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NOTE

Pressure-adaptive differences in lactate dehydrogenases of three hagfishes: *Eptatretus burgeri, Paramyxine atami* and *Eptatretus okinoseanus*

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Abstract The tolerance of abyssal pressures likely depends on adaptive modifications of fish proteins. However, structural modifications of proteins which allow functioning at high pressure remain unclear. We compared the activities of lactate dehydrogenase (LDH), an important enzyme in glycolytic reaction, in three hagfishes inhabiting different depths under increased pressure. LDH in *Eptatretus okinoseanus*, found at a depth of 1,000 m, was highly active at high pressure of 100 MPa maintaining the activity at 70% of that at 0.1 MPa. In contrast, LDH activity in *Paramyxine atami*, found at 250–400 m, decreased to 55% at 15 MPa, and that in *Eptatretus burgeri*, found at 45–60 m, was completely absent at 5 MPa. The result suggests that subunit interaction of the LDH-tetramer is more stable in *E. okinoseanus* than that in *P. atami* and *E. burgeri* under high-pressure conditions. We found six amino acid substitutions between the three LDH primary structures. Accordingly, these amino acid residues are likely to contribute to the stability of the *E. okinoseanus* LDH under high-pressure conditions.

Keywords Hagfish · Lactate dehydrogenase · High hydrostatic pressure · High-pressure adaptation

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The deep sea is characterized by low temperature (1–4°C), extremely high hydrostatic pressure, lack of sunlight, and relatively low influx of utilizable organic material derived from primary production in surface waters. Among such environmental factors, hydrostatic pressure is thought to have the greatest influence on the vertical distribution of organisms and speciation in the deep seas (France 1994; France and Kocher 1996; Morita 1999) and on the formation of protein complexes, e.g., enzyme–substrate or protein–protein interactions (Somero 1990). High-pressure habitats are very common in the biosphere, especially considering that 70% of the earth's surface is covered by oceans and nearly 79% of ocean water lies at depths greater than 1,000 m, where the pressure increases by approximately 0.1 MPa for every 10 m in depth. Many previous studies identified proteins from deep-sea fishes that function at high pressure (Somero 1990, 1992; Yancey et al. 2001), and hypothetical models for protein adaptation to deep-sea pressure have been proposed (Somero 1990). However, the primary structures of those proteins have not been determined in detail (Morita 2003). Somero (1992) studied the effects of pressure on the K_m values of many enzymes from shallow- and deep-living marine invertebrates and fishes. Malate dehydrogenase (MDH) from organisms adapted to pressure of 0.1 MPa increased with additional pressure, resulting in a concomitant increase in the K_m values for NADH (Siebenaller 1984). Morita (2003) cloned and sequenced the α-skeletal actin cDNA from *Coryphaenoides* sp. and found that some amino acid substitutions were responsible for the adaptation of α-actin to high pressure. Somero and Johns (2004) compared the kinetic properties and amino acid sequences of lactate dehydrogenase-A [L-LDH: NAD oxidoreductase, EC 1.1.1.27; (LDH-A_4)] with these of orthologues from congeners of Pacific damselfishes and found temperature-

adaptive changes in LDH-A_4 structure and function. In this study, we compared the kinetic properties and amino acid sequences of LDH-A_4 from three hagfishes and observed pressure-adaptive changes in LDH-A_4 structure and function.

Eptatretus okinoseanus and *Paramyxine atami* were collected from Suruga Bay, off Shizuoka, Japan, at 34–35′ N, 138–139′ E. *Eptatretus burgeri* was collected from Sagami Bay in the inlet off Misaki in Kanagawa prefecture, Japan, at 35.8′ N, 139.37′ E. The *E. burgeri* specimens were donated by the staff of the Tokyo University Marine Station, *E. okinoseanus* by Haruhisa Igarashi (Seisinmaru Ltd.) and *E. okinoseanus* and *P. atami* by Hisashi Hasegawa (Choukanemaru Ltd.). Skeletal muscles and hearts of the cyclostomata were washed with PBS buffer (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH7.4) pretreated with diethylpyrocarbonate to remove RNase and immediately frozen at −80°C.

The skeletal muscles of the three hagfishes were homogenized in a seven times of 0.25 M sucrose solution and LDH was purified with DEAE-cellulose and 5′-AMP-Sepharose 4B after salting-out by $(NH_4)_2SO_4$ and dialysis as described by Brodelius et al. (1973) with minor modification. Purity of the obtained sample was confirmed as a single peptide band by disc gel electrophoresis (Davis 1964) using 7% acrylamide gel stained with Coomassie Brilliant Blue (Chrambach et al. 1967). The disc was dipped in the reaction mixture described below and the LDH activity in the band was visualized by the absorption change of formazan added to the solution following the method of Kuroda and Yoshida (1986). Protein concentrations were determined using a Bio-Rad (California) protein assay kit I according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

