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Physiological roles of free D- and L-alanine in the crayfish *Procambarus clarkii* with special reference to osmotic and anoxic stress responses

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Abstract

Under hyper-salinity stress from freshwater to 17 and 25 ppt seawater, red swamp crayfish *Procambarus clarkii* largely accumulated D- and L-alanine together with glycine, L-glutamine, and L-proline in both muscle and hepatopancreas. The increases of D- and L-alanine in muscle were the highest in all amino acids and reached 6.8- and 5.4-fold, respectively, from freshwater to 25 ppt seawater. These results indicate that both D- and L-alanine are the most potent osmolytes for intracellular isosmotic regulation in crayfish as well as other crustaceans thus far examined. Under anoxia stress below 0.1 mg/l dissolved oxygen for 12 h and subsequent recovery in normoxia for 12 h in freshwater, 17 and 25 ppt seawater, muscle ATP decreased dramatically in all salinity levels and almost depleted in seawater. Along with the decrease of muscle glycogen level, the significant increase of L-lactate was found in muscle, hepatopancreas, and hemolymph for each salinity level, suggesting the transport of L-lactate from muscle into hepatopancreas via hemolymph. Under anoxia, D- and L-alanine also largely increased in both muscle and hepatopancreas for each salinity level. The increase was much higher in seawater than in freshwater. Thus, both D- and L-alanine are possible to be anaerobic end products during prolonged anaerobiosis of this species. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Several free D-amino acids have been known to exist in a wide spectrum of organisms from bacteria to mammals, although the concentration is low in most of the animals. In aquatic invertebrates, however, crustaceans (D'Aniello and Giuditta, 1980; Okuma and Abe, 1994a,b; Okuma et al., 1995; Okuma and Abe, 1998; Abe et al., 1999a,b) and several bivalve mollusks (Matsushima et al., 1984; Felbeck and Wiley, 1987; Matsushima and Hayashi, 1992; Willey and Felbeck,

1995; Okuma et al., 1998) have been clarified to contain a large amount of free D-alanine (3–50 $\mu\text{mol/g}$ wet wt.) in their tissues. This D-alanine has also been confirmed to be biosynthesized from L-form by alanine racemase (Matsushima et al., 1984; Fujita et al., 1997a). The enzyme has recently been isolated or partially purified from the muscle of black tiger prawn *Penaeus monodon* (Fujita et al., 1997b), crayfish *Procambarus clarkii* (Shibata et al., 2000), and bivalve mollusk *Corbicula japonica* (Nomura et al., 2001). These facts clearly indicate that free D-alanine in these invertebrates plays an important physiological role in the cell.

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One of the important roles has been revealed to be the role as a major osmolyte responsible for intracellular isosmotic regulation or cell volume regulation in these invertebrates (Matsushima et al., 1984; Okuma and Abe, 1994a; Abe et al., 1999a,b). There have been extensive research works from early 1950s on the osmolytes for isosmotic regulation in invertebrates (Schoffeniels, 1976; Hochachka and Somero, 1984). From these early works, alanine is recognized as one of the best and universal 'compatible osmolytes' (Schoffeniels, 1976) in the tissues of many invertebrates. At least a part of this alanine has recently been assigned to D-enantiomer. Thus, it is apparent that several invertebrates keep the alanine racemase gene during their long evolutionary time scale and employ D-alanine as a defensive osmolyte for daily or seasonal salinity changes in their environment.

This D-alanine is used much positively in some crustaceans. A strong hyper-osmoregulator, Japanese mitten crab *Eriocheir japonicus*, accumulate only D- and L-alanine and some inorganic ions in muscle during the maturation in freshwater river and during downstream spawning migration toward the sea (Abe et al., 1999a). Thus, D- and L-alanine play an important role in the adjustment of salinity tolerance in this species. These facts suggest that there may be some other physiological functions in D-alanine in some invertebrates having a large amount of D-alanine in their tissues.

Aquatic invertebrates encounter periodical environmental hypoxia and well survive hypoxic or anoxic conditions by metabolic arrest and/or anaerobic metabolism. During anaerobiosis, bivalve mollusks are well known to produce several anaerobic end products such as alanine, alanopine, succinate, and propionate (Hochachka, 1980; Hochachka and Somero, 1984). A half of the alanine increase during a week of anaerobiosis of hard clam *Meretrix lusoria* was attributed to D-alanine (Okuma et al., 1998). Therefore, it is quite possible for D-alanine to be produced in the tissues of some invertebrates during anaerobiosis.

