Acute Changes in Gill Na^+-K^+-ATPase and Creatine Kinase in Response to Salinity Changes in the Euryhaline Teleost, Tilapia (*Oreochromis mossambicus*)

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Accepted 10/17/01

ABSTRACT

Some freshwater (FW) teleosts are capable of acclimating to seawater (SW) when challenged; however, the related energetic and physiological consequences are still unclear. This study was conducted to examine the changes in expression of gill Na^+-K^+-ATPase and creatine kinase (CK) in tilapia (*Oreochromis mossambicus*) as the acute responses to transfer from FW to SW. After 24 h in 25 ppt SW, gill Na^+-K^+-ATPase activities were higher than those of fish in FW. Fish in 35 ppt SW did not increase gill Na^+-K^+-ATPase activities until 1.5 h after transfer, and then the activities were not significantly different from those of fish in 25 ppt SW. Compared to FW, the gill CK activities in 35 ppt SW declined within 1.5 h and afterward dramatically elevated at 2 h, as in 25 ppt SW, but the levels in 35 ppt SW were lower than those in 25 ppt SW. The Western blot of muscle-type CK (MM form) was in high association with the salinity change, showing a pattern of changes similar to that in CK activity; however, levels in 35 ppt SW were higher than those in 25 ppt SW. The activity of Na^+-K^+-ATPase highly correlated with that of CK in fish gill after transfer from FW to SW, suggesting that phosphocreatine acts as an energy source to meet the osmoregulatory demand during acute transfer.

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Physiological and Biochemical Zoology 75(1):29–36. 2002. 1522-2152/2002/7501-01042$15.00

Introduction

In teleosts, alterations of environment (water pH, salinity, or heavy metal pollution) cause physiological responses, such as secretion of hormones (growth hormone, prolactin, or cortisol); fluctuations of plasma ion, osmolality, and glucose; and changes in water balance and oxygen consumption rate (Potts et al. 1987; McCormick et al. 1989*b*; McCormick 1996; Lin et al. 2000). When a euryhaline teleost goes from hyperosmotic (seawater [SW]) to hypoosmotic (freshwater [FW]) media, it tends to lose ions and gain water. The reverse occurs after transfer from FW to SW. The physiological response is conducted to maintain a stable internal milieu via modulating the mechanisms of ionic regulation and water balance. Gills are the most important extrarenal organ responsible for osmoregulation in fish. Mitochondria-rich cells (MR cells; i.e., chloride cells) are the main site for active transport of ions in branchial epithelium (McCormick 1995). Na^+-K^+-ATPase, a universal membrane-bound enzyme, provides a driving force for water and ion transport in a variety of osmoregulatory epithelia, including fish gills. This enzyme is mainly located in the tubular system of the MR cells (Lee et al. 1998; Hwang et al. 1999) and plays a central role in the process of ion transport in gill MR cells in fishes (McCormick 1995). Increasing salinity causes the augmentation of Na^+-K^+-ATPase activity (Hwang et al. 1989; McCormick 1995), ouabain binding sites (McCormick and Bern 1989; Hwang et al. 1999), and Na^+-K^+-ATPase alpha subunit (Hwang et al. 1998; Lee et al. 1998) as well as morphological changes in MR cells (Hwang and Hirano 1985; Hwang 1987, 1990; Perry and Laurent 1993). Previous reports found that two periods of response exist in fish after transfer to SW. The first is a crisis phase in which fish are critically faced with the removal of water from gill and gut epithelia (dehydration), and the second phase is a stabilization phase during which fish recover from the altered internal milieu (Bath

and Eddy 1980; Hwang 1987; Hwang et al. 1989). However, the mechanisms of these two periods remain unclear.