The conversion of pyruvate to lactate by LDH was determined as described by Amador et al. (1963) with a slight modification in optimum pH and temperature for hagfishes, i.e., pH 6.2 and 30°C. A measure of 10 µl of LDH solution (6.0 µg/ml) was added to the reaction mixture (0.83 mM pyruvate, 0.13 mM NADH, 0.1 M MES buffer pH 6.2), and changes in the absorbance at 340 nm (A_{340}) was monitored using a spectrophotometer (Shimadzu 1600PC, Kyoto) equipped with a high-pressure optical cell (Abe and Horikoshi 1998). The activity was determined by the absorption decrease at 340 nm in 3 min after the addition of LDH under various pressure conditions. The Hill unit (Hill.U) is the amount of LDH which changes the optical density of NADH at 340 nm by 0.001 in 30 min (Hill 1956). The cDNA of LDHs was determined, and the accession numbers of the hagfish LDHs from *E. okinoseanus, P. atami* and *E. burgeri* are AB369246, AB369247 and AB369248, respectively.

The LDH activities of the three hagfishes under atmospheric pressure were measured and compared with those of other vertebrates. The specific activities of the three hagfishes at atmospheric pressure were 3.08×10^5 Hill.U/mg, 2.45×10^6 Hill.U/mg, 8.33×10^6 Hill.U/mg for *E. burgeri* LDH-A_4, *P. atami* LDH-A_4 and *E. okinoseanus* LDH-A_4, respectively. The specific activities of the hagfish LDH were very high in comparison with those of salmon, rabbit and human LDH ($3–6 \times 10^2$ Hill.U/mg) (Lim et al. 1975; Battellino et al. 1968; Pettit et al. 1981). The deep-sea hagfishes might have developed highly active LDHs to proceed with metabolism under harsh conditions such as low oxygen, low temperature and high hydrostatic pressure. The effect of high hydrostatic pressure on LDH activity was examined under pressures from 0.1 to 100 MPa (Fig. 1). The activity of LDH-A_4 from *E. burgeri* (shallow-sea species) was lost at pressure greater than 5 MPa. The activity of LDH-A_4 from *P. atami* (inhabiting at ∼400 m deep) was maintained at pressure of 10 MPa, but was decreased to about 55% at 15 MPa and to about 15% at 20 MPa. Among the three hagfishes, *E. okinoseanus* inhabit the deepest point around 700–1,000 m. Its LDH-A_4 appeared to be fully active at pressures up to 40 MPa and it maintained the activity of 70% even at 100 MPa. These results suggest that LDH-A_4 of *E. okinoseanus* is more adapted to high-pressure conditions than others. Schade et al. (1980) reported that porcine muscle LDH was completely dissociated from a tetramer to monomers at 150 MPa. Thus, LDH of the deep-sea hagfish has a structure resistant to pressure-induced subunit dissociation compared to that of the shallow-sea type hagfishes.

Morita (2003) performed structure-based analysis of the high-pressure adaptation of α-actin from two abyssal