In the present study, we investigated biochemical responses of a freshwater crayfish *Procambarus clarkii* to severe environmental stresses by both hyper-salinity and anoxia and a possibility of D-alanine as an osmolyte for intracellular isosmotic regulation and an anaerobic end product during anaerobiosis.

2. Materials and methods

2.1. Animals

Live specimens of red swamp crayfish *Procambarus clarkii*, weighing approximately 30–50 g, captured at a marsh pond in Ibaraki Prefecture were purchased from a local freshwater fish store and kept in an aerated freshwater aquarium (60 l) at 20 °C. They were fed with fish muscle daily. The animals were reared in the aquarium for at least 3 days and not fed for 2 days prior to the experiments.

2.2. Experimental procedure

All experiments were carried out at 20 °C. Salinity in the rearing water was adjusted using filtrated natural seawater (35 ppt, 1038 mosM/kg). At day 1 of the experiment, salinity was directly increased to 17 ppt (504 mosM/kg) seawater. After keeping the animals in 17 ppt seawater for 3 days, seawater was added gradually up to 25 ppt (740 mosM/kg) seawater taking 3 days and the animals were kept in the seawater for 3 days. Five individuals were used for the sample at 0, 17, and 25 ppt seawater acclimation steps.

After acclimation to each salinity condition, the animals were exposed to anoxia ($PO_2 < 0.1$ mg/l) for 12 h under constant bubbling of nitrogen gas. After anoxia, normoxic conditions were restored with vigorous aeration ($PO_2 = 6.8$ mg/l) for 8 or 12 h. Five individuals were employed for control, 12-h anoxia, and 8- or 12-h recovery. In a preliminary experiment for nucleotide determinations, five animals were taken out at every 4-h interval during anoxia and recovery.

2.3. Preparation of extracts

After decapitation of crayfish, tail muscle and hepatopancreas were excised and quickly freeze-clamped using an aluminum tong pre-cooled in liquid nitrogen. Hemolymph was also withdrawn for L-lactate analysis by inserting a needle into the pericardial cavity. The perchloric acid extract was prepared from a 1-g sample as described previously (Okuma and Abe, 1994b).

2.4. Determination of metabolites

Water content of muscle was determined by the conventional 105 °C drying method. The concen-

trations of glycogen and L-lactate were determined enzymatically according to the method of Keppler and Decker (1984) and Noll (1984), respectively. Nucleotides were determined on HPLC as described previously (Okuma and Abe, 1994b). Free amino acid analysis was performed on an amino acid autoanalyzer (L-8500A; Hitachi, Tokyo) for physiological amino acids. D-Alanine was analyzed according to the method of Nimura and Kinoshita (1986) with HPLC using *N*-acetyl-L-cysteine and *o*-phthalaldehyde as prelabeling reagents and fluorescence detection. All chemicals were of analytical grade and obtained from Sigma Chemicals (St. Louis) or Wako Pure Chemical Industries (Osaka).

2.5. Statistical analysis

Statistical comparisons of the data were performed using one-way ANOVA followed by post hoc comparison of means using Duncan's multiple range tests. All data were expressed as mean \pm S.D.

3. Results and discussion

3.1. Osmotic stress responses

Water contents of the crayfish muscle were $78.2 \pm 0.9\%$ in freshwater, $76.0 \pm 0.8\%$ ($P < 0.01$) in 17 ppt and $74.7 \pm 0.7\%$ ($P < 0.001$) in 25 ppt seawater ($n = 5$ each). The decrease of water content was approximately 4.5% from 0 to 25 ppt seawater, indicating rather small dehydration during salinity stress in this species as previously shown by Okuma and Abe (1994a). Thus, all the following data were expressed as $\mu\text{mol/g}$ wet wt.

To examine the muscle energy states of crayfish during osmotic stress, the levels of nucleotides were determined (Fig. 1a). Of the dominant nucleotides, AMP, ADP, and ATP, found in the crayfish muscle, ATP was predominant in all salinity levels. The muscle energy charge was kept to be approximately 0.7 during salinity stress. Thus, the muscle energy status was well maintained even in 25 ppt seawater. The levels of ATP and ADP as well as total nucleotides significantly increased along with the salinity stress as previously reported (Okuma and Abe, 1994a). The increase of ADP, ATP, and total nucleotides from 0 to 25 ppt seawater was 1.7-, 1.7- and 1.6-fold, respectively, and clearly exceeded the muscle dehydration. An opposite tendency was found in L-lactate level (Fig. 1c),