Creatine kinase (CK; EC 2.7.3.2) is used to transfer energy between phosphagens in the reversible reaction: ADP + phosphocreatine → ATP + creatine. The function of the phosphocreatine in excitable cells (particularly muscle and heart) appears to be that of an acute energy source for both aerobic and anaerobic metabolism. Phosphocreatine/creatine kinase is present in some excitable and nonexcitable tissues of fish with high and fluctuating energy demands, such as the electric organ of *Narcine brasiliensis* (Blum et al. 1991), shark rectal gland (Friedman and Roberts 1992), and *Gillichthys mirabilis* gills (Kültz and Somero 1995). Surprisingly, there are no data regarding the changes in branchial (gill) parameters related to the physiological roles of phosphocreatine/creatine kinase after short-term salinity transfer. The aim of this study was to analyze the changes in tilapia (*Oreochromis mossambicus*) gill parameters (Na^+-K^+-ATPase activity and CK activity) as a result of acute responses to transfer from FW to SW.

Material and Methods

Animals

Tilapia (*Oreochromis mossambicus*) were originally obtained from the Tainan Fish Culture Station of the Taiwan Fisheries Research Institute. Euryhaline tilapia are able to live in both SW and FW. All fish were maintained in a freshwater recirculating tank with a 12L : 12D photoperiod at 25°–28°C at the Institute of Zoology, Academia Sinica, Taipei, Taiwan. The SW was prepared by the addition of artificial sea salt to FW. The sampled fish were about 5–7 cm in total length and 2.5–4.0 g in body weight. Tilapia were transferred directly from FW to 25 or 35 ppt SW at various times by nylon-net capture. During the experimental period (0.5–24 h), fish were reared in 25 or 35 ppt SW tanks without feeding. Fish were anesthetized with ice and killed immediately. The gills were removed and weighed. The tissue was homogenized in homogenization solution (100 mM imidazole-HCl buffer, pH 7.0, 5 mM Na_2EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate) with a motorized Teflon pestle at 600 rpm for 20 strokes on ice. After centrifugation (12,000 rpm for 30 min at 4°C), the supernatant was kept at −70°C until assay. Total protein was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif.) and calculated using bovine serum albumin (Sigma, St. Louis) as a standard.

Na^+-K^+-ATPase Activity

Na^+-K^+-ATPase activity was determined following Hwang et al. (1988). In brief, the sample (10 μL, ~80–100 μg total protein) was prepared in a 1.5-mL vial with 500 μL reaction buffer (100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, and 5 mM Na_2ATP). After incubation at 37°C for 30 min, the vial was put on ice, and 200 μL 30% trichloroacetic acid was added to stop the reaction. The vials

