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Effects of hypoxic and osmotic stress on the free D-aspartate level in the muscle of blood shell *Scapharca broughtonii*

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Summary. Blood shell, *Scapharca broughtonii*, contains large quantities of free D-aspartate comparable to free L-aspartate in its tissues. When the shell was reared in hypoxic seawater, D-aspartate as well as L-aspartate in the foot muscle decreased rapidly, and their total level became about one-fourth within 24 hr. None of the other amino acids examined showed a similar behavior, but many of them rather increased during the same period. The increase in L-alanine was especially remarkable and was almost equal to the sum of the decrease in aspartate enantiomers. When the shell that had been acclimated to hypoxic seawater for 96 hr was transferred to normoxic seawater, all the amino acid levels mostly returned to the control levels within 96 hr. In contrast to these effects of hypoxic stress, hyperosmotic stress of 150% seawater had no effect on the D- and L-aspartate levels in the same tissue. These results suggest that D-aspartate is involved in anaerobic energy metabolism of this bivalve as well as L-aspartate, whose vital role in anoxia-tolerant bivalves is well known.

Keywords: Anaerobic metabolism – *Scapharca broughtonii* – D-Aspartate – Aspartate racemase – Hypoxic stress – Osmotic stress

Introduction

Although D-amino acids were once believed to be unnatural products, their occurrence is recognized in a wide variety of living organisms from bacteria to mammals in these days. Especially, several bivalves have been reported to contain large quantities of free D-alanine or D-aspartate in their tissues (Felbeck and Wiley, 1987; Okuma et al., 1998). Of these two D-amino acids, D-alanine is most likely to serve as an osmoregulator since the level of D-alanine as well as that of L-alanine increased with increasing environmental salinity in the tissues of the brackish-water bivalve *Corbicula japonica* (Matsushima et al., 1984) and the hard clam *Meretrix lusoria* (Okuma et al., 1998). This behavior of D-alanine in response to the osmotic stress has been observed also in an intertidal sipunculid *Phascolosoma arcuatum* (Low et al., 1996) and some crustaceans (Matsushima et al., 1984; Okuma and Abe, 1994; Okuma et al., 1998; Abe et al., 1999a, b; Fujimori and Abe, 2002). On the other hand, the other D-amino acid, D-aspartate has not been extensively studied yet with respect to the physiological role, which is thus a remaining question.

We have previously reported that the blood shell, *Scapharca broughtonii*, contained substantial amounts of D-aspartate together with about equal amounts of L-aspartate in all the tissues examined, and that the activity of aspartate racemase, which catalyzes the interconversion of the enantiomers, was localized in the foot muscle and mantle (Watanabe et al., 1998). Further, we have recently demonstrated that the activity of the racemase purified from the foot muscle shows high sensitivity to some nucleotides such as ATP and AMP similar to that of phosphofructokinase I (Shibata et al., 2003b). This finding suggests that the enzyme may be involved in regulation of energy metabolism of *S. broughtonii*.

Aspartate has been known to function as a main substrate of anaerobic energy metabolism during the initial stage of anoxia in many molluscs including bivalves with high tolerance for anoxia (Collicutt and Hochachka, 1977; Gäde and Meinardus, 1981; Meinardus and Gäde, 1981; Zwaan et al., 1982; Eberlee et al., 1983; Gäde, 1983; Korycan and Storey, 1983; Sandra et al., 1983; Zwaan and Dando, 1984; Zwaan and Putzer, 1985; Isani et al., 1989; Brooks et al., 1991; Cortesi et al., 1992; Zwaan

et al., 1995). One of those bivalves studied well is *Scapharca inaequivalvis* that is closely related to *S. broughtonii*, which is also tolerant of anoxic conditions. We have therefore postulated that L-aspartate, which was often expressed as ''aspartate'' in these previous reports, plays a similar role in *S. broughtonii*, and D-aspartate participates as a storage form of L-aspartate which can be rapidly produced from the D-enantiomer by the action of aspartate racemase.

