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Comparative sequence analysis of myosin heavy chain proteins from congeneric shallow- and deep-living rattail fish (genus *Coryphaenoides*)

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SUMMARY

The evolutionary adaptations of functional genes to life at high pressure are not well understood. To elucidate the mechanisms of protein adaptation to high pressure, we cloned the myosin heavy chain (MyHC) cDNA from skeletal muscle of two deep-sea fishes, *Coryphaenoides yaquinae* and *C. armatus*, and two non-deep-sea fishes, *C. acrolepis* and *C. cinereus*. The MyHCs of deep-sea fishes have a unique structure in two loop regions, loop-1 and loop-2, in comparison with those of non-deep-sea fishes. The loop-1 region of deep-sea fishes has a Pro residue and the loop-2 region, which is an actin-binding site, is shorter than the same region in non-deep-sea fishes. The amino acid substitution in the loop-1 region is expected to be mainly involved in ATPase activity, whereas the deletion in the loop-2 region affects the association of MyHC with actin filaments at high pressure. In addition, the MyHC of deep-sea fishes has biased amino acid substitutions at core positions in the coiled-coil structure of the rod region. These amino acid substitutions are likely to decrease the cavities in the coiled-coil structure and consequently make the structure more compact and unaffected by high pressure. Together, these results indicate that amino acid substitutions can adaptively alter the pressure sensitivity of a protein even if they do not directly influence core structure.

Key words: adaptive evolution, myosin heavy chain, high pressure adaptation, deep-sea fish.

INTRODUCTION

The deep-sea environment is characterized by low temperature, lack of light and extremely high hydrostatic pressure. Hydrostatic pressure increases by approximately 0.1 MPa with every 10 m of depth in the ocean (Saunders and Fofonoff, 1976) and high hydrostatic pressure influences the physiological and biochemical processes of marine organisms (Siebenaller and Somero, 1989; Somero, 1992; Gibbs, 1997). An important parameter to study in the context of pressure is the volume change that accompanies biochemical events such as protein–ligand or protein–protein interactions. The sign of volume change in a reaction determines the reaction's sensitivity to pressure: when the reaction volume increases or decreases, high hydrostatic pressure will, respectively, inhibit or enhance the reaction.

Species of the genus *Coryphaenoides*, commonly known as rattails or grenadiers, have been extensively studied as an excellent model to elucidate adaptation to the deep sea because of their widespread bathymetric distribution from 200 to 6400 m in depth (Iwamoto and Stein, 1974; Smith, 1978; Morita, 1999). A good study system for efficiently investigating an environmental adaptation is to compare closely related species inhabiting different environments (Siebenaller and Somero, 1989). Although many previous studies have identified proteins from deep-sea fish that function at high hydrostatic pressure, only α-actin protein has been examined at the level of the amino acid sequence (Morita, 2000; Morita, 2003). The polymerization of globular (G)-actin to filamentous (F)-actin is accompanied by an increase in total volume (Ikkai and Ooi, 1966). Interestingly, the increase in volume with the polymerization of α-actin from two deep-sea fishes, *C. armatus* and *C. yaquinae*, is much smaller than that from non-deep-sea fishes, *C. acrolepis* and *C. cinereus*, which is advantageous for a deep-sea environment (Morita, 2003; Swezey and Somero, 1985).

Myosin plays a central role in a molecular motor in biological motility (Harrington and Rodgers, 1984; Warrick and Spudich, 1987). In particular, conventional myosin (class II) is the major component of the thick filaments of muscle cells, and consists of two myosin heavy chains (MyHCs), two essential light chains (ELCs) and two regulatory light chains (RLCs). The N-terminal portion, subfragment-1 (S-1), of MyHC forms a globular head including actin- and ATP-binding sites, whereas the C-terminal portions (rod region) of the two MyHCs associate to form a coiled-coil rod that is involved in filament formation under physiological ionic conditions (Harrington and Rodgers, 1984; Lowey et al., 1969). Myosin functions as an actin-based molecular motor that transduces chemical energy obtained by ATP hydrolysis into mechanical work (Spudich, 1994). Although it is unclear what influence high pressure has on this function, high pressure is known to dissociate the complex between F-actin and heavy meromyosin (HMM) (Ikkai and Ooi, 1969). In a previous study, no amino acid substitutions in the MyHC-binding sites of deep-sea fish actin were found (Morita, 2003). Therefore, the deep-sea fish MyHCs are expected to have amino acid substitutions in the F-actin binding sites.