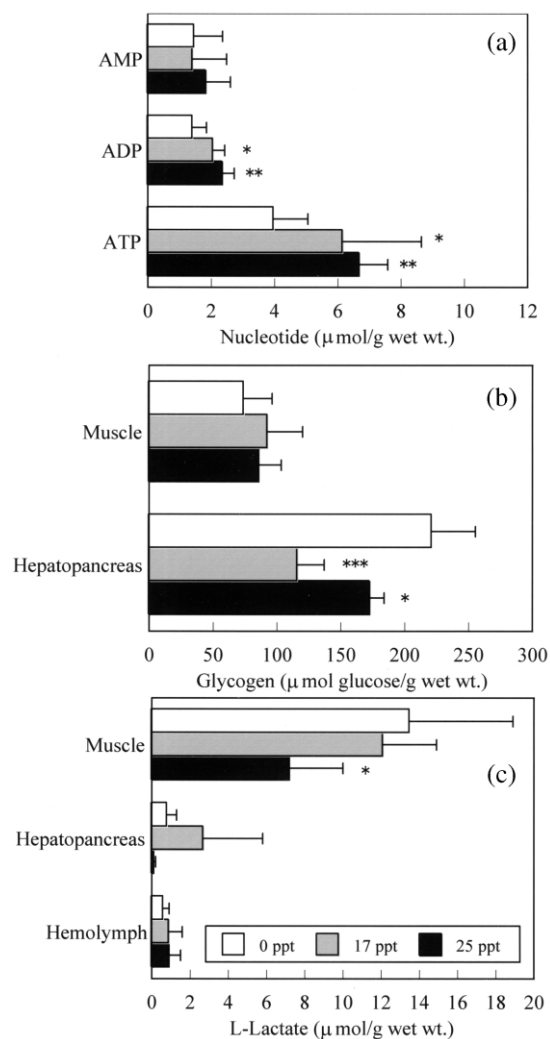


Fig. 1. Changes in the concentrations of nucleotides (a), glycogen (b), and L-lactate (c) in the muscle of crayfish during seawater acclimation. Mean \pm S.D. expressed as $\mu\text{mol/g}$ wet wt. for five animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

which was highest in freshwater and decreased with increasing salinity. Thus, this phenomenon may stem from the activity state of the animals in water. Crayfish was highly active in freshwater and the activity level would be suppressed in seawater due to the salinity stress and/or light anesthesia with magnesium ions in seawater. Magnesium ion has been known to be an anesthetizing agent in invertebrates and was reported to prevent the lactate increase during anoxia in the shrimp *Crangon crangon* (Sartoris and Pörtner, 1997).

Although the glycogen level in muscle was not

Table 1
Changes of major amino acid osmolytes during seawater acclimation of crayfish

Amino acid	Muscle			Hepatopancreas		
	FW	17 ppt-SW	25 ppt-SW	FW	17 ppt-SW	25 ppt-SW
Taurine	4.3±1.8	6.0±2.8	5.7±3.6	13.3±5.0	8.1±2.1	28.1±16.3
L-Glutamate	3.1±2.1	4.1±2.2	4.1±1.3	5.4±2.1	6.1±1.2	5.5±0.8
L-Glutamine	26.0±4.4	29.8±7.3	42.9±2.3***	2.3±1.0	4.8±2.7	6.7±3.8
L-Proline	5.9±3.1	12.6±4.9	22.6±4.7***	1.7±0.3	4.5±2.7	13.2±3.5***
Glycine	15.6±5.7	23.8±2.5	38.1±12.1**	8.3±4.4	28.0±7.9**	30.4±7.4***
D-Alanine	2.6±0.8	10.1±3.2***	17.6±2.5***	1.0±0.1	4.9±2.0**	7.7±2.4***
L-Alanine	4.2±1.0	11.9±3.8**	19.0±2.6***	2.2±0.5	6.0±2.2*	9.2±2.9***
Total alanine	6.8±1.8	21.9±6.9**	36.6±5.0***	3.2±0.6	10.9±4.2*	17.0±5.3***
L-Arginine	48.6±3.7	61.2±4.7***	61.6±3.5***	7.5±3.1	11.2±3.7	9.1±3.3
Others	36.0±10.6	52.8±11.9*	58.0±9.6*	19.1±3.1	19.4±6.0	30.1±6.1*
Total	146.6±6.3	212.1±29.8***	269.7±19.4***	60.4±5.3	93.2±15.4*	140.1±29.6***

Values are mean ± S.D. expressed as $\mu\text{mol/g}$ wet wt.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

changed largely from freshwater to 25 ppt seawater, the level in hepatopancreas was significantly lower in seawater than in freshwater (Fig. 1b). The glycogen level in hepatopancreas was lowest in 17 ppt seawater. This tendency was also found in another batch of crayfish as shown below for anoxia response. Thus, it is possible that glycogen in hepatopancreas is used as a carbon source to synthesize free amino acids as osmolytes in seawater and the amino acids are transported into muscle. While L-lactate in muscle decreased with increasing salinity, that in hepatopancreas and hemolymph was not changed and kept at a low level during seawater acclimation (Fig. 1c), suggesting the low activity level of the animal in each salinity level.

