more dilute salinities of 17 and 5 ‰ were prepared using the same ionic composition as described above with the addition of deionized water to dilute the saline solution to 720 and 640 mosmol l^{-1}, respectively. Osmolarity was measured using a VAPRO 5520 vapor-pressure osmometer.

The isolated muscle was tied at resting length to a plastic capillary tube using 3-0 surgical suture. The muscle was then placed in a 10 mm diameter NMR tube and connected to a superfusion system with peristaltic pumps that continuously washed the tissue with oxygenated saline solution at a flow rate of 10 ml min^{-1}. The temperature was maintained at 20 °C with a 1016S Isotemp recirculating water bath. The muscle preparation and superfusion tubing were lowered into the NMR magnet where the sample was 'mated' with a 10 mm NMR probe. Experiments were designed to simulate the ionic environment experienced by the muscle *in vivo* during a rapid change in environmental salinity. AK flux was therefore measured under control, hypo-osmotic and hyperosmotic conditions (Table 1). For example, a muscle from a control animal was superfused with a medium equivalent to the osmolarity existing in the animal's blood after the 7-day exposure (e.g. an animal exposed to a 5 ‰ environmental salinity and the muscle preparation exposed to a superfusion medium of 640 mosmol l^{-1}; Piller et al., 1995). In contrast, a hyperosmotic shock treatment involved superfusing the muscle in a saline solution with an osmolarity greater than that existing in the animal's blood after the 7-day exposure period (e.g. an animal exposed to a 5 ‰ environmental salinity and the muscle preparation exposed to a superfusion medium of 960 mosmol l^{-1}). This example would simulate a rapid move from a salinity of 5 to 35 ‰.

NMR spectra were obtained using a Bruker 400 MHz DMX spectrometer housed in the Department of Chemistry at the University of North Carolina at Wilmington. The sample was tuned to the phosphorus precessional frequency of 162 MHz and shimmed on the residual proton signal arising from water to optimize magnetic field homogeneity. Initial spectra were obtained to ensure tissue viability. These spectra were acquired using a 45 ° (25 μs) excitation pulse and a 1 s relaxation delay. Ninety scans were acquired, and 25 Hz exponential line-broadening was applied before Fourier transformation.

To calculate AK flux from the saturation transfer data, it is necessary to determine the actual concentrations of arginine phosphate and ATP at the time the NMR measurement is made. As stated above, we directly measured the arginine phosphate levels spectrophotometrically (and we inferred the amount of ATP from the NMR spectrum) at the three acclimation salinities. However, changing the osmolarity of the superfusion medium may lead to energetic challenges to the tissue that result in changes in the concentrations of arginine phosphate and ATP. Therefore, for a subset of hyperosmotic and hypo-osmotic treatment groups, a time series of spectra was acquired to assess new steady-state concentrations of metabolites and ensure stable levels of high-energy phosphates during the experiment. The same parameters were used as previously described for initial spectra. In addition, the intracellular pH (pHi) of the muscle preparation was determined in each experiment from the chemical shift of the P_i peak relative to that of arginine phosphate (Kinsey and Moerland, 1999).

The pseudo-first-order unidirectional rate constants in the forward and reverse directions ($k_{forward}$ and $k_{reverse}$) were measured for the AK reaction using a saturation transfer method (Briggs et al., 1985; Graham et al., 1986). A 10 s low-power pulse was used to saturate selectively either the resonance for arginine phosphate or that for γ-ATP. This was followed immediately by a 90 ° (45 μs) broadband excitation

Table 1. *Treatments used for experiments included a 7-day exposure to one of three acclimation salinities and, at the time of each experiment, isolation of the muscle and exposure to one of three experimental osmolarities*

Acclimation salinity (‰)	Osmolarity (mosmol l^{-1})	Experimental osmolarity (mosmol l^{-1})			Predicted [organic osmolyte]
		960	720	640	
35	1000	Control	Hypo-osmotic	Hypo-osmotic	High
17	486	Hyperosmotic	Control	Hypo-osmotic	Intermediate
5	143	Hyperosmotic	Hyperosmotic	Control	Low

Control groups were those in which the osmolarity of the superfusion medium matched that observed for hemolymph from acclimated animals, based on literature values.

To simulate a change in environmental salinity, the superfusion medium was changed to an osmolarity that was greater than (hyperosmotic) or less than (hypo-osmotic) that of the blood in the acclimated state.

Organic osmolyte concentrations are predicted to be proportional to the acclimation salinity, but not to vary during the time course of the hypo- or hyperosmotic experiments.

pulse that allowed for signal detection. One hundred and twenty-four scans were averaged, so that each spectrum was collected in 21 min. To measure the forward reaction, the γ-ATP resonance was saturated; for the reverse reaction, the arginine phosphate resonance was saturated. These spectra were compared with control spectra in which no peak was saturated. Here, the saturating pulse was applied at the opposite side of the unsaturated peak at the same frequency offset as in the corresponding saturated spectrum. This controls for possible partial saturation of the unsaturated peak during selective irradiation. The total experimental time was 84 min, which included the two saturated and the two control spectra. Peaks were integrated to determine the area under each peak using Bruker X-Win NMR software. The rate constant k was calculated from the expression:

$$k = [(1 - (M^+/M^0)](T_1)^{-1}, \quad (2)$$

where M^+/M^0 is the ratio of the magnetization of the unsaturated metabolite resonance in the presence of the saturating irradiation at the exchanging site to the magnetization of the metabolite resonance in the control spectrum. M^+ and M^0 were determined by integrating the area under each peak in the NMR spectrum. T_1 is the longitudinal relaxation time constant for the unsaturated resonance in the presence of saturating irradiation at the exchanging site. The T_1 values used were 2 s for arginine phosphate and 0.9 s for ATP, which were previously measured in crustacean muscle at 20 °C (Butler et al., 1985) and are very similar to the values measured by Briggs et al. (1985) in crab leg muscle at 23 °C. The reaction flux is determined by multiplying the rate constant, $k_{forward}$ or $k_{reverse}$, by the concentration of arginine phosphate or γ-ATP, respectively.