In this study, the MyHC cDNA from two deep-sea fishes, *C. yaquinae* and *C. armatus*, and two non-deep-sea fishes, *C. acrolepis* and *C. cinereus*, were cloned and sequenced to test this hypothesis. The MyHCs from the deep-sea fishes have unique amino acid substitutions, suggesting a new mechanism of protein function at high hydrostatic pressure.

MATERIALS AND METHODS

Materials

C. yaquinae (Iwamoto et Stein), *C. armatus* (Hector) and *C. acrolepis* (Bean) were collected using large pots with long lines by

the R/V Soyo-maru of the National Research Institute in the Fisheries Research Agency of Fisheries Science, and *C. cinereus* (Gilbert) was collected using trawl nets by the R/V Wakataka-maru of the Tohoku National Fisheries Research Institute in the Fisheries Research Agency. The sampling locations were 39–58.10′N, 154–59.50′E, 5600 m for *C. yaquinae* (habitat depth about 4000–6400 m); 44–00.70′N, 145–22.20′E, 3940 m for *C. armatus* (habitat depth about 2700–5000 m); 41–40.20′N, 142–57.40′E, 180 m for *C. acrolepis* (habitat depth about 180–2000 m); and 38–02.13′N, 142–22.05′E, 997 m for *C. cinereu* (habitat depth about 455–1270 m) (Nakabo, 2000). Sampled fish were rapidly frozen after collection and stored below –80°C until use.

Isolation of myosin cDNAs

Poly(A)$^+$ mRNA was extracted from the dorsal skeletal muscle of *C. yaquinae*, and single-strand cDNA was synthesized as described previously (Morita, 2000). In order to obtain the partial nucleotide sequences of MyHC cDNA from *C. yaquinae*, reverse transcription PCR (RT-PCR) was performed using sense (primer 1: 5′-GGC-TGCCATTTACCTCCGGAAGCCAGAGAG-3′) and antisense (primer 2: 5′-CTGATGAATTTACCAAAACGGGAGGAGTTG-3′) primers, which were synthesized using the sequence corresponding to positions 36–758 of walleye pollack MyHC cDNA (GenBank accession number AB017819). The RT-PCR conditions were 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The reaction mixture (100 μl) contained 2 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} each dNTP, 100 pmol of each primer, 2.5 units of ExTaq DNA polymerase and 100 ng of cDNA. DNA sequence analysis revealed that the nucleotide sequence of the RT-PCR product was homologous to that of the walleye pollack MyHC gene.

In order to determine the 5′ non-coding region nucleotide sequence of *C. yaquinae* MyHC, 5′ rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE system, version 2 (Gibco BRL, Gaithersburg, MD, USA). The antisense primer (5′-AACGCTCTGCAAGGTTATACAACACAGAGGC-3′) for 5′ RACE was synthesized by using the nucleotide sequences determined from the RT-PCR product. To obtain PCR products for all four species used in this study, including the full-length myosin cDNA, long PCR was performed using the *Not*I–d(T)$_{18}$ primer and a sense primer (5′-TGACTGCAGGGCTGGTTGTTACGACT-3′) synthesized using the nucleotide sequences determined by the 5′ RACE strategy. The long-PCR conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 7 min. The reaction mixture (100 μl) contained 2 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} each dNTP, 100 pmol of each primer, 2.5 units of ExTaq DNA polymerase and 100 ng of cDNA. The amplified fragments were purified on a 1.0% low-melting point agarose gel. Each purified fragment was verified by direct nucleotide sequencing of both sense and antisense strands with sequencing primers synthesized using the nucleotide sequences of walleye pollack MyHC cDNA and the first RT-PCR product described above. Some sequencing primers were also synthesized using the nucleotide sequences determined by the direct nucleotide sequencing of long-PCR products. The GenBank accession numbers of MyHC nucleotide sequences obtained in this study are *C. yaquinae* AB330139, *C. armatus* AB330140, *C. acrolepis* AB330141 and *C. cinereus* AB330142.