As shown in Table 1, several free amino acids increased with increasing salinity both in muscle and hepatopancreas as osmolytes for intracellular isosmotic regulation. In the muscle of freshwater crayfish, the major free amino acids were L-arginine, L-glutamine, glycine, D- and L-alanine, and L-proline. Along with the hyper-salinity stress, total concentration of free amino acids increased almost twice from 0 to 25 ppt seawater. L-Glutamine, L-proline, glycine, and D- and L-alanine were the dominant amino acid osmolytes increased with the increase of salinity. Thus, these amino acids were considered to be the major osmolytes for the intracellular isosmotic regulation in this species. These results were in good agreement with those

reported for muscle (Okuma and Abe, 1994a), and the total D-amino acid increased during seawater acclimation of crayfish was confirmed to be D-alanine in the present experiment. Of these osmolytes, D- and L-alanine showed the largest increase, 6.8- and 4.5-fold increase, respectively, from 0 to 25 ppt seawater followed by L-proline (3.8-fold). L-Arginine also increased significantly from 0 to 17 ppt seawater. However, this L-arginine mainly consisted of phosphoarginine in muscle and would not work as an osmolyte when crayfish is alive. The other amino acids also increased significantly and included L-serine, L-threonine, and L-asparagine. These amino acids, however, were low in amounts as L-glutamate (Table 1) and showed large individual variation. Although L-glutamate level was low under salinity stress, this amino acid has been known to have a pivotal role in the biosynthesis of non-essential amino acids as a precursor for L-proline and L-glutamine and an amino group donor for glycine and L-alanine (Schoffeniels, 1976). Thus, the L-glutamate level should be high during the initial stage of seawater acclimation.

In hepatopancreas, the concentration of total free amino acids also increased over twice from 0 to 25 ppt seawater (Table 1). The increase of L-proline was the largest (7.8-fold) in this tissue followed by D-alanine (7.7-fold). Although the increase of L-glutamine was 2.9-fold from 0 to 25 ppt seawater, this increase was not significant

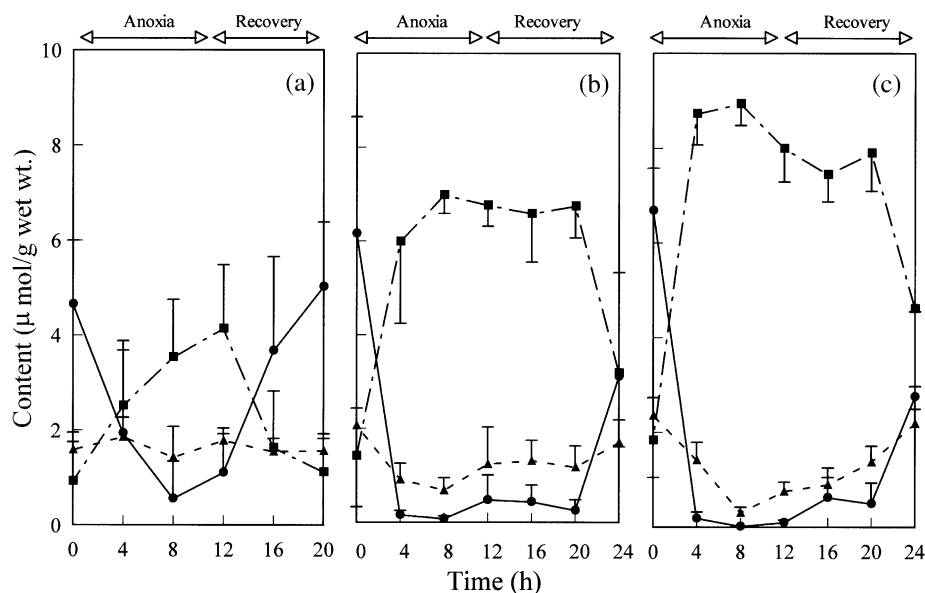


Fig. 2. Changes of ATP (●), ADP (▲), and AMP (■) levels in crayfish muscle during anoxia and recovery in 0 (a), 17 (b), and 25 (c) ppt seawater ($n=5$ each). Anoxic ($PO_2 < 0.01$ mg/l) and recovery ($PO_2 = 6.8$ mg/l) period were shown on the top of each graph.

statistically. Taurine was also high in 25 ppt seawater but not significant because of large individual differences.