Arginine kinase activity assays

Maximal AK activity was measured spectrophotometrically to determine its salinity-dependence and to compare *in vitro* activity with *in vivo* flux. It was assumed that, during the 84 min experiments, there would be no significant osmolarity-induced changes in the total amount of AK, so activity was measured for the control animals only. We believe that this assumption is valid because, to our knowledge, there is no evidence of AK induction by salinity or any other environmental variable in crustacean muscle. This is probably because the principal function of AK in crustacean muscle is temporal ATP buffering (see Discussion), and upregulation of the enzyme would not enhance this function. Further, AK occurs in very high concentration in crustacean muscle, so if protein turnover in general were affected by the experimental treatments, the percentage change in AK concentration is likely to be relatively small.

The tissue was homogenized in 9 vols of 100 mmol l^{-1} glycine and 10 mmol l^{-1} β-mercaptoethanol (pH 8.6). The homogenate was centrifuged in a Beckman J2-21M/E for 10 min at 12 000 ***g*** at 4 °C. Assays were performed using a Pharmacia Ultrospec 4000 spectrophotometer with the temperature maintained at 20 °C. The activity was measured by enzymatically linking the AK reaction to the oxidation of NADH, which was monitored at a wavelength of 340 nm. The assay medium was 65 mmol l^{-1} Tris/HCl (pH 8.0), 38 mmol l^{-1} KCl, 13 mmol l^{-1} magnesium acetate, 5 mmol l^{-1} ATP, 1.25 mmol l^{-1} phosphoenolpyruvate, 0.25 mmol l^{-1} NADH and excess pyruvate kinase/lactate dehydrogenase. Basal ATPase activity was initiated by the addition of the extract. After a steady-state trace had been recorded, 10 mmol l^{-1} arginine was added to the mixture to initiate arginine kinase activity.

^{1}H-NMR

^{1}H-NMR was used to measure the relative concentration of organic osmolytes in muscle tissue under each experimental condition. Tissue was prepared following the same time course of osmolarity exposure as in the saturation transfer experiments. Since the ^{1}H-NMR spectra could be collected in several minutes, the samples were not superfused. The sample was placed in a 5 mm NMR tube, shimmed to the proton signal arising from water and tuned to 400 MHz. The water resonance was suppressed using a 5 s presaturation pulse. The excitation pulse was 11 μs, the relaxation delay was 5 s and 128 scans were acquired. The total experiment time was 12 min. A 0.50 Hz exponential line-broadening function was applied before Fourier transformation.

Statistical analyses

Normality of data was determined using a χ^2 goodness-of-fit test, and homogeneity of variances was assessed using Bartlett's test. Two-way analysis of variance (ANOVA) was used to test all NMR-derived data for significant effects of acclimation salinity, experimental osmolarity of the medium and for interaction of these two parameters. One-way ANOVA was used to test for significant effects of acclimation salinity for NMR-derived data from control animals and for spectrophotometrically measured arginine phosphate concentrations and AK activities. Student's *t*-tests were used for pairwise comparisons. Linear regression analysis was used to assess the dependence of AK rate constants and flux rates on the extent of osmotic stress. The level of significance for analysis was $P<0.05$, and the data are reported as means ± S.E.M. All data were analyzed using SAS-JMP statistical software version 4.04 (SAS Institute, Cary, NC, USA).

Results

Metabolite concentrations

NMR time series indicated that the high-energy phosphate profiles in the isolated muscle exhibited long-term stability even during the most extreme hypo- or hyperosmotic treatments (Fig. 1). No changes in the peak areas for arginine phosphate, γ-ATP or P_i were seen for any of the treatments. Therefore, the arginine phosphate concentrations that were measured spectrophotometrically were considered to be constant within the three acclimation salinities for a period of at least 2 h.