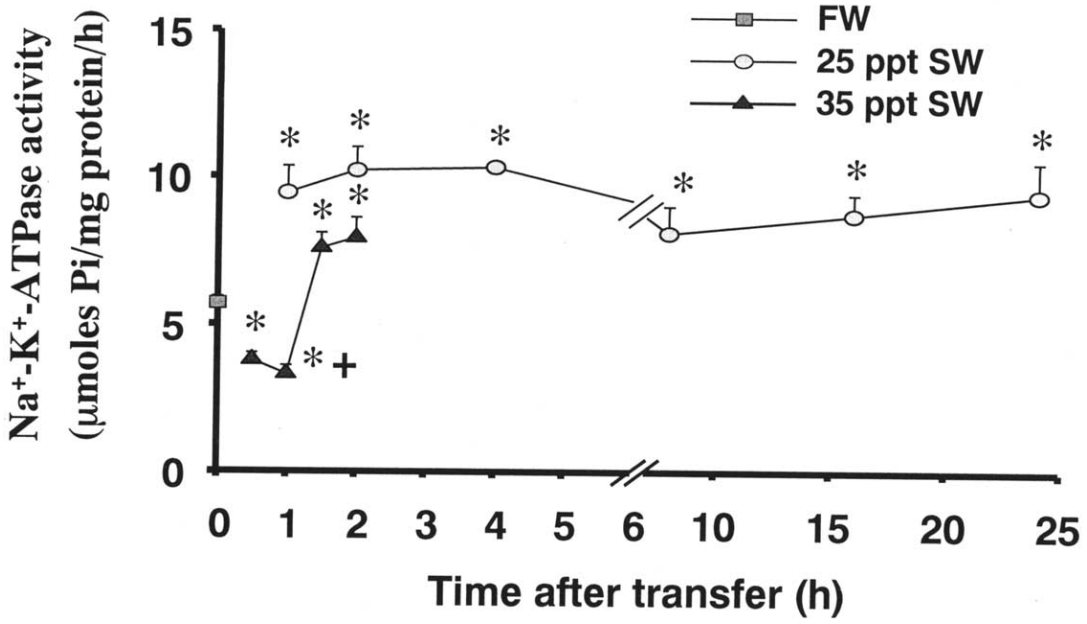

Figure 1. The acute responses of Na^+-K^+-ATPase activity in tilapia gill at various times after transfer from FW to SW (25 or 35 ppt). An asterisk represents a significant difference ($P < 0.05$) between the FW control and 25 or 35 ppt SW. A plus sign represents a significant difference ($P < 0.05$) between 25 and 35 ppt.

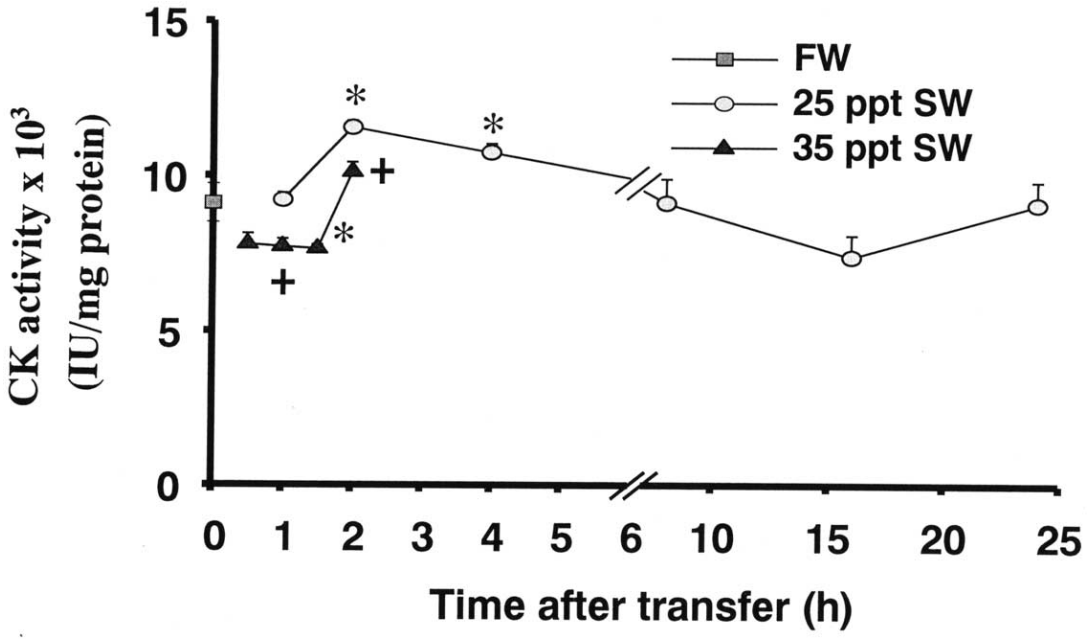

Figure 2. The acute responses of creatine kinase activity in tilapia gill at various times after transfer from FW to SW (25 or 35 ppt). An asterisk represents a significant difference ($P < 0.05$) between the FW control and 25 or 35 ppt SW. A plus sign represents a significant difference ($P < 0.05$) between 25 and 35 ppt SW.

were centrifuged at 4,000 rpm at 4°C for 10 min, and the supernatant (500 μL) and the phosphate standard (160, 80, 40, 20, 10, 5, and 0 μg/mL) were added with 2.5% acid molybdate solution, 10% SDS, and 0.025% 1-amino-2-naphthol-4-sulformic acid). After incubation at 20°C for 30 min, the inorganic phosphate production was measured at 700 nm following the method described by Peterson (1978). The enzyme activity of Na^+-K^+-ATPase was defined as the difference between the inorganic phosphate liberated in the presence and absence of 0.5 mM ouabain in the reaction mixture and calculated by comparison with the concentrations of inorganic phosphate standard. The data were expressed in micromoles of inorganic phosphate released per milligram of protein per hour.