RESULTS

The long-PCR strategy yielded full-length MyHC cDNA for all species. The nucleotide sequence of the coding region of full-length MyHC showed high homology between the two deep-sea fishes (99.7%) and between the two non-deep-sea fishes (99.6%). There was also high homology in this region between the deep-sea fishes and the non-deep-sea fishes (97.1% to 98.6%). Amino acid sequence homology of the full-length MyHC cDNA was 99.2% between the two deep-sea fishes, as well as between the two non-deep-sea fishes. Amino acid sequence homology varied from 95.4% to 96% between the deep-sea and non-deep-sea fishes. These comparisons are summarized in Table 1. MyHC is mainly composed of two parts: the subfragment-1 (S-1) head and the rod (Harrington and Rodgers, 1984; Lowey et al., 1969). The S-1 head contains ATP-binding sites I–III, actin-binding sites I–IV, a reactive lysyl residue (RLR) region, two light chain-binding regions and two loop regions. Each region, except for the two loop regions, showed high homology to that of the other species (Table 1). The sequences of ATP-binding sites I and III, actin-binding sites I–III and the RLR region were identical between deep-sea and non-deep-sea fish MyHCs, and only one substitution, Lys to Arg, was found in ATP-binding site II. The RLC binding site and the ELC binding site of the deep-sea fish MyHCs showed, respectively, 88.9% and 92.3% homology to the corresponding region of the non-deep-sea fish MyHCs.

Table 1. Comparison of the nucleotide sequence and deduced amino acid sequence of MyHC

	C. armatus	*C. acrolepis*	*C. cinerus*
C. yaquinae	99.7/99.2 (99.1, 99.6, 99.8)	98.6/95.4 (95.5, 98.3, 98.9)	97.1/95.0 (95.1, 97.9, 98.8)
C. armatus	–	97.2/96.0 (96.1, 97.9, 98.8)	97.2/95.6 (95.7, 98.3, 98.9)
C. acrolepis	–	–	99.6/99.2 (99.1, 99.6, 99.8)

MyHC, myosin heavy chain.
Numbers to the left and right of the solidus represent the percentage homology of the nucleotide sequence in the coding region and amino acid sequence, respectively.
Numbers in parentheses represent the percentage homology of the amino acid sequence of, from left to right, S-1 (subfragment-1), S-2 (subfragment-2) and LMM (light meromyosin).

The loop-1 and loop-2 regions of deep-sea fish MyHC, which are known as the 25–50 kDa and 50–20 kDa (actin-binding site IV) junctions, respectively, showed less conservation to the MyHCs of other species (Harrington and Rodgers, 1984; Mornet et al., 1979; Goodson et al., 1999; Gauvry et al., 2000). These loops are known to include a large number of Lys residues. Indeed, there are four, three and three Lys residues in the loop-1 region of rabbit, walleye pollack and white croaker MyHC, respectively. Deep-sea and non-deep-sea fish MyHCs also have four Lys residues in the loop-1 region. There is variation in the net charge (from +1 to +2) and the

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                  203        Loop-1
Deep-sea fishes   G G E K K K D E P G K I Q G
Non deep-sea fishes G G E K K K E E A G K I Q G

                  619        Loop-2
Deep-sea fishes   M P V L Y P T I V E D - A T K K G G K K K G G S M
Non deep-sea fishes M P V L Y P T I V E D T A T K K G G K K K G G S M
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Fig. 1. Comparison of the deduced amino acid sequences of the loop-1 and loop-2 regions of myosin heavy chain (MyHC) from deep-sea and non-deep-sea fishes. Numbers indicate the position of each left-hand amino acid residue. A dash corresponds to a gap in the amino acid sequence.