The increase of D-alanine in both tissues was the highest in all amino acid osmolytes, indicating the most important osmolyte for isosmotic regulation in this species. The percentage of D-alanine to total alanine significantly elevated from 37.5% in freshwater crayfish muscle to 48.1% in 25 ppt seawater one. Hepatopancreas showed the same tendency as muscle. The major role of D-alanine as an important compatible osmolyte has been reported in the other invertebrates such as a crab species (Abe et al., 1999a,b), a bivalve mollusk (Matsushima et al., 1984), and a sipunculid worm (Low et al., 1996). These data indicate that aquatic invertebrates containing a large amount of free alanine in tissues possibly contain D-alanine and employ it for tissue isosmotic regulation.

3.2. Anoxic stress responses

In the natural habitat, red swamp crayfish usually burrows in mud on the bottom of a marsh pond during hot daytime and breaths air on the surface of water at night. Thus, the species is considered to have high anoxia tolerance. After acclimation to 0, 17, and 25 ppt seawater, the animals were exposed to anoxia below 0.1 mg/l

of dissolved oxygen for a 12-h duration. Almost all crayfish survived this severe anoxia. During anoxia, ATP dramatically decreased and AMP largely increased in the muscle of crayfish regardless of salinity level (Fig. 2). The energy charge dropped from 0.72–0.76 to 0.29 in freshwater, 0.12 in 17 ppt, and 0.05 in 25 ppt seawater. During recovery in normoxic conditions for 8 h, the energy charge almost restored the control level (0.75) in crayfish acclimated to freshwater. In 17 and 25 ppt seawater, however, the energy charge did not restore the control level even after 12 h of recovery and reached only 0.47 and 0.40, respectively. All the ATP decreased during anoxia changed to AMP. ADP also declined during anoxia in 17 and 25 ppt seawater crayfish. These data suggest that the animal received some stress during hyper-salinity acclimation even though it easily acclimated to 17 ppt seawater. It is marvelous that crayfish can survive even when the muscle energy charge is lowered below 0.1. To our knowledge, such a low energy charge has never been reported for live animals. In a related species *Orconectes limosus*, the muscle energy charge has been reported to be maintained up to 12 h of anoxia (Gade, 1984). An isopod *Cirolana borealis* has also been reported to keep the energy charge of the whole animal throughout 48 h of anoxia (Skjoldal and Bakke,