Creatine Kinase Activity

CK activity was measured using a commercial kit (Sigma) and followed the manuals with minor modification. The reactions are (1) ADP + phosphocreatine → ATP + creatine and (2) creatine + α-naphthol + diacetyl → colored complex. The diluted samples (10 μL) were prepared in a 1.5-mL tube; then 50 μL phosphocreatine solution and 20 μL ADP-glutathione solution (start reaction) were added to each tube and mixed vigorously. A serial creatine standard without the addition of phosphocreatine solution, ADP-glutathione solution, and p-hydroxymercuribenzoate was set up simultaneously. The reactions including standards were incubated at 37°C for 30 min. Afterward, 20 μL p-hydroxymercuribenzoate was added to stop the reaction, and then 100 μL α-naphthol solution, 100 μL diacetyl solution, and 700 μL redistilled water were added and mixed well. After incubation at 37°C for 20 min, the tubes were centrifuged at 3,500 rpm for 5 min. The absorbance of the supernatant was measured at 520 nm. The CK activity of an unknown sample was calculated by comparison to concentrations of a creatine standard. The data were expressed in micromoles of creatine released per milligram of protein.

Western Blotting

Homogenate of gill (total protein 100 μg) was mixed with an equal volume of 2 × electrophoresis sample buffer containing DTT (1,4-Dithiothreithol), and the proteins were separated by electrophoresis on a 4%–12% gradient polyacrylamide slab gel (NuPAGE, NOVEX, San Diego, Calif.) and electrophoretically transferred to polyvinylidene difluoride membrane (Amersham Life Science, Piscataway, N.J.). The blots were incubated overnight in 3% NET buffer (0.25% gelatin, 50 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, pH 7.5, 0.05% Tween-20) and washed three times in PBST buffer (0.01 M phosphate, 0.09% NaCl, pH 7.5, 0.05% Tween-20). Filters were incubated 1 h with first antibody for muscle form (MM) CK monoclonal antibody (Biogenesis, Poole, U.K.) in 1 : 2,000 dilution and for rabbit anti-brain form (BB) CK (Biogenesis) in 1 : 1,000 dilution.