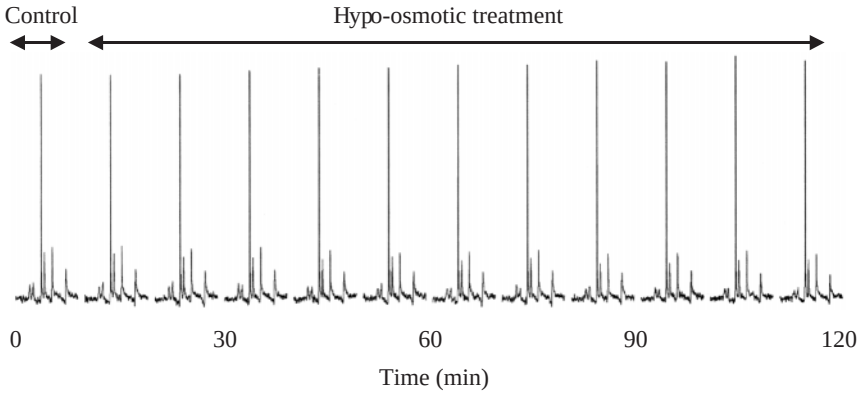

Fig. 1. Representative ^{31}P-NMR spectra arranged in time series during an extreme hypo-osmotic treatment. From left to right, the peaks are sugar phosphate, inorganic phosphate (P_i), arginine phosphate (tallest peak) and the γ, α and β peaks of ATP. The tissue was acclimated to 35‰ salinity and exposed to a medium simulating a transfer to 5‰. Each spectrum was collected over a period of 10 min, so the total experimental time was 2 h. The first spectrum was collected under control conditions in which the tissue was superfused with a medium of 960 mosmol l^{-1}, and all subsequent spectra were collected while the muscle was superfused with a medium of 640 mosmol l^{-1}. Several such stability experiments were conducted for the hypo- and hyperosmotic treatments and, in each case, the arginine phosphate, ATP and P_i peak areas remained constant. This indicates that the tissues were not energetically compromised during the flux experiments.

Table 2 includes the concentrations of arginine phosphate, γ-ATP and P_i for the control treatments, determined spectrophotometrically (arginine phosphate) and using NMR (γ-ATP, P_i). In addition, concentrations are presented for the hypo- and hyperosmotic treatments, which were derived exclusively from the NMR data using the control concentrations of arginine phosphate, to illustrate the stability of the concentrations of high-energy phosphate compounds (Table 2). One-way ANOVA indicated that muscle arginine phosphate concentration varied significantly across the experimental salinities from 11.9 μmol g^{-1} wet mass for animals acclimated to 17‰ to 17 μmol g^{-1} wet mass for animals acclimated to 35‰. Animals from 5‰ salinity had intermediate muscle arginine phosphate concentrations. Two-way ANOVA was used to test for an effect of acclimation salinity, experimental osmolarity or an interaction of the two on the other high-energy phosphate concentrations. There were no significant differences in the levels of γ-ATP or P_i or in the arginine phosphate/γ-ATP or γ-ATP/P_i concentration ratios across either acclimation salinity or experimental osmolarity. Further, acclimation salinity or experimental osmolarity did not significantly affect pHi (Table 2).

Table 2. *Metabolite levels and pHi measured using coupled enzyme assays (for arginine phosphate) and ^{31}P-NMR (for all other variables)*

	Controls			Hypo-osmotic treatment			Hyperosmotic treatment		
Acclimation salinity (‰)	35	17	5	35	35	17	17	5	5
Experimental osmolarity (mosmol l^{-1})	960	720	640	640	720	640	960	720	960
AP concentration* (N=4 for each)	17.0±0.9	11.9±1.3	14.4±0.9	17.0±0.9	11.9±1.3	14.4±0.9	17.0±0.9	11.9±1.3	14.4±0.9
[γ-ATP] (N)	3.6±0.3 (6)	3.6±0.5 (5)	3.9±0.5 (7)	3.8±0.2 (6)	5.4±0.6 (5)	3.6±0.5 (3)	3.3±0.3 (5)	4.4±0.4 (5)	4.4±0.6 (5)
[P_i]	3.6±1.1	4.9±1.9	6.7±0.8	4.0±1.3	4.7±1.0	3.5±0.5	3.5±0.5	3.9±0.6	5.2±1.3
[AP]/[γ-ATP]	4.9±0.3	3.5±0.4	4.0±0.5	4.5±0.3	3.3±0.4	3.4±0.4	3.7±0.3	3.4±0.3	3.5±0.5
[γ-ATP]/[P_i]	1.0±0.2	1.1±0.2	0.6±0.03	1.4±0.3	1.0±0.1	1.0±0.2	1.0±0.1	1.2±0.1	1.1±0.4
pHi	7.3±0.1	7.2±0.1	7.0±<0.1	7.2±<0.1	7.2±0.1	7.2±<0.1	7.2±0.1	7.1±0.1	7.2±<0.1

Values are means ± S.E.M.

Arginine phosphate (AP) concentrations were measured only for control treatments and were assumed to be independent of experimental osmolarity (see text and Fig. 1). Therefore, the same values are presented for the control, hypo- and hyperosmotic treatments.

All other concentrations were derived from NMR peak areas using AP as a reference. The N-values for the NMR-derived data and for pHi are as indicated for [γ-ATP]. The asterisk indicates that there was a significant effect of acclimation salinity on AP concentrations, and Student's t-tests revealed that the AP concentrations at each of the three acclimation salinities was significantly different from those of the other two salinities.

P_i, inorganic phosphate.

AP, γ-ATP and P_i concentrations are given as μmol g^{-1} wet mass.