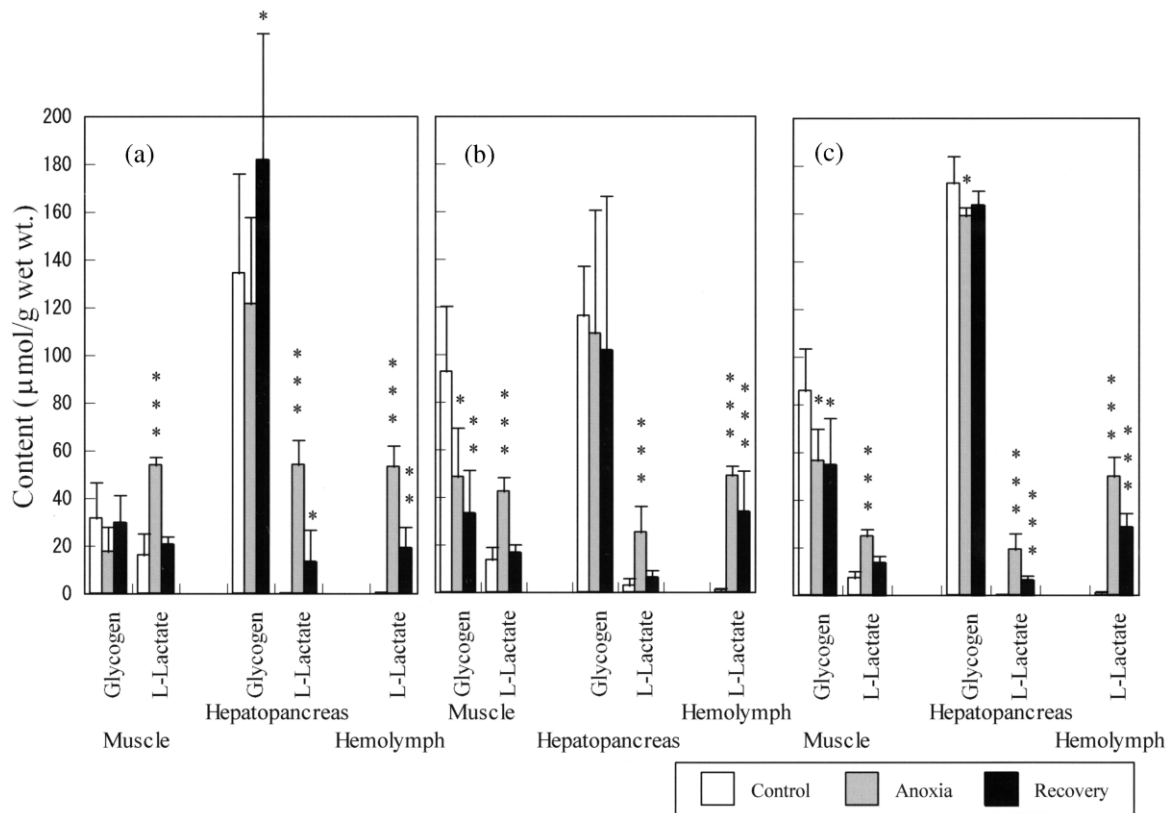


Fig. 3. Changes of glycogen and L-lactate contents in crayfish during anoxia and recovery in 0 (a), 17 (b), and 25 (c) ppt seawater ($n=5$). Refer to the legend of Fig. 1 for significance.

1978). Thus, the anoxia tolerance differs largely in crustacean species.

During anoxia and recovery, muscle glycogen decreased significantly in seawater acclimated crayfish (Fig. 3). However, in freshwater crayfish, of which the glycogen level was low compared with the others, the muscle glycogen level did not change significantly, although it decreased during anoxia and increased during recovery. In contrast to muscle, the glycogen in hepatopancreas was high in amount in every case and did not change largely during anoxia and recovery. The initial level of L-lactate was low in all body parts regardless of salinity level. The lactate level in muscle increased significantly during anoxia and returned almost to the control level during subsequent recovery in all salinity levels (Fig. 3). This is also true for the levels in hepatopancreas. In both tissues, the initial control level of L-lactate and the increase during anoxia was highest in freshwater and was suppressed in 17 and 25 ppt seawater. It is in good agreement with the lactate levels during

seawater acclimation shown in Fig. 1. In contrast, lactate increased in hemolymph almost to the same level as in hepatopancreas in all salinities and was still higher than the control level even after recovery. These data suggest the washout of lactate from muscle into hemolymph and transport it to hepatopancreas. Thus, L-lactate is considered to be a major anaerobic end product of this species, as reported previously (Gade, 1984).

During anaerobiosis, however, D- and L-alanine increased in both muscle and hepatopancreas (Fig. 4). In muscle, D- and L-alanine increased even after recovery in freshwater and 17 ppt seawater. The increase was low in freshwater because of small amounts of both D- and L-alanine in the control. In 25 ppt seawater, D- and L-alanine significantly increased during anoxia and decreased after recovery. The total increase of D- and L-alanine in 25 ppt seawater reached as high as 20 $\mu\text{mol/g}$ wet wt. in muscle after anoxia. In hepatopancreas, D- and L-alanine increased during anoxia and decreased after recovery irrespective of