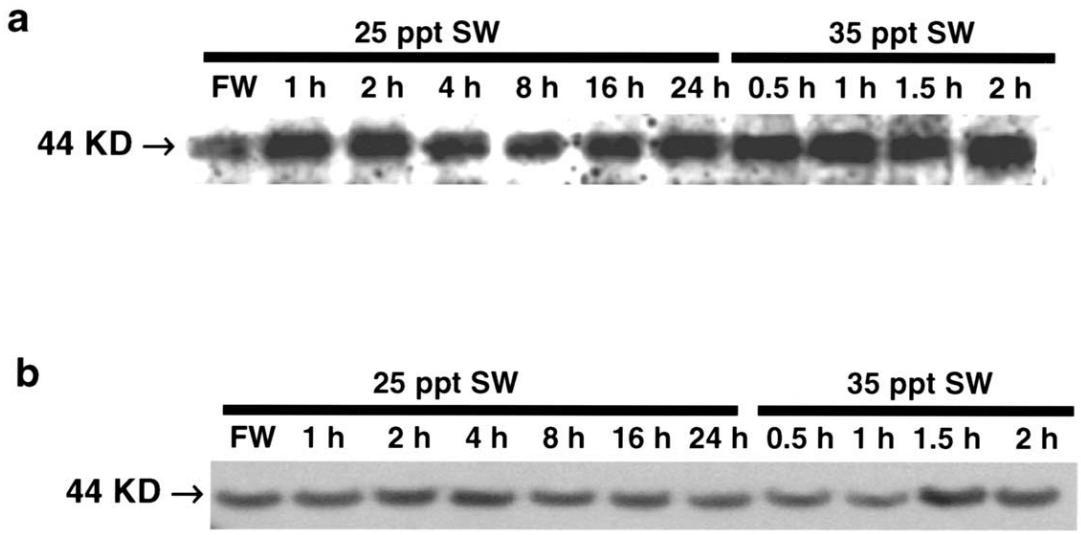

Figure 3. Western blots of the acute responses of tilapia gill (*a*) muscle-type creatine kinase (MM form, typical) and (*b*) brain-type creatine kinase (BB form, Western blot) at various times after transfer from FW to SW (25 or 35 ppt).

Mouse muscle or brain tissues were applied as a positive control to check molecular weight and immunoreactivity. After washing three times with PBST buffer, immunoreactive proteins were visualized using an enhanced chemiluminescent (ECL) system (Pierce, Rockford, Ill.) according to the instructions supplied by the manufacturer. The differences in the band intensity for FW and SW were compared using densitometry (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, Calif.).

Statistics

All biochemical data were expressed as mean ± SEM. Differences among the groups were examined by a one-way ANOVA, and means among them were compared with the FW control by least significant difference test. The values of each group as compared with FW were shown at the $P < 0.05$ significance level. The relationships of CK and Na^+-K^+-ATPase activity were tested by correlation analysis.

Results

Na^+-K^+-ATPase

Tilapia died 4 h after direct transfer from FW to 35 ppt SW. Results were in agreement with the previous report (Hwang et al. 1989). After transfer to 25 ppt SW, gill Na^+-K^+-ATPase activities were elevated within 1 h and reached higher ($P < 0.05$) levels within 24 h compared to those of FW fish (Fig. 1). Fish in 35 ppt SW did not increase gill Na^+-K^+-ATPase activities until 1.5 h after transfer, and then the activities were not significantly different from those in 25 ppt SW (Fig. 1).

Creatine Kinase

After transfer to SW, gill CK activities of 25 ppt SW fish were elevated within 2 h and thereafter gradually returned to the level of FW control fish (Fig. 2). In the 35 ppt SW group, gill CK activities did not increase until 2 h after the transfer. In addition, the gill CK activity in 35 ppt SW was significantly lower than that in 25 ppt SW at the same time (Fig. 2). Western blot of muscle-type CK (MM form; Fig. 3*a*) showed high association with salinity change (Fig. 4). Both 25 and 35 ppt SW fish kept higher protein levels of CK than FW control fish right after transfer until the end of the experiment (Fig. 3*a*). In addition, the level in 35 ppt SW was higher than that in 25 ppt SW. However, it was not the case in brain-type CK (BB form; Fig. 3*b*). No significant elevation in BB form was found in 25 ppt SW after transfer, while in 35 ppt SW significant increase in BB form occurred only about 1.5–2 h after transfer (Fig. 5). The correlation coefficient between CK activity and Na^+-K^+-ATPase activity was 0.89 ($P < 0.05$) within 4 h after transfer from FW to 25 ppt SW and was 0.81 within 8 h as well. In addition, CK activity and CK content (MM form) showed high correlation ($r = 0.91$, $P < 0.05$ within 4 h, and $r = 0.95$, $P < 0.05$ within 8 h) after transfer from FW to 25 ppt SW.

Discussion

The notable finding of this study is the correlated changes in the activity between gill Na^+-K^+-ATPase and CK following transfer from FW to SW. This suggests the possibility that CK