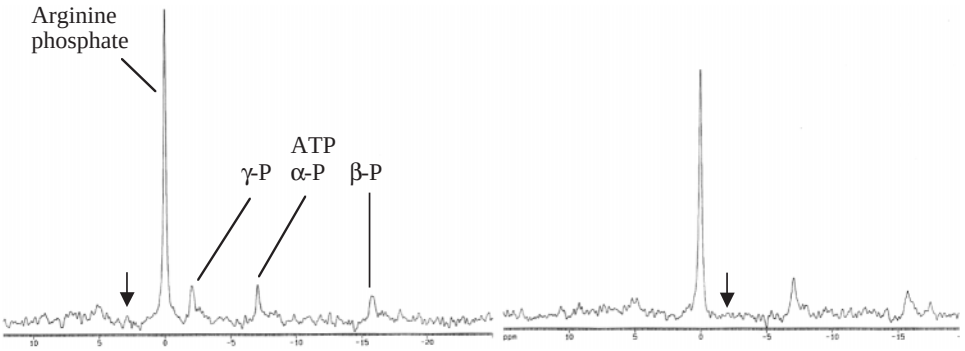

Fig. 2. Typical spectra used for the measurement of the pseudo-first-order unidirectional rate constant for the forward direction ($k_{forward}$) of the arginine kinase reaction. The spectrum on the left is a control in which the saturating irradiation is applied at a frequency offset from the arginine phosphate resonance equivalent to that between the arginine phosphate and γ-ATP resonances (vertical arrow). The spectrum on the right demonstrates that, when the γ-P of ATP is saturated with radiation, a decrease in the arginine phosphate peak is induced that is proportional to the rate of transfer of the phosphate from arginine phosphate to ADP (forward arginine kinase reaction). To measure $k_{reverse}$, the arginine phosphate peak is saturated.

Arginine kinase fluxes

The exchange of the high-energy phosphate catalyzed by AK is apparent from the reduction in amplitude of the arginine phosphate resonance when the γ-ATP peak is saturated, as illustrated in the representative spectra presented in Fig. 2. The forward ($k_{forward}$) and reverse ($k_{reverse}$) pseudo-first-order unidirectional rate constants and the corresponding forward and reverse fluxes for control treatments are presented in Table 3. Among the control animals only, a one-way ANOVA indicated that there was no significant effect of acclimation salinity on rate constants or flux measurements. When the control, hypo-osmotic and hyperosmotic data were analyzed together, two-way ANOVA indicated a significant effect of acclimation salinity for $k_{reverse}$, forward flux and reverse flux. This analysis revealed no significant effect of experimental osmolarity.

However, careful inspection of the data did reveal a general pattern in which mean values for rate constants and flux rates were relatively high for the hypo-osmotic treatments and relatively low for the hyperosmotic treatments (Table 3). This pattern is more clearly observed by plotting the rate constants and fluxes against the difference in experimental osmolarity from the control value (Fig. 3). The most hyperosmotic treatments can be seen to have rate constants and flux rates that are approximately 1.7 times lower than those of the most hypo-osmotic treatments. Linear regressions of both rate constants and both fluxes against the difference from control osmolarity were significant in all cases except for $k_{forward}$ (Fig. 3). The ratio of forward flux to reverse flux (F/R) ranged from 0.81 to 1.08 and had a grand mean of 0.98±0.07 (N=37), which is very close to the expected value of 1 for an enzyme-catalyzed reaction at equilibrium (Table 3).

Table 3. *Arginine kinase rate constants, flux measurements and* in vitro *arginine kinase activity for each treatment group*

	Controls			Hypo-osmotic treatment			Hyperosmotic treatment		
Acclimation salinity (‰)	35	17	5	35	35	17	17	5	5
Experimental osmolarity (mosmol l^{-1})	960	720	640	640	720	640	960	720	960
N	6	5	7	5	5	3	5	5	4
$k_{forward}$ (s^{-1})	0.08±0.01	0.11±0.02	0.10±0.01	0.11±0.01	0.12±0.01	0.08±0.02	0.09±0.01	0.10±0.02	0.09±0.03
$k_{reverse}$* (s^{-1})	0.47±0.06	0.39±0.02	0.39±0.07	0.51±0.04	0.49±0.04	0.43±0.11	0.35±0.04	0.32±0.05	0.29±0.04
Forward flux* (μmol s^{-1} g^{-1} wet mass)	1.35±0.11	1.32±0.19	1.42±0.16	1.83±0.13	2.10±0.15	0.97±0.24	1.06±0.14	1.43±0.25	1.23±0.36
Reverse flux* (μmol s^{-1} g^{-1} wet mass)	1.69±0.28	1.50±0.29	1.82±0.30	1.88±0.12	2.41±0.12	1.59±0.55	1.19±0.21	1.48±0.25	1.43±0.22
F/R	0.81±0.05	1.08±0.29	1.00±0.30	0.98±0.06	0.86±0.06	0.95±0.50	1.03±0.24	1.06±0.24	1.04±0.11
In vitro arginine kinase activity* (μmol s^{-1} g^{-1} wet mass) (N=4)	12.8±1.0	18.7±1.2	16.1±1.6						

For the rate constants and flux measurements, the asterisk indicates a significant effect of acclimation salinity when all the data are analyzed (when analyzed alone, control values showed no significant effect of salinity).

For *in vitro* arginine kinase activity, the asterisk indicates a significant effect of acclimation salinity, and Student's *t*-tests demonstrated that *in vitro* arginine kinase activity in muscle from animals acclimated to 35‰ was significantly lower than that from animals acclimated to 17‰.

F/R, ratio of forward to reverse flux.