To test this hypothesis, we examined the changes in the contents of several amino acids, including D-aspartate in the foot tissue of *S. broughtonii* under hypoxic stress and subsequent recovery from the stress. In addition, we also examined the effect of hyperosmotic stress on the contents of these amino acids to find out whether D- and L-aspartate are involved in isosmotic regulation just like D- and L-alanine in some bivalves and crustaceans.

Materials and methods

Animals and acclimation to experimental conditions

Living specimens of the blood shell, *Scapharca broughtonii*, collected in the coast of Miyagi Prefecture, Japan were purchased from a fisherman. Animals of shell length approximately 8 cm were selected and kept in aquariums (about 25 animals/aquarium) each containing 30 l of artificial seawater, which was well aerated to contain dissolved oxygen above 6 mg/l at a temperature of 17–20°C. A complete water exchange was carried out on the second day, then every other day, before the animals were used for experiments at least 5 days later. To eliminate a possibility of incorporation of external amino acids, the animals were not fed throughout the experimental period.

Hypoxic stress and recovery from the stress

To impose hypoxia stress, each animal was placed separately in a bottle (about 8 cm diameter × about 18 cm height) containing 600 ml of the seawater, which was flushed with nitrogen until the dissolved oxygen became less than 0.06 mg/l. Immediately the bottles were tightly sealed off, and groups of the animals were sampled after 0, 12, 24, 48 and 96 hr.

For recovery experiments, animals were first held for 96 hr under the hypoxic condition and then returned to the aerated seawater aquarium, and sampled after 96 hr.

Exchange of the seawater was carried out daily and every other day, on hypoxic condition and aerobic condition, respectively. Animals, kept in the aerated seawater aquarium were taken at the same time as the sampling for hypoxia and recovery experiments, and were used as ''Control''.

Sampled animals were quickly opened, and only more the intensely colored outside part of the foot muscle, as described in our previous study (Watanabe et al., 1998), was dissected out. The muscle was washed in ice-cold saline to eliminate adherent mud, blotted with tissue paper, and stored frozen at −35°C until used.

Osmotic stress

To impose hyperosmotic stress, the salinity in seawater was increased stepwise using an artificial seawater salt mixture. Animals acclimated to the normal salinity (930 mOsm; 100% seawater) were transferred to a plastic vessel containing 125% seawater, and after three days, a number of animals were sampled. The remaining animals were then acclimated to 150% seawater, and sampled after three days. Throughout the experiment, the seawater was exchanged daily. Sampled animals were treated in the same manner as mentioned above.

Extraction of free amino acids from the foot muscle

Neutralized perchloric acid extract of the foot muscle was prepared by the method described previously (Watanabe et al., 1998). In brief, the tissues were homogenized first in 5 volumes of 5 mM potassium phosphate buffer (pH 7.4) containing 25 mM KCl, then further homogenized after addition of 5 volumes of 16% perchloric acid. After centrifugation, the supernatant solution was neutralized with KOH. The suspension was centrifuged to remove $KClO_4$, and the supernatant was concentrated to dryness under reduced pressure at 40°C. The resultant residue was dissolved in 100 μM L-cysteic acid (adjusted to about pH 7 with KOH), which was employed as the internal standard solution, and a portion of the mixture was subjected to amino acid analysis by HPLC.

HPLC analysis

The free amino acids extract, obtained from the procedure described above, was derivatized with *o*-phthaldialdehyde (OPA) and *N*-acetyl-L-cysteine (NAC), according to the method described by Aswad (1984). The fluorescent derivatives produced were separated and detected using an HPLC system consisting of a Model L-7100 low-pressure gradient unit (Hitachi, Tokyo, Japan), a Model FS-8020 fluorescence detector (Tosoh, Tokyo, Japan), a Model D-2500 chromato-integrator (Hitachi, Tokyo, Japan), a Model L-5090 degasser (Hitachi, Tokyo, Japan), and J' sphere-ODS-M80 column (4.6 × 250 mm) (YMC, Kyoto, Japan). Use of solvents and elution of the derivatives were performed under the same conditions as in our previous study (Watanabe et al., 1998).