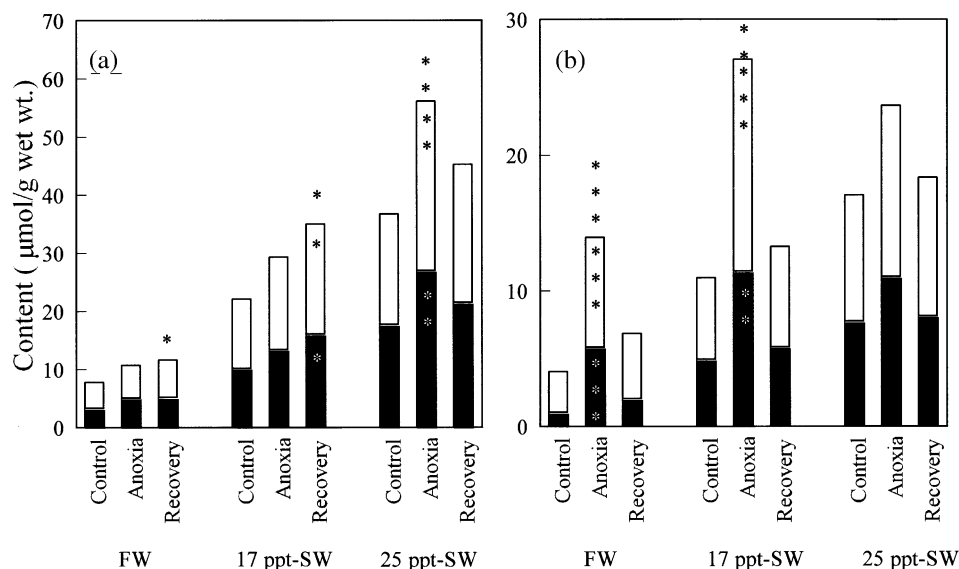


Fig. 4. Changes of D- (■) and L- (□) alanine in muscle (a) and hepatopancreas (b) of crayfish during anoxia and recovery in 0, 17 and 25 ppt seawater ($n=5$). Refer to the legend of Fig. 1 for significance.

the salinity levels, although their concentrations were lower in hepatopancreas than those in muscle. However, the total increase of D- and L-alanine reached 17 $\mu\text{mol/g}$ wet wt. in 17 ppt seawater. The percentage of D-alanine to total alanine in the muscle of freshwater crayfish was also significantly elevated from 40.8 to 47.2% and dropped to 45.9% after recovery, while that in seawater acclimated crayfish was almost kept at a constant value of approximately 43 and 46% in 17 and 25 ppt seawater, respectively. This is also true in hepatopancreas. The percentage in freshwater crayfish increased from 25.2 to 41.3% during anoxia and decreased to 29.5% after recovery, while the percentage did not change in 17 and 25 ppt seawater. Other than D- and L-alanine, several amino acids such as glycine, L-arginine, and L-glutamine were found to increase during anoxia or recovery. However, their increases were at random and no systematic change occurred during anoxia and recovery.

From these data, it is quite possible that D- and L-alanine are one of the anaerobic end products in crayfish during anaerobiosis. This is the first report describing the increases of D- and L-alanine during anaerobiosis other than lactate in crustaceans. In the other species of invertebrates, however, there have been several reports on the accumulation of D- and L-alanine during anaerobiosis. Schöttler et al. (1984) clarified the increase of D- and L-

alanine in body-wall musculature and gut tissues of the lugworm *Arenicola marina*. Pörtner et al. (1986) also reported that L-alanine rather than the D-form increased in the muscle of sipunculid *Sipunculus nudus* throughout anaerobiosis and was converted to D-alanine during recovery. Okuma and Abe (1998) revealed that D- and L-alanine increased in the tissues of hard clam *Meretrix lusoria* during anoxia for 7 days. Thus, it is possible that aquatic invertebrates extensively employ D-alanine as well as L-form as a major osmolytes for intracellular isosmotic regulation and as an anaerobic end product.

Acknowledgments

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References

- Abe, H., Okuma, E., Amano, H., Noda, H., Watanabe, K., 1999a. Role of free D- and L-alanine in the Japanese mitten crab *Eriocheir japonicus* to intracellular osmoregulation during downstream spawning migration. *Comp. Biochem. Physiol.* 123A, 55–59.
- Abe, H., Okuma, E., Amano, H., Noda, H., Watanabe, K., 1999b. Effects of seawater acclimation on the levels of free D- and L-alanine and other osmolytes in the Japanese mitten crab *Eriocheir japonicus*. *Fish. Sci.* 65, 949–954.