Table 2. Comparison of length, charge and number of Pro residues in loop-1 and loop-2 regions of MyHC

No.	Common name	Species	Accession no.	Loop-1			Loop-2		
				Length	Charge	No. of Pro	Length	Charge	No. of Pro
1	Rattail/grenadier (deep-sea fish)	*Coryphaenoides yaquinae*	AB330139*	14	1	1	24	3	2
2	Rattail/grenadier (deep-sea fish)	*Coryphaenoides armatus*	AB330140*	14	1	1	24	3	2
3	Rattail/grenadier (non-deep-sea fish)	*Coryphaenoides acrolepis*	AB330141*	14	1	0	25	3	2
4	Rattail/grenadier (non-deep-sea fish)	*Coryphaenoides cinerus*	AB330142*	14	1	0	25	3	2
5	Walleye pollack	*Theragra chalcogramma*	BAA33452	16	0	0	26	3	3
6	Chum salmon	*Oncorhynchus keta*	BAC00871	13	1	1	25	4	3
7	White croaker	*Pennahia argentata*	BAB12571	11	1	0	24	3	3
8	Greater amberjack	*Seriola dumerilii*	BAA92289	14	2	1	30	4	0
9	Antarctic rock cod	*Notothenia coriiceps*	CAC27776	9	1	1	24	3	2
10	Blacksided hawkfish (tropical fish)	*Paracirrhites forsteri*	CAC59753	14	2	2	28	4	0
11	Carp-10**	*Cyprinus carpio*	BAA22067	14	2	2	26	3	1
12	Carp-20**	*Cyprinus carpio*	BAA22068	14	2	2	25	3	1
13	Carp-30**	*Cyprinus carpio*	BAA22069	14	2	4	27	4	0
14	Chinese perch	*Siniperca chuatsi*	AAS0050	14	2	2	29	4	0
15	Zebra fish	*Danio rerio*	NP_694514	15	2	0	26	3	2
16	Zebra fish	*Danio rerio*	AAY26547	16	2	1	26	3	2
17	Chicken	*Gallus gallus*	NP_001013415	14	2	0	28	3	0
18	Rabbit	*Rattus norvegicus*	U32574	15	2	0	28	3	0

*Nucleotide accession no.
**Numbers refer to acclimation temperatures of the carp (°C).

number of residues (9 to 15) in loop-1 of MyHC from different species. The loop-1 regions of both deep- and non-deep-sea fish MyHCs have a net charge of +1 and contain 14 amino acid residues (Fig. 1 and Table 2). Interestingly, whereas non-deep-sea fishes have no Pro residue in the loop-1 region, deep-sea fishes do have a Pro residue, similar to salmon and carp. In the loop-2 region, both deep-sea and non-deep-sea fishes have five Lys residues (Fig. 1). These Lys residues, which are highly conserved in the C-terminal portion of the loop-2 region, interact with negatively charged residues located in the N-terminal subdomain-1 of actin (Joel et al., 2000). The net charges in the loop-2 region of other fishes range from +3 to +5. Both deep-sea and non-deep-sea fishes also have +3 net charges in the loop-2 region. It is noteworthy that the loop-2 region is shorter in deep-sea fish relative to other species, with the exception of white croaker (Fig. 1 and Table 2).

The rod region, which is composed of the S-2 and light meromyosin (LMM) regions, has a coiled-coil structure formed by two α-helices. The amino acid sequence homology of the S-2 regions between the two deep-sea fishes and between the two non-deep-sea fishes was 99.6% in both cases. Furthermore, the amino acid sequence homology between deep-sea fishes and non-deep-sea fishes was 97.9–98.3% (Table 1). The S-2 regions of deep-sea and non-deep fish show features typical of α-helical coiled-coil proteins with a seven residue repeating pattern, and can be divided into 20 zones every 28 residues (McLachlan and Karn, 1982). In addition, both S-2 regions have a skip residue (Thr) at the 350th residue, similar to other fishes (Iwami et al., 2002). The amino acid sequence of the LMM region between the two deep-sea fishes and between the two non-deep-sea fishes showed 99.8% homology in both cases. The amino acid sequence homology between the deep-sea fishes and the non-deep-sea fishes was 98.6–98.9% (Table 1). The amino acid sequence of the deep-sea fish LMM region also has a coiled-coil motif, which can be divided into 21 zones of 28 residues with a seven residue repeating pattern, as found in other fish species (McLachlan and Karn, 1982). Furthermore, the LMM region of both deep-sea and non-deep-sea fishes has three skip residues (Glu, Glu and Gly) at the 14th, 211th and 436th residues, similar to LMMs of other fishes (Iwami et al., 2002). The fish LMM region characteristically possesses a higher frequency of Gly substitutions compared with that of rabbit and chicken. These Gly residues are considered to be responsible for the heat instability of the tail region of fish MyHCs, because the small side chain of Gly residues makes α-helices unstable (Iwammi et al., 2002; Gekko and Hasegawa, 1986). There was no difference in the number of Gly residues in the LMM region between deep- and non-deep-sea fishes.