Statistical analysis

Statistical significance was determined by Student's *t*-test for unpaired comparison. All data were expressed as mean ± S.D.

Results

Effect of hypoxia stress and recovery on the contents of free amino acids

Figure 1A–I show the effects of hypoxia and subsequent recovery on the contents of 9 amino acids, which were well separated and determined, independent of the presence of unknown contaminants in the tissue extracts.

The contents of all amino acids in the control animals remained nearly constant throughout the experiment, suggesting that starvation hardly influenced the levels of the amino acids during the experimental period. Of the amino acids examined, only D- and L-aspartate decreased during hypoxia (Fig. 1A and B): both of them decreased rapidly during the first 24 hr, and then continued to decrease slowly. The net decreases in D- and L-aspartate contents were approximately 4.0 μmol/g (74%) and 4.1 μmol/g (89%), respectively, at 96 hr of the hypoxia period. The total level of the enantiomers was about one-fourth of the

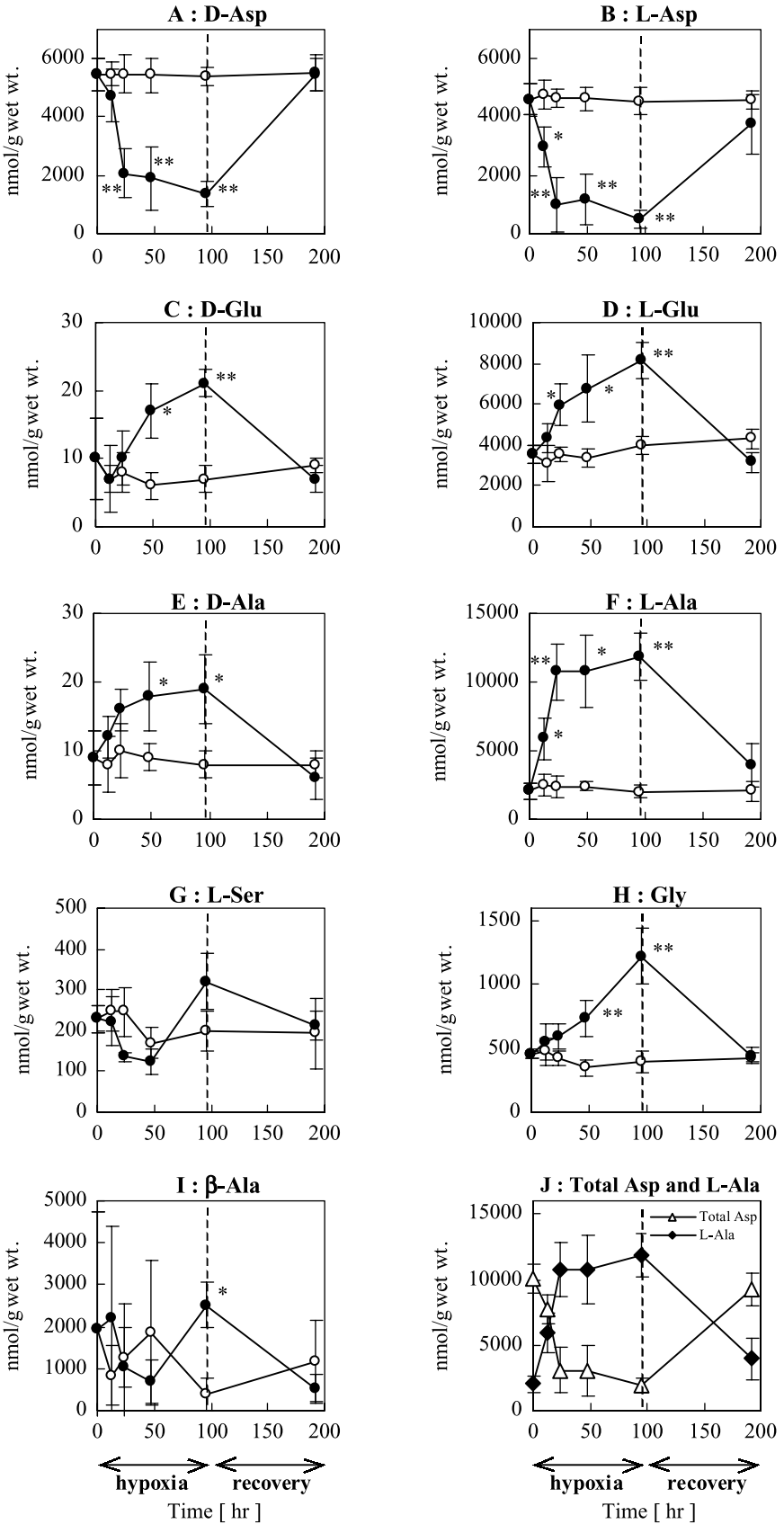

Fig. 1. Effect of hypoxia and recovery on the contents of free amino acids in the foot muscle of *Scapharca broughtonii*. (○): Control (aerobic condition); (●): Hypoxic stress followed by recovery from the stress. Vertical bars represent standard deviation (S.D.). Each value is mean ± S.D. (n = 3–5). $^{*}P < 0.01$, $^{**}P < 0.001$ (vs. controls)

aerobic control level after 24 hr. Their levels lowered during hypoxia were nearly restored to the control levels after 96 hr of aerobic recovery. It is also noted that the D-aspartate level was slightly higher than L-aspartate level by about 1 μmol/g all the time, whether or not the animals were under hypoxic stress, and the two aspartate levels changed in parallel throughout the experiments.