- D'Aniello, A., Giuditta, A., 1980. Presence of D-alanine in crustacean muscle and hepatopancreas. *Comp. Biochem. Physiol.* 66B, 319–322.
- Felbeck, H., Wiley, S., 1987. Free D-amino acids in the tissues of marine bivalves. *Biol. Bull.* 173, 252–259.
- Fujita, E., Okuma, E., Abe, H., 1997a. Occurrence of alanine racemase in crustaceans and the changes of the properties during seawater acclimation of crayfish. *Comp. Biochem. Physiol.* 116A, 83–87.
- Fujita, E., Okuma, E., Abe, H., 1997b. Partial purification and properties of alanine racemase from the muscle of black tiger prawn *Penaeus monodon*. *Fish. Sci.* 63, 440–445.
- Gade, G., 1984. Effects of oxygen deprivation during anoxia and muscular work on the energy metabolism of the crayfish, *Orconectes limosus*. *Comp. Biochem. Physiol.* 77A, 495–502.
- Hochachka, P.W., 1980. Living without Oxygen: Closed and Open Systems in Hypoxia Tolerance. Harvard University Press, Cambridge.
- Hochachka, P.W., Somero, G.N., 1984. Biochemical Adaptation. Princeton University Press, Princeton.
- Low, W.P., Ong, W.T., Ip, Y.K., 1996. Different physiological functions of free D- and L-alanine in three body parts of the intertidal sipunculid *Phascolosoma arcuatum*. *J. Comp. Physiol.* B 165, 558–564.
- Keppeler, D., Decker, K., 1984. Glycogen. In: Bergmeyer, H.U. (Ed.), *Methods in Enzymatic Analysis*, 3rd ed., vol. 6, Verlag Chemie GmbH, Weinheim pp. 11–17.
- Matsushima, O., Hayashi, Y.S., 1992. Metabolism of D- and L-alanine and regulation of intracellular free amino acid levels during salinity stress in a brackish-water bivalve *Corbicula japonica*. *Comp. Biochem. Physiol.* 102A, 465–471.
- Matsushima, O., Katayama, H., Yamada, K., Kado, Y., 1984. Occurrence of free D-alanine and alanine racemase activity in bivalve mollusks with special reference to intracellular osmoregulation. *Mar. Biol. Lett.* 5, 217–225.
- Nimura, N., Kinoshita, T., 1986. *o*-Phthalaldehyde-*N*-acetyl-L-cysteine as a chiral derivatization reagent for liquid chromatographic optical resolution of amino acid enantiomers and its application to conventional amino acid analysis. *J. Chromatogr.* 352, 169–177.
- Nomura, T., Yamamoto, I., Morishita, F., Furukawa, Y., Matsushima, O., 2001. Purification and some properties of alanine racemase from a bivalve mollusc *Corbicula japonica*. *J. Exp. Zool.* 289, 1–9.
- Noll, F., 1984. L-Lactate. In: Bergmeyer, H.U. (Ed.), *Methods in Enzymatic Analysis*, 3rd ed., vol. 6, Verlag Chemie GmbH, Weinheim pp. 582–588.
- Okuma, E., Abe, H., 1994a. Total D-amino and other free amino acids increase in the muscle of crayfish during seawater acclimation. *Comp. Biochem. Physiol.* 109A, 191–197.
- Okuma, E., Abe, H., 1994b. Simultaneous determination of D- and L-amino acids in the nervous tissues of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high-performance liquid chromatography. *J. Chromatogr. B* 600, 243–250.
- Okuma, E., Abe, H., 1998. Effects of starvation and D- or L-alanine administration on the free D- and L-alanine levels in the muscle and hepatopancreas of the crayfish, *Procambarus clarkii*. *Comp. Biochem. Physiol.* 120A, 681–686.
- Okuma, E., Fujita, E., Amano, H., Noda, H., Abe, H., 1995. Distribution of free D-amino acids in the tissues of crustaceans. *Fish. Sci.* 61, 157–160.
- Okuma, E., Watanabe, K., Abe, H., 1998. Distribution of free D-amino acids in bivalve mollusks and the effects of physiological conditions on the levels of D- and L-alanine in the tissues of the hard clam, *Meretrix lusoria*. *Fish. Sci.* 64, 606–611.
- Pörtner, H.O., Vogeler, S., Grieshaber, M.K., 1986. Recovery from anaerobiosis in the intertidal worm *Sipunculus nudus*. I. Restoration of aerobic, steady-state energy metabolism. *J. Exp. Biol.* 122, 37–50.
- Sartoris, F.J., Pörtner, H.O., 1997. Increased concentrations of haemolymph Mg^{2+} protect intracellular pH and ATP levels during temperature stress and anoxia in the common shrimp *Crangon crangon*. *J. Exp. Biol.* 200, 785–792.
- Schoffeniels, E., 1976. Adaptation with respect to salinity. *Biochem. Soc. Symp.* 41, 179–204.
- Schöttler, U., Wienhause, G., Westermann, J., 1984. Anaerobic metabolism in the lugworm *Arenicola marina* L.: the transition from aerobic to anaerobic metabolism. *Comp. Biochem. Physiol.* 79B, 93–103.
- Shibata, K., Shirasuna, K., Motegi, K., Kera, Y., Abe, H., Yamada, R., 2000. Purification and properties of alanine racemase from crayfish *Procambarus clarkii*. *Comp. Biochem. Physiol.* 126B, 599–608.
- Skjoldal, H.R., Bakke, T., 1978. Anaerobic metabolism of the scavenging isopod *Cirolana borealis* Lilljeborg. Adenine nucleotides. In: McLusky, D.S., Berry, A.J. (Eds.), *Physiology and Behaviour of Marine Organisms*, Pergamon Press, Oxford pp. 67–74.
- Wiley, S., Felbeck, H., 1995. D-Alanine metabolism in the lucinid clam *Lucinoma aequizonata*. *J. Comp. Physiol.* B. 164, 561–569.