The amino acid sequence of proteins with the coiled-coil structure comprises a characteristic seven residue repeat pattern, designated a, b, c, d, e, f and g. The amino acid residues at the a- and d-positions are in the core of the coiled-coil as an internal hydrophobic seam. On the other hand, the c and the g residues are usually charged and form a salt bridge between the helices of the two monomers (Atkinson and Stewart, 1992). The total volume of the amino acid residues in the rod region of deep-sea fish MyHCs is smaller than that in other species except for walleye pollack MyHC. In particular, the total volume at the a-position located in the core of the coiled-coil is smallest among those in other species (Table 3). Interestingly, substitutions to amino acids with small side chains were frequently found in the a-position of the rod region of deep-sea fish MyHC (Table 4).

DISCUSSION

High hydrostatic pressure influences biochemical processes accompanied by volume changes such as protein–protein interactions, and also shifts the equilibrium of the protein complex towards dissociation (Siebenaller and Somero, 1989; Somero, 1992; Gibbs, 1997; Silva et al., 1996). MyHC binding to F-actin functions

as a molecular motor by transducing chemical energy from ATP hydrolysis into mechanical work (Spudich, 1994). Because high pressure dissociates the complex between F-actin and HMM (Ikkai and Ooi, 1969), this binding increases the volume. A previous study revealed that actin protein from deep-sea fishes had no substitutions in the contact regions with MyHC relative to non-deep-sea fishes (Morita, 2003). The deep-sea fish MyHC had therefore been predicted to have amino acid substitutions in the F-actin contact regions. DNA sequence analyses in this study showed that the MyHC of deep-sea fishes relative to that of non-deep-sea fishes has a shorter loop-2 region, which is one of the four contact regions for F-actin.

The α-actin protein from deep-sea fishes has three unique amino acid substitutions: Val54Ala or Leu67Pro, Gln137Lys and Ala155Ser. Two of the substitutions, Gln137Lys and Ala155Ser, prevent the dissociation reaction of ATP and Ca^{2+} from being influenced by high pressure. The Val54Ala or Leu67Pro substitution in the β-sheet structure in subdomain-2, which interacts with two other actin proteins, is considered to reduce the increase in volume associated with actin polymerization and enable the actin protein to polymerize at high pressure without a large increase in volume (Morita, 2003). Although the advantage of the shorter loop-2 region of MyHC from deep-sea fishes is not fully understood, the small number of amino acid residues would surely also decrease the space occurring between MyHC and F-actin at their binding. The loop-2 region is thought to be regulated by the level of actin-activated Mg-ATPase activity, because actin-activated Mg-ATPase activity of *Dictyostelium* chimeric myosin containing a foreign myosin loop-2 region has been shown to reflect the activity level of the donor MyHC (Uyeda et al., 1994; Murphy and Spudich, 1999).

The loop regions of MyHC have been proposed to be a major determinant of the kinetic properties of myosin (Spudich, 1994). The loop regions of MyHC are hypervariable and the properties of the loops (net charge, charge distribution and length) have been used as a molecular tool to study evolutionary or phylogenetic relationships among MyHCs from different species or among MyHC gene families within a species (Goodson et al., 1999; Gauvry et al., 2000). In particular, the loop properties in fish MyHC have been investigated in relation to the water temperature in their habitat (Watabe et al., 1998).