In contrast to these behaviors of aspartate enantiomers, many of the other amino acids increased under the hypoxic condition. In particular, the content of L-alanine increased remarkably during the initial stage of hypoxia (Fig. 1F) and became about 5 times higher than the aerobic control level after 24 hr. The level of L-alanine thus elevated also returned nearly to the control level after the aerobic recovery. When the time course of L-alanine content was compared with that of the total of D- and L-aspartate as in Fig. 1J, they appeared to be mirror images of each other, indicating a stoichiometric relationship between the two quantities.

D-Glutamate, L-glutamate, D-alanine and glycine also increased significantly during hypoxia, but not so remarkably as L-alanine: their increase was 2–3 times compared to the control and at most about 4.5 μmol/g wet wt. in quantity (Fig. 1C–E and H). No significant changes were found in L-serine and β-alanine levels throughout the period (Fig. 1G and I). All the amino acid levels changed during hypoxia mostly returned to the control levels after the recovery.

Effect of hyperosmotic stress on the contents of free amino acids

Figure 2 shows the changes in the contents of free amino acids in the foot muscle of *S. broughtonii* during stepwise acclimation to high salinity seawater. Exposure to 125% and 150% seawater caused no significant changes in the D- and L-aspartate levels. D-Glutamate (data not shown), L-glutamate and D-alanine (data not shown) levels also did not change significantly during this stress, whereas L-alanine, L-serine, glycine and β-alanine levels increased markedly with increasing salinity: the increases of the four amino acids at 150% seawater were 4, 3.4, 9.8 and 38 times, respectively, compared to the normal seawater.