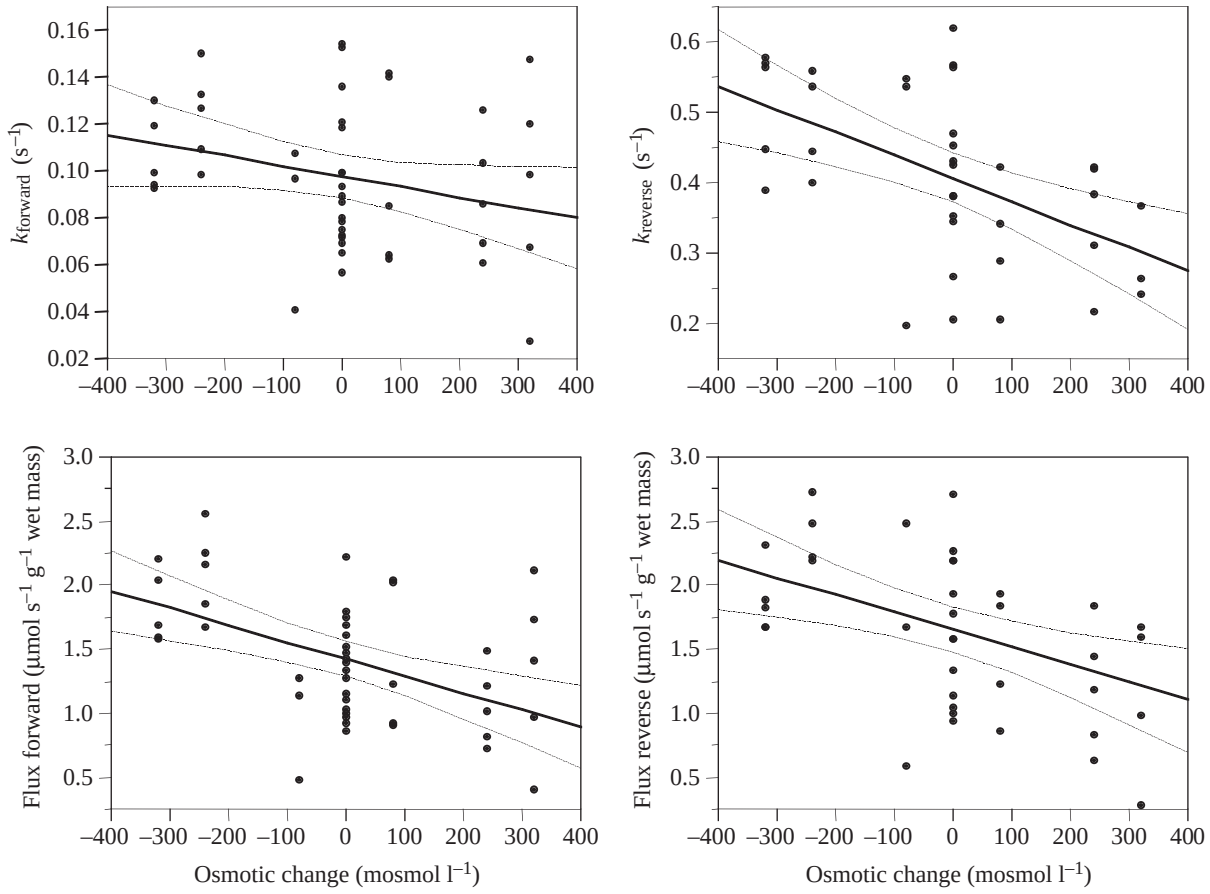

Fig. 3. Linear regression analysis of arginine kinase rate constants and flux rates against the extent of the hypo- or hyperosmotic treatment (osmotic change). The osmotic change was determined by subtracting the control osmolarity from the experimental osmolarity. Negative values indicate a hypo-osmotic treatment, positive values indicate a hyperosmotic treatment and zero indicates controls. For example, the –320 mosmol l^{-1} osmotic treatment results when muscle from an animal exposed to 35‰ salinity is superfused with a medium of 640 mosmol l^{-1} (640–960=–320). Regression lines are shown with 95% confidence limits. Regression equations are as follows: $k_{forward}$=–4.4×$10^{-5}$$O$+0.10, r^2=0.07, P=0.07 (not significant); $k_{reverse}$=–3.3×$10^{-4}$$O$+0.41, r^2=0.28, P<0.01; forward flux=–1.3×$10^{-3}$$O$+1.43, r^2=0.24, P<0.01; reverse flux=–1.4×$10^{-3}$$O$+1.66, r^2=0.22, P<0.01, where O is osmotic change.

Arginine kinase activity assays

In vitro maximal AK activities (measured in the forward direction) were approximately 10 times greater than the forward flux values determined in the muscle tissue (Table 3). Maximal AK activity was also higher in animals acclimated to more dilute media than in animals from full-strength sea water. Animals acclimated to a salinity of 35‰ had the lowest AK activity, while animals acclimated to a salinity of 17‰ had the highest AK activity. Interestingly, the maximal AK activity was inversely proportional to the arginine phosphate concentration (Tables 2, 3).

^{1}*H-NMR*

A dominant peak that demonstrated salinity-induced variation was observed at a chemical shift of 3.2 p.p.m. in ^{1}H-spectra (Fig. 4). We cannot attribute this large resonance to any of the amino acids that have been shown to be important osmolytes in blue crab muscle (Gérard and Gilles, 1972), although the triplet CH_2 peak of arginine probably contributes slightly to the large peak at 3.2 p.p.m. The peak has a chemical shift consistent with a methylamine compound such as betaine (including the presence of the small peak at 3.85 p.p.m.; Fig. 4), and NMR spectra collected from a muscle extract before and after it was spiked with betaine support this conclusion. To our knowledge, betaine has not previously been reported to occur in abundance in blue crab muscle, but it is an important osmolyte in the euryhaline crab *Eriocheir sinensis* (Bricteux-Grégoire et al., 1962). Despite some uncertainty in the assignment of this peak, we believe that it represents an important organic osmolyte on the basis that its concentration is both high and sensitive to salinity. The relative concentration of this resonance was determined by normalizing the peak area to the cumulative area of all other proton peaks, excluding that of water. Although some of the other small resonances visible in the ^{1}H-spectra may also be derived from regulated osmolytes, the peaks were too small and broad to estimate concentration changes reliably in the living muscle preparation.