The loop regions in deep-sea fish MyHC have a characteristic structure: the loop-2 region is shorter than that in non-deep-sea fishes and the loop-1 region has a Pro residue. The Pro residue uniquely constrains rotational freedom through the creation of a pyrrolidine ring between the α-carbon and the amide nitrogen of the peptide backbone. The net charge, charge distribution and length of the loop-1 region are involved in ATPase activity and sliding velocity by controlling ATP binding, ATP hydrolysis or ADP release rate (Spudich, 1994; Goodson et al., 1999; Murphy and Spudich, 1998; Rovner et al., 1997). A longer or more positively charged loop region has a faster rate of ADP release, more rapid shortening velocity and higher ATPase activity. By contrast, a loop with a shorter length or more Pro residues, which makes the structure more rigid, retards *in vitro* motility and reduces the rate of ADP release (Sweeney et al., 1998). The shorter length of the loop-2 region and the presence of a Pro residue in the loop-1 region of MyHC from deep-sea fishes would make the respective loop structure rigid and consequently would maintain the structure at high pressure. In the case of actin protein from deep-sea fishes, the tight binding of ATP and Ca^{2+} maintains the structure from the inside (Morita, 2003).

Table 3. Summed volumes of amino acid residues at each position in the rod region

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
a	14031.7	14031.7	14052.6	14052.6	14032.1	14172.5	14227.5	14271.4	14205.0	14247.3	14277.8	14274.3	14348.1	14266.1	14267.9	14284.6	14262.2	14307.4
b	12258.5	12288.2	12245.3	12275.0	12291.2	12280.4	12227.1	12364.2	12294.0	12273.7	12310.3	12371.2	12377.2	12294.0	12350.9	12383.5	12526.3	12523.6
c	12582.7	12582.7	12587.3	12587.3	12514.6	12622.3	12584.2	12585.9	12613.7	12580.6	12568.8	12662.7	12596.8	12585.9	12600.8	12600.8	12816.2	12710.9
d	13604.7	13604.7	13648.7	13648.7	13582.8	13638.1	13666.3	13559.8	13565.5	13580.6	13546.2	13549.7	13554.5	13572.3	13543.5	13549.3	13710.1	13709.5
e	13058.0	13058.0	13039.6	13039.6	13008.7	13014.2	13042.7	13131.9	13035.7	13036.4	13029.6	13027.0	13083.9	13128.1	13053.8	13051.5	13141.6	13134.1
f	12826.8	12826.8	12859.4	12859.4	12792.0	13012.8	12944.2	12896.1	12931.9	12844.0	12882.5	12865.8	12898.4	12875.4	12948.6	12931.0	12938.7	12956.7
g	12886.0	12888.3	12901.0	12903.3	12921.3	12810.5	12905.0	12900.4	12870.3	12907.7	12959.7	12942.8	12908.1	12883.6	12876.4	12892.9	13127.4	13118.9
Total	91248.4	91280.4	91333.9	91365.9	91142.7	91550.8	91597.0	91709.7	91516.1	91470.3	91574.9	91693.5	91767.0	91605.4	91641.9	91693.6	92522.5	92461.1

a–g indicate amino acid residue positions within the seven residue repeat pattern. To compare the same number of amino acid residues, the sequences from the last a-position were deleted.
Species numbers 1–18 are the same as in Table 2.
Data are in ml mol^{-1}.

Table 4. Percentage of substitutions to amino acids with smaller volume residues out of the total substitutions at each position within the rod region in comparison with that of *C. yaquinae*