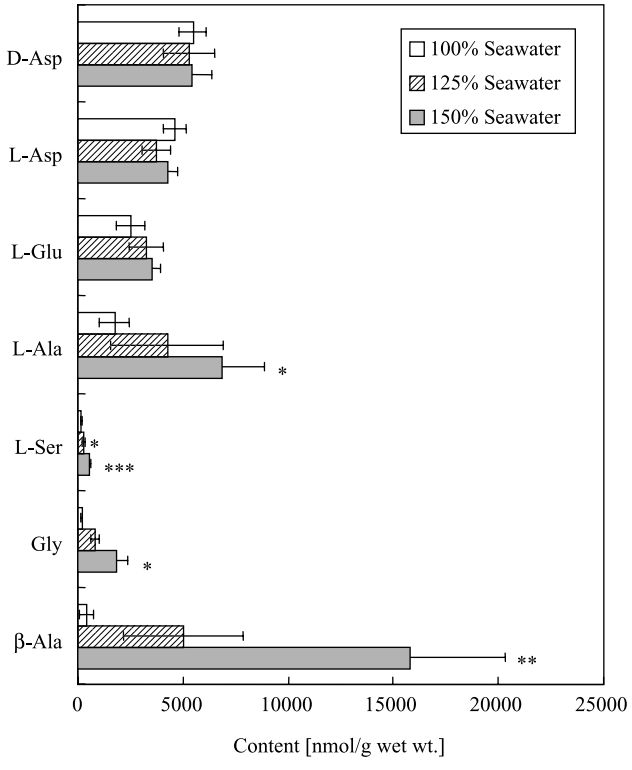

Fig. 2. Effect of hyperosmotic stress on the contents of free amino acids in the foot of *Scapharca broughtonii*. Vertical bars represent standard deviation (S.D.). Each value is mean ± S.D. (n = 3–8). $^{*}P < 0.02$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. 100% seawater)

Discussion

With regard to the physiological role of D-aspartate in *S. broughtonii*, we have previously proposed the hypothesis that D-aspartate serves as a storage form of L-aspartate, which functions as a main substrate of anaerobic energy metabolism in this anoxia-tolerant bivalve (Shibata et al., 2003b). This hypothesis is based on previous findings that L-aspartate functions as a main substrate for anaerobic energy metabolism during initial stage of anoxia in many molluscs including bivalves with high tolerance for anoxia (Collicutt and Hochachka, 1977; Gäde and Meinardus, 1981; Meinardus and Gäde, 1981; Zwaan et al., 1982; Eberlee et al., 1983; Gäde, 1983; Korycan and Storey, 1983; Sandra et al., 1983; Zwaan and Dando, 1984; Zwaan and Putzer, 1985; Isani et al., 1989; Brooks et al., 1991; Cortesi et al., 1992; Zwaan et al., 1995), and our findings that substantial amounts of D-aspartate occur together with nearly equal amounts of L-aspartate in *S. broughtonii* (Watanabe et al., 1998), and that the bivalve also contains aspartate racemase that catalyzes the interconversion of aspartate enantiomers (Watanabe et al., 1998; Shibata et al., 2003a), and that the enzyme activity is increased by AMP and decreased by ATP, suggesting that the enzyme is involved in energy metabolism (Shibata et al., 2003b).

The present study has been conducted, on one hand, to test this hypothesis and, on the other hand, to examine the possibility that D-aspartate serves as an osmolyte like D-alanine. D-Alanine is the D-amino acid most abundantly found in invertebrates and its main physiological role is established to serve as an osmolyte for isosmotic regulation, since its content increases under hyperosmotic stress in some bivalves and crustaceans (Matsushima et al., 1984; Okuma and Abe, 1994; Okuma et al., 1998; Abe et al., 1999a, b; Fujimori and Abe, 2002). The experimental results obtained in this study showed that D-aspartate as well as L-aspartate decreased markedly under hypoxic stress in the muscle of *S. broughtonii* and returned to the control level during the recovery period (Fig. 1A and B), whereas hyperosmotic stress imposed by up to 150% seawater did not influence the D-aspartate level (Fig. 2).