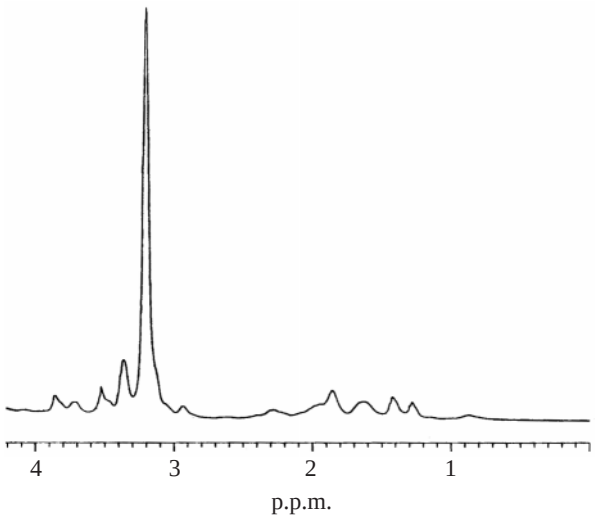

Fig. 4. Example of a ^{1}H-NMR spectrum of a living muscle from a blue crab that had been acclimated to a salinity of 35‰. The large peak at 3.2 p.p.m. varied in amplitude with salinity and has been tentatively assigned to betaine. Note the small peak at 3.85 p.p.m., which is also indicative of betaine.

Two-way ANOVA of the ^{1}H-NMR results indicated that the concentration of betaine changed significantly as a function of acclimation salinity but not as a function of experimental osmolarity (Fig. 5). As expected, the concentration of this osmolyte was highest in muscle from animals acclimated to 35‰ and lowest in animals acclimated to 5‰. The mean values were approximately 40 % lower in muscle from animals acclimated to 5‰ than for those acclimated to 35‰. Therefore, the 7-day salinity acclimation period induced changes in the concentration of this osmolyte (and probably others), but during the relatively short time course of the saturation transfer experiments the concentration was unaltered.

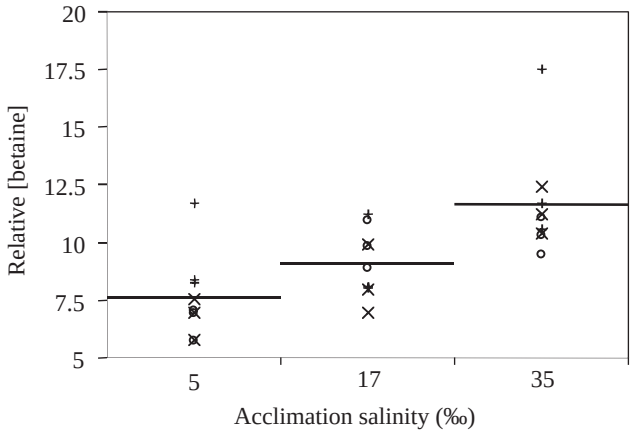

Fig. 5. The effect of acclimation salinity and experimental osmolarity on the relative concentration of betaine in crab muscle. +, muscles exposed to 960 mosmol l^{-1}; ×, muscles exposed to 720 mosmol l^{-1}; ○, muscles exposed to 640 mosmol l^{-1}. The horizontal bars are mean values for each acclimation salinity. The units on the *y*-axis are arbitrary. Two-way ANOVA indicated a significant effect of acclimation salinity but no effect of experimental osmolarity. *N*=3 for each treatment.

Discussion

Euryhaline organisms have evolved complex phenotypes for tolerating changes in environmental salinity, and the integrated response encompasses adaptations at the behavioral, physiological and molecular level. Despite the obvious effectiveness of the osmoregulatory machinery of the blue crab, changes in environmental salinity still lead to dramatic changes in hemolymph osmolarity and, subsequently, in the intracellular composition of inorganic ions and organic solutes (Lynch et al., 1973; Gérard and Gilles, 1972; Mantel and Farmer, 1983). For cellular function to be maintained, it is necessary to preserve enzyme structure and catalytic capacity in spite of these changes in the intracellular environment.

The present study found that the levels of the high-energy phosphate compounds (Fig. 1) and pHi were not altered by hypo- or hyperosmotic treatments. The stability of the levels of arginine phosphate, ATP and P_i during even the most extreme treatments implies that there is not a substantial energetic cost in terms of whole-muscle ATP demand during osmotic stress. A sizable increase in ATP demand would be manifested as decreased [ATP]/[P_i] and [arginine phosphate]/[ATP] ratios (for reviews, see van den Thillart and van Waarde, 1996; Wasser et al., 1996). However, we cannot rule out the possibility that there may have been an undetectable localized perturbation to high-energy phosphate concentrations in regions near the sarcolemmal membrane, where regulatory ion pumps may be active (Combs and Ellington, 1997).

In vitro AK activities and arginine phosphate concentrations were consistent with values found from oxidative tissues from other invertebrates (Tables 2, 3) (for a review, see Ellington, 2001). Both AK activities and arginine phosphate concentrations were different for each acclimation salinity, but the differences were not proportional to salinity (i.e. the lowest-salinity treatment, 5‰, had intermediate values for both measurements). It is interesting that the values for AK activity and arginine phosphate levels were inversely related at the three acclimation salinities, although we do not know the biological significance of this relationship. *In vitro* AK activity for the forward reaction was approximately 10 times higher than forward flux measured in intact muscle (Table 3). This discrepancy has been reported previously for AK in magnetization transfer experiments (Briggs et al., 1985; Platzer et al., 1999). It has been suggested that the extraction procedure for the *in vitro* assays may liberate compartmentalized AK that has a lower activity in resting muscle (Briggs et al., 1985). In contrast, Graham et al. (1986) found that the phasic adductor muscle in scallop had flux values that were in accord with *in vitro* maximal activity values.