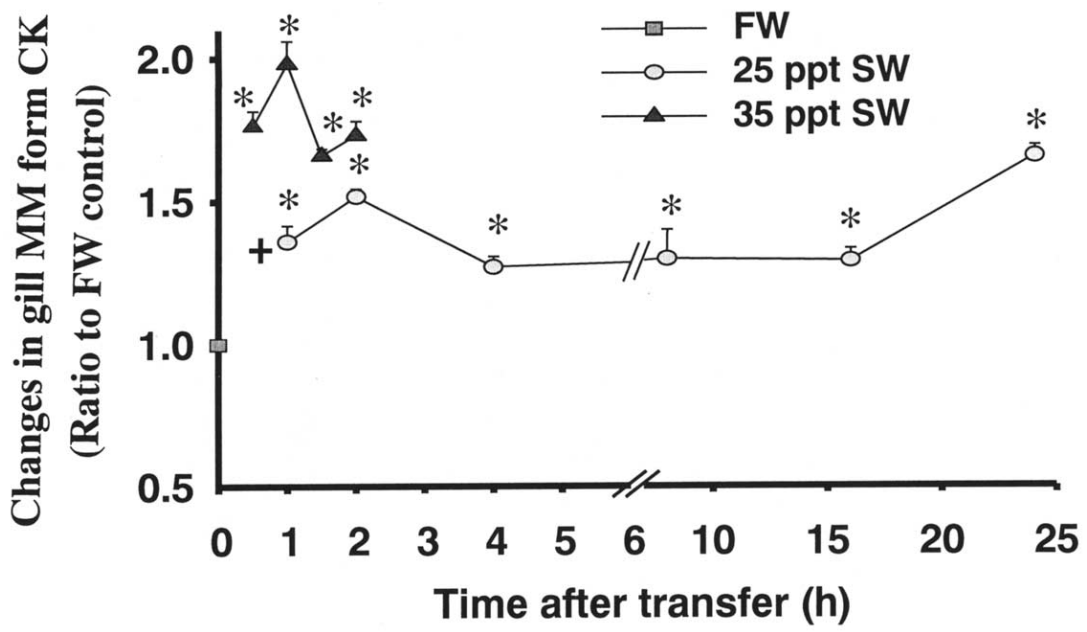

Figure 4. The alterations of muscle-type creatine kinase (MM form, Western blot) in tilapia gill at various times after transfer from FW to SW (25 or 35 ppt). An asterisk represents a significant difference ($P < 0.05$) between the FW control and 25 or 35 ppt SW. A plus sign represents a significant difference ($P < 0.05$) between 25 and 35 ppt SW.

is able to provide energy for Na^+-K^+-ATPase to conduct osmoregulation during acute salinity challenges.

According to the current model for MR cells in the gill of SW teleosts, Na^+-K^+-ATPase provides a driving force for active excretion of Cl^- to compensate for the passive Cl^- influx following the ionic gradient between plasma and environment (Karnaky 1986; McCormick 1995). Therefore, stimulation of activity and protein expression of gill Na^+-K^+-ATPase is correlated with environmental salinity (Kültz and Jürss 1993; McCormick 1995; Hwang et al. 1998, 1999; Lee et al. 1998; Lin et al. 2000). The activities and contents (MM form) of gill CK and Na^+-K^+-ATPase activities were stimulated in SW groups compared with those in the FW control group. Surprisingly, the increase of gill Na^+-K^+-ATPase activities was similar to the trend of elevated gill CK (MM form) contents after transfer from FW to 25 ppt SW. However, there is a significant decrease in gill Na^+-K^+-ATPase and CK after exposure to 35 ppt SW. Lower CK is insufficient to provide energy for a sodium pump to maintain ion balance. This may be one reason why fish died 4 h after direct transfer from FW to 35 ppt SW. Previous studies found that tilapia would die 6 h after direct transfer from FW to 30 ppt SW as a result of severe dehydration but survive well with an initial moderate dehydration in 20 ppt SW (Hwang et al. 1989). It was suggested that preacclimation to 20 ppt SW permit tilapia not only to survive against sublethal dehydration but also to have sufficient time to activate salt-secreting mechanisms for subsequent exposure to 30 ppt SW (Hwang et al. 1989). In our study, both the 25 and 35 ppt SW groups presented higher enzyme activities than the FW control; however, the 35 ppt SW group was not higher than the 25 ppt SW group. This could be water and ion imbalance in cells (including gill MR cells) due to the severe dehydration on direct transfer to 35 ppt SW, which may impair the function (i.e., enzyme activity) of Na^+-K^+-ATPase and CK. Moreover, the impairment in the enzyme activities due to intracellular hydromineral imbalance may be the reason for death in 35 ppt SW. Occurrence of abnormal gill MR cells at 5 h (just before death) in 30 ppt SW (Hwang 1987) provides further evidence to support this inference.