In view of the previous report (Okuma et al., 1998) that acclimation to 150% seawater largely increased D-alanine in the hard clam *Meretrix lusoria*, it seems unlikely that the main physiological role of D-aspartate in *S. broughtonii* is to serve as an osmolyte for isosmotic regulation like D-alanine. By contrast, L-alanine, L-serine, glycine and β-alanine levels increased markedly under high salinity stress in the foot muscle of *S. broughtonii* (Fig. 2). In particular, β-alanine, a nonprotein amino acid, showed the largest increase (about 38 times) and amount (about 15 μmol/g wet wt.) of 9 amino acids examined at 150% seawater. Therefore, it is very likely that not aspartate but these amino acids play the role as main osmolytes in this bivalve.

On the contrary, the observed behaviors of aspartate enantiomers during hypoxia and recovery indicate that their levels were lowered as they were consumed for anaerobic energy metabolism and restored by resumption of aerobic metabolism, thus supporting the hypothesis described above. It is also pointed out that D- and L-aspartate levels changed closely in parallel both in the period of hypoxia and recovery. This indicates that the enantiomers were interconverted rapidly enough to keep their balance at all times, supported by the action of aspartate racemase, so that the enantiomers might function as if a unified entity.

Comparison of the behaviors of the amino acids given in Fig. 1A–I indicates that only aspartate enantiomers decreased during hypoxia to serve for anaerobic metabolism, while many other amino acids increased as end products. Of these end products, L-alanine showed an overwhelmingly large increase. Similar increase in L-alanine as a major end product has been previously reported for anaerobic metabolism in many anoxia-tolerant invertebrates which utilize L-aspartate as a major substrate (Collicutt and Hochachka, 1977; Meinardus and Gäde, 1981; Zwaan et al., 1982; Eberlee et al., 1983; Gäde, 1983; Korycan and Storey, 1983; Sandra et al., 1983; Zwaan and Dando, 1984; Zwaan and Putzer, 1985; Isani et al., 1989; Brooks et al., 1991; Cortesi et al., 1992; Zwaan et al., 1995). Moreover, a 1:1 stoichiometry has been observed between L-aspartate utilization and L-alanine accumulation especially in the muscle tissues (Meinardus and Gäde, 1981; Zwaan et al., 1982; Eberlee et al., 1983; Korycan and Storey, 1983; Zwaan and Dando, 1984; Zwaan and Putzer, 1985; Isani et al., 1989; Brooks et al., 1991).

Different from this previous observation, the present results show a 1:1 stoichiometry between the decrease in the total of D- and L-aspartate and the increase in L-alanine during hypoxia, and between their changes in the reverse direction during the recovery period as illustrated in Fig. 1J. This stoichiometry seems to be in agreement with the active function of D-aspartate that virtually shares with L-aspartate the role in anaerobic energy metabolism in *S. broughtonii*. The same role is apparently assumed only by L-aspartate in the invertebrates previously studied, although little is known about D-aspartate in those animals.

It may be emphasized that the behaviors of D- and L-aspartate in the blood shell seem to be nearly identical: they are present at about a same concentration, being consumed and restored in parallel during hypoxia and recovery, respectively. One important difference between them is that D-aspartate has to be converted to the L-enantiomer by aspartate racemase before it is utilized, since there appears no other way of utilization in this bivalve lacking D-aspartate oxidase (Watanabe et al., 1998).

These considerations lead to a conclusion that the present study confirms the hypothesized role of D-aspartate as a storage form of L-aspartate that acts as a main substrate of anaerobic energy metabolism in this anoxia-tolerant bivalve. To our knowledge, this is the first report demonstrating the role of D-aspartate in anaerobic metabolism in *S. broughtonii*.

It should be pointed out that L-glutamate also showed a large and significant increase during hypoxia. This may reflect that L-aspartate utilization and L-alanine production during anaerobiosis are linked via L-glutamate through the action of two glutamate transaminases as discussed previously (Collicutt and Hochachka, 1977; Zwaan et al., 1982; Zwaan and Dando, 1984; Zwaan and Putzer, 1985).

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