Enzyme *F*/*R* flux ratios measured using NMR magnetization transfer methods have frequently been found to deviate from a

value of 1, and this phenomenon has been observed for AK. In muscle from the shore crab *Carcinus maenas*, *F*/*R* ratios were nearly 2, and Briggs et al. (1985) suggested that the cause was either metabolic compartmentation or multi-site exchange that led to an underestimation of reverse flux. In the present study, the AK *F*/*R* flux ratios in muscle were very close to 1, with a grand mean of 0.98±0.07 (Table 3). An *F*/*R* ratio of 1 would be expected for an enzyme near equilibrium, and our results are similar to that found in scallop phasic adductor muscle (Graham et al., 1986).

The major finding of the present study is that AK flux in muscle varies over a 1.7-fold range under different osmotic conditions (Table 3; Fig. 3). The results suggest an effect of both acclimation salinity and experimental osmolarity; however, only the former effect was significant in the two-way ANOVA. It should be reiterated that one-way ANOVA of the three control treatments alone did not indicate a significant effect of acclimation salinity. The similarity of the control values indicates that AK function is independent of salinity in crabs that have been exposed to a given salinity long enough to reach a new 'acclimated' osmotic steady state. However, the significant acclimation salinity effect found across all treatments suggests that salinity history may play a role in the response of AK to hypo- or hyperosmotic conditions.

The rate constants and fluxes were plotted as a linear function of the extent of osmotic stress, with the highest values observed in the most hypo-osmotic treatments and the lowest values found in the most hyperosmotic treatments (Fig. 3). Linear regression was used here for statistical convenience and because a reasonably good fit was attained, but we do not mean to imply that this relationship is truly linear over a broad osmotic range. Although the mechanism by which AK flux was altered in our experiments is not known, the systematic manner in which flux was affected by the extracellular osmotic conditions may offer some insight into possible causes. Three scenarios that might lead to a change in the AK flux measurements include (i) a change in the muscle energetic state, (ii) a change in cell volume that would alter substrate concentrations and (iii) a direct effect of inorganic ions and/or organic osmolytes on AK function.

Since ATP, ADP and arginine phosphate are all substrates for the AK reaction, changes in the energetic state of the cell associated with osmotic stress might be expected to alter AK flux. As stated above, however, our hypo- and hyperosmotic treatments do not appear to be an energetic challenge to the muscle as a whole on the basis of the stability of the levels of high-energy phosphate compounds (Fig. 1; Table 2). A second reason why energetic changes should not affect AK flux is that the enzyme has a high activity in the dark levator muscle (Table 3), and it is likely that the major functional role of AK in this tissue is temporal ATP buffering. This assumption is based on the fact that, while AK is localized to mitochondria in tissues from arthropods (Ellington, 2001), the vast majority of activity is associated with the cytosolic form of AK (Doumen and Ellington, 1990). Furthermore, mitochondrial AK does not appear to be coupled functionally to oxidative phosphorylation in the manner of mitochondrial creatine kinase so that AK flux does not respond directly to ATP demand (Doumen and Ellington, 1990). The available evidence therefore suggests that AK is a simple equilibrium enzyme, and that the flux rate should not be susceptible to moderate changes in energetic demand. A similar argument might be made regarding the concentration of arginine, which is both a substrate for the AK reaction and a salinity-sensitive osmolyte in blue crab muscle (Gérard and Gilles, 1972). However, the K_m of AK for arginine is typically less than 1 mmol l^{-1} (Platzer et al., 1999; Suzuki et al., 2000), which is well below the concentration of arginine supported by the AK reaction at equilibrium (Teague and Dobson, 1999). Nevertheless, to establish definitively the envelope of expected flux values on the basis of *in vivo* metabolite concentrations would require a detailed kinetic analysis similar to those offered for creatine kinase by McFarland et al. (1994) and van Dorsten et al. (1997).

Although the levels of high-energy phosphate compounds remained constant in the NMR measurements (Fig. 1), changes in cell volume associated with the hypo- and hyperosmotic treatments would alter the cellular concentrations of AK substrates. The most extreme treatments in the present study simulated a change in the hemolymph osmolarity of approximately 300 mosmol l^{-1}. Lang and Gainer (1969) examined cell volume regulation in blue crab muscle using osmotic treatments of the same magnitude as our most extreme tests. It was found that acute hyperosmotic shock led to an immediate reduction in cell size to 80–85 % of the initial cell volume, and this reduced volume was maintained for at least 5 h. Hypo-osmotic treatments led to an immediate and larger increase in cell size, but was followed by a fairly rapid (1 h) adjustment of cell volume to approximately 110 % of the initial volume. Cells were then maintained for at least several hours at this partially corrected volume (Lang and Gainer, 1969).