CK appears to be a good indicator in critical phases of brain development (cichlid fish and clawed toads) and reacts very sensitively during this period to a change in environmental conditions (Slenzka et al. 1993). Cholinergic stimulation of fish electric organ would cause a rapid turnover of phosphocreatine, which is mainly due to Na^+-K^+-ATPase activity (Blum et al. 1990). Muscle-type CK of rat heart is functionally coupled to Na^+-K^+-ATPase activity, providing ATP for the ATPase reaction (Saks et al. 1977, 1991). Kültz and Somero (1995) used the CK inhibitor iodoacetamide in their experiments and concluded that CK may provide an energy source for ion transport in gill of *Gillichthys mirabilis*; however, they did not find any direct evidence relating the increase of gill CK and Na^+-K^+-ATPase when fish were transferred from 36 to 60 ppt SW. In our study, CK activity of tilapia gill was in concert with the alterations of

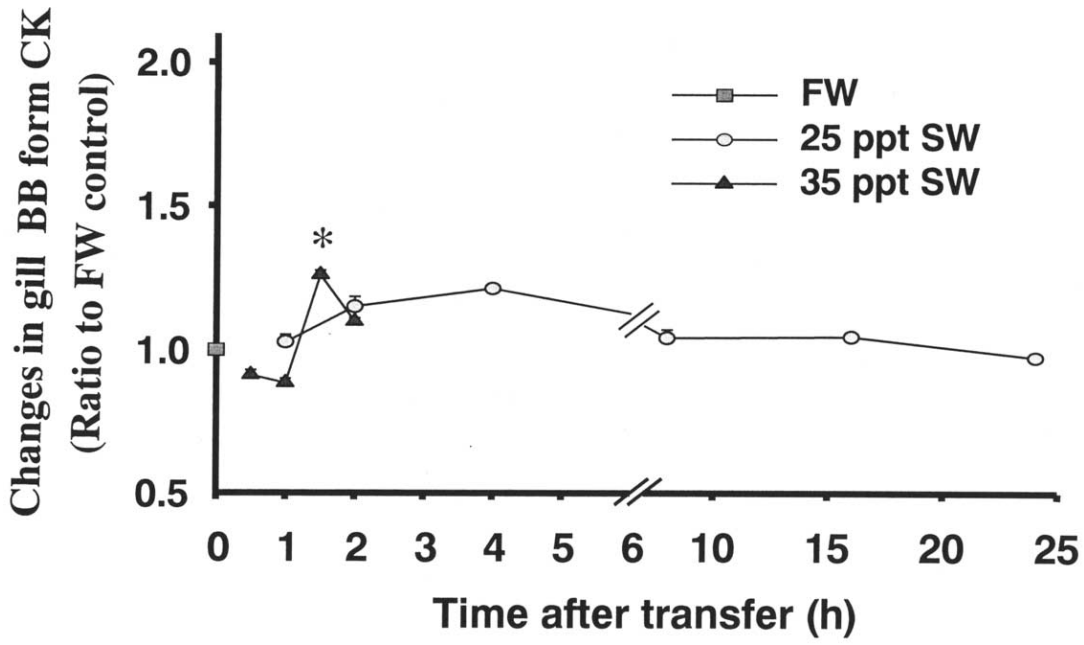

Figure 5. The alterations of brain-type creatine kinase (BB form, Western blot) in tilapia gill at various times after transfer from FW to SW (25 or 35 ppt). An asterisk represents a significant difference ($P < 0.05$) between the FW control and 25 or 35 ppt SW.