Position	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
a	0	100	100.0	50.0	64.7	66.7	60.0	56.3	69.2	60.0	60.0	75.0	64.3	68.8	73.3	60.0	58.8
b	100	0	66.7	60.0	55.6	25.0	60.0	50.0	50.0	46.2	53.3	58.3	50.0	50.0	53.3	63.6	69.6
c	0	50.0	50.0	0	45.5	50.0	50.0	58.3	54.5	46.2	64.3	42.9	50.0	33.3	33.3	68.2	50.0
d	0	50.0	50.0	36.4	47.4	45.0	33.3	47.1	43.8	31.8	36.4	29.4	35.3	31.3	38.9	54.1	48.7
e	0	0	0	37.5	27.3	35.7	58.3	36.4	37.5	43.8	42.9	46.7	50.0	42.9	42.9	53.6	50.0
f	0	100.0	100.0	44.4	72.2	62.5	57.1	73.3	50.0	55.0	55.0	58.8	53.8	81.3	84.6	54.5	59.4
g	100	66.7	75.0	53.8	26.3	42.9	55.6	47.1	50.0	55.6	50.0	52.9	47.4	50.0	53.3	64.7	60.6

a–g indicate amino acid residue positions within the seven residue repeat pattern.
Species numbers are the same as in Table 2.
Data are percentages.

The rod region consisting of the S-2 and LMM regions shows a coiled-coil structure formed by two α-helices. High pressure would compress the cavities present in the coiled-coil structure. Although actin protein also has a large cavity in the protein pocket, this cavity is maintained in deep-sea fish by tight binding of ATP and Ca^{2+} (Morita, 2003). In this study, we found that MyHCs from deep-sea fishes have a high substitution rate, greater than 50% at the a-position located in the core of the coiled-coil to amino acids with smaller side chains (Table 4). These substitutions decrease the cavities in the coiled-coil structure and consequently would make the structure compact, rigid and unaffected by high pressure.

The deep-sea environment is also characterized by low temperature (about 4°C). Protein loop regions have been shown to influence the enzymatic characteristics and functional properties of several catalytic proteins (Diggle et al., 1995; Salinelli et al., 1996). In order to adapt to a cold environment, the loop structure requires greater flexibility and efficiency at low temperatures (Fields and Houseman, 2004). A shorter length loop and hence higher charge density, or a loop without a Pro residue, is expected to exhibit the cleft opening and closing associated with protein function more readily and frequently. Indeed, the MyHC from Antarctic fish has a shorter loop-1 region (Gauvry et al., 2000). Carp, *Cypinus carpio*, has MyHC isoforms that are adapted to different water temperatures (Watabe et al., 1998). The 10°C type MyHC isoform obtained from 10°C-acclimated carp has fewer Pro residues than the 30°C type isoform obtained from 30°C-acclimated carp (Table 2). However, the loop-1 region of MyHC from deep-sea fishes is the same length as that of non-deep-sea fish MyHC, and also has a Pro residue. In conclusion, the MyHC of deep-sea fishes exhibits adaptations to high pressure, but does not appear to have the characteristics observed previously in a cold-adapted MyHC. This finding suggests that pressure is a more challenging abiotic factor than temperature for this protein.

It is believed that new species occur when the gene flow between different populations is interrupted. High pressure is considered to enhance the speciation process as a barrier to gene flow even in the absence of absolute geographic isolation because high pressure has various effects on biochemical and physiological processes (France and Kocher, 1996). Hydrostatic pressure is one of several factors that determine the distribution patterns of marine organisms (Morita, 1999). Adaptation in conserving protein function is categorized into extrinsic adaptation and intrinsic adaptation (Somero, 2003). The former is defined as adjustments in the composition of the cellular fluids to maintain protein function, such as trimethylamine-*N*-oxide for high pressure adaptation (Yancey et al., 2002; Samerotte et al., 2007). The latter is accomplished through genetic differences inducing amino acid substitution, such as the actin protein of deep-sea fishes adapted for high pressure (Morita, 2003). This previous study agrees with Perutz's theory about protein adaptation, which states that a few amino acid substitutions in key positions, not needed for core structure or basic function, enable the protein to adapt to a new environment (Morita, 2003; Perutz, 1983; Weeds and Taylor, 1975). In this study, we have shown that deep-sea fish MyHC has one amino acid substitution in the loop-1 region, one amino acid deletion in loop-2 of the S-1 region and biased amino acid substitutions at a specific position in the rod region (Fig. 1 and Table 4). Although the number of substitutions found in the rod region is large because of the repeat structure, these observations are also consistent with Perutz's theory.

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