If similar cell volume changes occurred in our study, then this would lead to a dampening of the osmotic effects on AK flux demonstrated in Fig. 3. Hypo-osmotic treatments would lead to swelling and a reduction in substrate concentration, which would lead to reduced flux. In contrast, cell shrinking under hyperosmotic treatments would increase flux. The rate constants would not be affected by volume changes. If we assume volume changes comparable with that described above and adjust the levels of arginine phosphate and γ-ATP accordingly, the pattern observed in Fig. 3 remains the same. However, the difference in flux between the extreme hypo- and hyperosmotic treatments is reduced by approximately 50 %. If these volume-adjusted data are re-analyzed in a two-way ANOVA, there is still a significant effect of acclimation salinity for the forward flux measurements as described above, but this effect is no longer significant for the reverse flux. Although this conservative analysis represents an overestimate of the effects of volume change on most of our data, it indicates that cell volume changes are probably responsible in part for the patterns observed in Fig. 3.

It is also likely that changes in the composition of the intracellular environment contribute to the observed osmotic

effects on AK function. In their analysis of blue crab muscle, Lang and Gainer (1969) found that inorganic ion concentrations could not be adequately explained by volume changes alone. Intracellular K^+ concentration was particularly variable, and its concentration decreased from 187 to 105 mmol l^{-1} following hypo-osmotic incubation. Gérard and Gilles (1972) measured intracellular concentrations of Na^+, K^+ and Cl^- in *C. sapidus* muscle during hypo-osmotic shocks comparable with our intermediate treatments and found that these three ions showed a cumulative decrease of 55 mosmol kg^{-1} intracellular water at the lower salinity. A number of studies have shown that changes in the concentrations of Na^+, K^+ and Cl^- over similar ranges may lead to moderate or even dramatic effects on enzyme catalytic capacity *in vitro* (Bowlus and Somero, 1979; Yancey et al., 1982; Yancey, 1994). In the present study, our hyperosmotic treatments tended to reduce flux (Table 3; Fig. 3). Under these conditions, AK would be exposed to relatively high concentrations of inorganic ions, which may disrupt protein function, and relatively low concentrations of organic osmolytes, which may help stabilize protein structure (Yancey et al., 1982; Yancey, 1994, 2001). In contrast, hypo-osmotic treatments tended to yield higher AK fluxes (Table 3; Fig. 3). Here, the compatible solute concentration is presumably high and the inorganic ion concentration is relatively low.

The ^{1}H-NMR data suggest that this interpretation of the organic osmolyte concentrations in our treatments is accurate. The peak tentatively assigned to betaine changed in amplitude with acclimation salinity as expected (Fig. 5), but it did not change during the time course of our hypo- or hyperosmotic treatments. Lacking a complete organic osmolyte response, it appears that, for cell volume regulation purposes (or as a result of cell volume changes), concentrations of potentially perturbing inorganic ion were relatively high during the short-term hyperosmotic treatments and relatively low during the hypo-osmotic treatments (Table 1; Pierce, 1982).

An interesting alternative hypothesis is that planar anions, such as Cl^-, may inhibit the enzyme *in vivo* by stabilizing the abortive dead-end complex, enzyme·MgADP·arginine. This effect has been demonstrated for the creatine kinase reaction *in vivo* (McFarland et al., 1994), and it could be argued that AK is similarly inhibited under hyperosmotic conditions, while inhibition is relieved in the hypo-osmotic treatments. However, the anion stabilization of the dead-end complex is less pronounced in AK than in creatine kinase, and the most likely anion candidate with respect to osmoregulation, Cl^-, has to our knowledge not been shown to stabilize the AK complex *in vitro* (Anosike and Watts, 1976). We therefore conclude that the non-specific ionic effects described above are a more likely modulator of enzymatic flux in blue crab muscle.

The observed changes in AK flux raise a question as to the physiological relevance of salinity-induced changes in the osmotic state of blue crab muscle. Our hypo- and hyperosmotic treatments simulated rapid changes in environmental salinity that temporarily disrupted the balance between levels of inorganic ions and organic osmolytes found in the acclimated state. While small estuaries can undergo dramatic shifts in salinity over a short period, the typical daily variation in salinity is likely to be less than that simulated in our most extreme osmotic challenges. Also, in using isolated muscle preparations, we have removed the gill osmoregulatory machinery that may serve as a temporal buffer of the osmolarity of the hemolymph. Even if the environmental salinity is changed instantaneously, blue crab hemolymph osmolarity may change more gradually because of the ion-transporting capacity of the gills (Gilles, 1979; Towle et al., 1994). Therefore, our most extreme treatments may represent an osmotic condition that exceeds that routinely experienced in nature. In fact, the range of AK flux is quite small across the more modest osmotic treatments used in this study (Table 3; Fig. 3). In this light, the changes in AK flux that we observed should probably be considered to be fairly modest, perhaps even indicating a remarkable preservation of AK function during dramatic changes in the extracellular environment.

AK was selected as a model enzyme for examining the effect of osmotic challenges on enzyme function because it is a tractable system using non-invasive magnetization transfer methods. However, AK may not be an ideal enzyme for this purpose. The bulk of AK in arthropod muscle cells is thought to be a soluble monomeric protein (Ellington, 2001). However, because of the disruptive effects of inorganic ions on protein–protein interactions (Yancey et al., 1982), multimeric enzymes may be more susceptible to intracellular osmotic perturbations. Therefore, non-invasive measurements of enzymes that are composed of multiple subunits and of those that are functionally localized to specific regions within the cell would be beneficial.

We thank Dr Robert Roer, Dr Thomas Shafer and two anonymous reviewers for valuable comments on this manuscript. Funding was provided by the National Science Foundation (DBI-9978613) and the UNCW Center for Marine Science. This is contribution number 264 from the UNCW Center for Marine Science.

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