salinity. The higher correlation ($r = 0.89$ to 0.81) between CK activity and Na^+-K^+-ATPase activity was present within 8 h after transfer from FW to 25 ppt SW. Moreover, CK content was highly associated with CK activity in our study. These data indicate that CK might play a certain role in SW transfer as well, particularly during the early stage. However, interactions from other physiological factors during acute SW challenges or other stresses should also be considered. High salinity induces increased gill Na^+-K^+-ATPase activity in Atlantic salmon, but it does not induce a substantial increase in the metabolic capacity of gill (McCormick et al. 1989*a*). Exercise and adrenaline are known to increase gill permeabilities to water and oxygen (Randall et al. 1967; Wood and Randall 1973*a*, 1973*b*; Isaia 1984). The osmotic imbalance is associated with the increased ventilation and gill perfusion (Randall et al. 1972; Wood and Randall 1973*a*, 1973*b*). Catecholamines, produced during exercise and in response to stress, not only increase gill permeabilities to water and oxygen but also inhibit salt extrusion in marine fishes (Zadunaisky 1984). Metabolism and energy partitioning in different salinities may be influenced by other interactive physiological processes indirectly related to the energetics of osmoregulation, including changes in cortisol levels (Morgan and Iwama 1996), tissue permeabilities to water and ions (Isaia 1984; Rankin and Bolis 1984), gill ventilation, perfusion, functional surface area, and permeabilities (Jones and Randall 1978; Rankin and Bolis 1984).

Three types (brain, muscle, and mitochondria) of CK have been demonstrated to exist in teleosts (Rottiers et al. 1992; review by Coppes et al. 1990). By using muscle-type (MM) and brain-type (BB) specific antibodies to do a Western blot of CK, we found that MM-type CK of gill is altered by salinity after transfer from FW to SW. This is the first study to demonstrate that CK in gill can be influenced by salinity, directly or indirectly, especially MM-type CK. Moreover, in combination, MM- and BB-specific antibodies with fluorescein to stain the gill and localize the cell type, confocal microscopic data confirmed the existence of MM- and BB-type CK in the pavement and MR cells of tilapia gill (data not shown). The presence of MM-type CK, BB-type CK, and mitochondrial CK is demonstrated, and MM-type CK was predominant in *G. mirabilis* gill (Kültz and Somero 1995). Our Western data also showed the predominance of the MM-type CK in tilapia (*Oreochromis mossambicus*) gill after comparisons of MM- and BB-type CK. High CK activity is present in trout spermatozoa and is named as an s-CK isozyme (Saudrais et al. 1996), but a previous study showed the absence of s-CK in gill (Garber et al. 1990). Marine fish, red sea bream (*Pagrus major*), and Pacific mackerel (*Scomber japonicus*) possess less thermostable muscle CK than carp (*Cyprinus carpio*; Nakagawa and Nagayama 1991). Total CK activity decreased significantly (20%) in *O. mossambicus* brain after exposure to hypergravity for 7 d (Slenzka et al. 1993). We found that the alterations of brain CK activity in tilapia were also in response to salinity change (Weng et al. 2002). This suggests that CK plays a ubiquitous role in meeting the energy

demand for homeostasis during environmental change. Recently, three muscle CK isoforms of carp (*C. carpio*) have been cloned and characterized. The expression of different isoforms might also be linked to acclimation and environmental temperature (Sun et al. 1998). Interestingly, the alteration of MM-type CK after SW transfer seems to be newly synthesized protein involved in the process of osmoregulation. From this point of view, we propose that the promoter of MM-type CK may possess stress elements that are responsive to stress (e.g., salinity change) and have their own binding proteins. Moreover, the existence of transactivating factors (stress-responsive factors) or early-response genes in salinity-induced gill may be involved in the early stage of adaptation, and thus fish become more easily adapted to SW. Further studies will be necessary to test these hypotheses. We may also investigate the signal pathways that mitogen-activated protein kinases (MAPs or ERKs) or stress-activated protein kinase (see review in Tibbles and Woodgett 1999) play in response to environmental stress by using ERK-related specific antibodies.

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