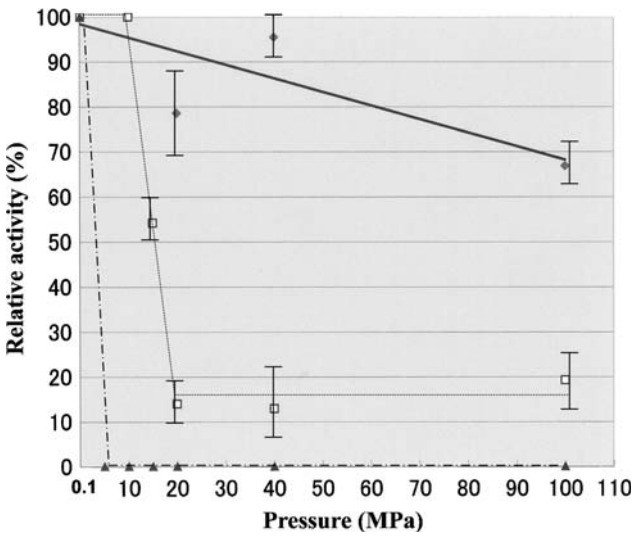

Fig. 1 Effects of hydrostatic pressure on LDHs from three hagfishes

Coryphaenoides species. He found three amino acid substitutions that distinguished these abyssal actins from orthologues from nonabyssal *Coryphaenoides*. The substitutions Q137K and A155S prevent the dissociation reactions of ATP and Ca^{2+} from being influenced by high pressure. In particular, the lysine residue at position 137 results in a much smaller apparent volume change in the Ca^{2+} dissociation reaction. Another substitution, V54A or L67P, reduces the volume change associated with actin polymerization and has a role in maintaining the DNase I activity of actin at high pressure. There were differences in six amino acid residues (6, 10, 20, 156, 269 and 341) when comparing the LDHs of the three hagfishes. There were two types of amino acid variation among the three hagfishes (Fig. 2), one type (type A: residues 10, 20 and 269) represents the difference between *E. okinoseanus* and the other two hagfishes, and the other type (type B: residues 6, 156 and 341) represents that between *E. burgeri* and the other two. *P. atami* shows intermediate properties among the three hagfishes, along with its intermediate habitat depth. Four of the amino acid residues (6, 10, 20 and 341) occur where the four monomers combine to form tetramers and amino acid residues (156 and 269), which are in the neighborhood of the active site and may control enzymatic activity. The relationship of pressure-tolerance and amino acid substitution has been studied in malate dehydrogenase in hyperthermophilic microorganisms, *Pyrococcus furiosus* and *Pyrococcus abyssi* (Giulio et al. 2005). They showed the list starting with the most piezophilic residue: Arg > Gly > Ser > Asp > Val > Glu > Lys > Ala > Asn > Leu > His > Met > IIe > Thr > Phe > Pro > Cys > Gln > Trp > Tyr. In the previous discussion, we postulated the dissociation of LDH under high pressure. In the three hagfishes studied, four of the six amino acid substitutions followed the general rule of pressure tolerance and were found in type-A substitutions at positions 10, 20 and 269.

The effects of high temperature on LDH activity were also determined. At 60°C, LDH activity decreased to 10% in *E. burgeri*, whereas 36 and 33% of the activity remained in *P. atami* and *E. okinoseanus*, respectively. Thus, the heat stability and pressure tolerance of LDH-A_4 from the three hagfishes differed depending on the depth of their habitats. Somero (2004) reported the structures of LDHs relevant to their heat stability of Pacific Damselfishes (*Chromis punctipinnus*). Heat stability of Pacific Damselfishes LDH was achieved by the amino acid substitution from Thr to Ala at the 219th position, in the region of a key "hinge" for the moving α1G–α2G helix and also in one of the most solvent-exposed loops of the tetrameric enzyme. The increased hydrophobicity of this loop resulting from the substitution could potentially force the monomer to remain closer to the overall molecule and result in a less flexible "hinge" for the movement of the α1G–α2G helix. The structures conferring heat stability and pressure tolerance were also estimated. In all three hagfish LDHs in the present study, the 219th amino acid was Leu and not relevant to the difference in heat stability among them. The additional region (amino acids 220–227), specific and common to hagfish LDHs, is not responsible for heat stability.

In this study, some pressure tolerance structures of the LDHs from hagfishes were postulated. They must be examined further to determine the effects of amino acid substitutions. The structural relevance of pressure tolerance to heat tolerance in LDH is also should be clarified. We previously reported on the use of the new site-directed mutagenesis technique (Kashiwagi et al. 2006). The mechanism of adaptation of LDHs from deep-sea hagfishes

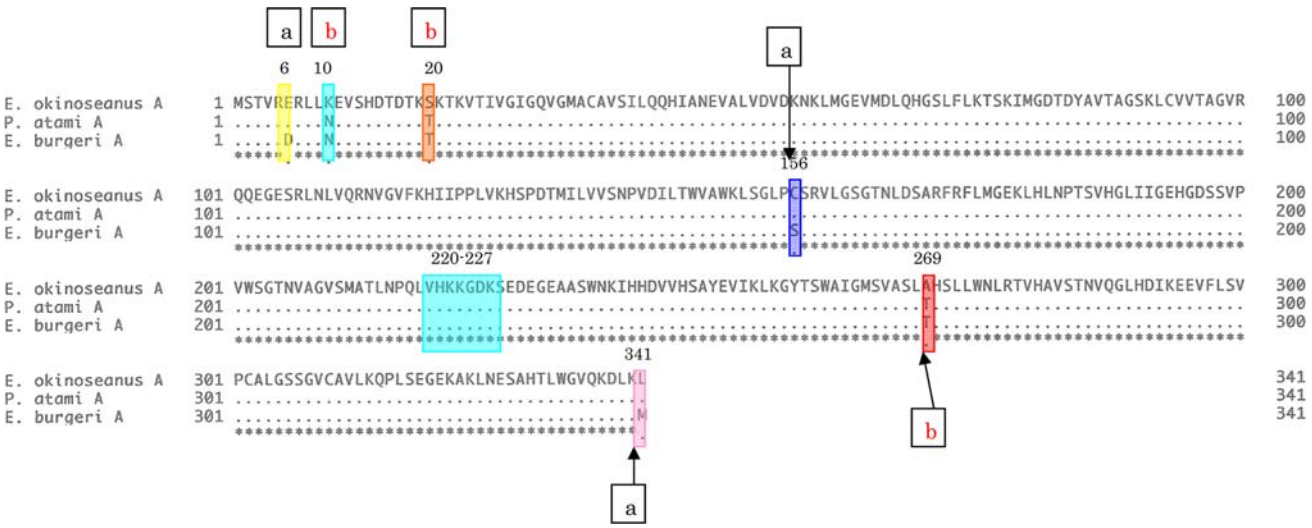

Fig. 2 Differences in amino acid sequences of LDH-A_4 from *E. okinoseanus*, *P. atami* and *E. burgeri*

to high pressure at the amino acid sequence level will be clarified in an ongoing experiment using that method.

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