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Research Article

Notable diversity in hemoglobin expression patterns among species of the deep-sea clam, *Calyptogena*

K. Kawano [a], N. Iwasaki [b] and T. Suzuki [a, *]

[a] Laboratory of Biochemistry, Faculty of Science, Kochi University, Kochi 780-8520 (Japan), Fax: +88 844 8356, e-mail: suzuki@cc.kochi-u.ac.jp
[b] Usa Marine Biological Institute, Kochi University, Kochi 781-1164 (Japan)

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Abstract. The deep-sea clams *Calyptogena nautilei* and *C. tsubasa*, which live in the cold-seep area at a depth of 3570 m in the Nankai Trough, Japan, have abundant hemoglobins (Hbs) in erythrocytes, similar to other *Calyptogena* species. We determined the cDNA-derived amino acid sequences of Hbs from two *Calyptogena* species. *C. tsubasa* was found to contain two dimeric Hbs, Hb I consisting of 145 amino acid residues and Hb II with 137 residues, similar to known Hbs from *C. soyoae* and *C. kaikoi*. Sequence identity was over 90% among the orthologous chains of *Calyptogena* Hbs. On the other hand, surprisingly, *C. nautilei* contained two monomeric Hbs, Hb III containing 141 residues and Hb IV with 134 residues. In addition, Hbs III and IV showed only 33–42% sequence identity with Hbs I and II from other *Calyptogena* species. The distal (E7) histidine, one of the functionally important residues of the heme protein, is replaced by glutamine in all Hb chains of *Calyptogena* species. A phylogenetic analysis indicated that *C. nautilei* Hb III is closer to Hb I from other *Calyptogena* species. We suppose that a Hb gene was duplicated at least three times in an immediate ancestor of *Calyptogena* and, presumably depending on physiological conditions different Hb sets are being expressed: dimeric Hbs I and II in *C. soyoae*, *C. kaikoi* and *C. tsubasa*, and monomeric Hbs III and IV in *C. nautilei.*

Key words. Hemoglobin; cDNA sequence; *Calyptogena nautilei*; evolution; deep sea.

Unique biological communities are present around areas of hydrothermal vents or cold seeps at depths of 1100–6000 m [1, 2], in which the most conspicuous animals are the giant clam *Calyptogena* and the giant tube worm *Riftia*. These invertebrates are sustained by a mutual symbiosis with sulfide-oxidizing bacteria [3], and their blood, containing abundant hemoglobins (Hbs), is believed to facilitate O_2 transport to the site of carbon fixation [4, 5].

The subunit structure and amino acid sequences of Hbs from the deep-sea cold-seep clams *Calyptogena soyoae* and *C. kaikoi* have been published previously [6–8]. These species have two homodimeric Hbs (Hb I and II) in erythrocytes. *C. kaikoi* contains two kinds of abundant myoglobins in the dark-red-colored adductor muscle, and the myoglobins are identical to Hbs I and II by amino acid sequence analysis [8]. The Hb sequences of *Calyptogena* show low homology with other globins (at most 20% identity) and lack the N-terminal extension of seven to nine amino acid residues characteristic of all molluscan Hbs. Although the subunit assembly of molluscan Hb is known to be 'back-to-front' relative to mammalian Hb [9], *C. soyoae* Hb is unlikely to undergo such subunit assembly because it lacks homology in the sequence involving the back-to-front subunit interface [6, 7]. Many of the molluscan intracellular globin genes have a characteristic four-exon/three-intron structure, with the precoding and the two conventional introns widely conserved in animal

* Corresponding author.

globin genes. Interestingly, the *C. soyoae* Hb gene has no preceding intron but instead contains an additional intron in the A-helix region, together with the two conventional introns [10].

C. soyoae Hb is autoxidized 1300 times faster than human Hb under the same conditions (37 °C, pH 7.2). However, *Calyptogena* oxyHb is stable enough to act as an O_2 carrier, since the autoxidation rate at near physiological temperature (3 °C) is about three times lower than that of human Hb at 37 °C [6].

C. nautili and *C. tsubasai*, living in the cold-seep area at 1.5 °C at a depth of 3570 m in the Nankai Trough, Japan, also have abundant Hbs in erythrocytes. Here we report the subunit structure and cDNA-derived amino acid sequences of the Hbs, and show that a quite different Hb set is being expressed in *C. nautilei*, compared with that of other *Calyptogena* species.

Materials and methods

Materials

C. nautilei and *C. tsubasa* clams (15–20 cm long) were collected from the cold-seep area located at 32°21.2′ N, 134°31.9′ E at a depth of 3570 m in the Nankai Trough, Japan, by the unmanned submersible Kaiko during May 2001 [11]. As soon as the clams were brought to the surface, they were stored at –70 °C until required.

Sequence determination of *C. nautilei* Hbs

The blood (hemolysate) of *C. nautilei* was centrifuged at 12,000 rpm for 10 min at 2 °C, and the resultant Hb solution was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol (DTT). The Hb solution was then applied to a TSK-Gel DEAE-5PW (7.5 × 75 mm; Tosoh) column and eluted with a linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) over 60 min at a flow rate of 1 ml/min. The isolated chains (III and IV) were pyridylethylated and the amino acid sequences of the entire proteins were determined by an automated protein sequencer (Applied BioSystems 476 A). Chain IV was digested further with lysyl endopeptidase (Wako) in 50 mM Tris-HCl (pH 8.0), and the peptides were separated by reverse phase chromatography using a Cosmosil 5 C_{18}-300 column (2.5 × 150 mm). Subsequently, some of the isolated peptides were sequenced.

mRNA was prepared from the blood of *C. nautilei* with a QuickPrep Micro mRNA Purification Kit (Pharmacia). The single-stranded cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Pharmacia) using the oligo-dT adaptor as a primer. The 3′ regions of Hb III and IV cDNAs were amplified by PCR for 30 cycles, each cycle consisting of 0.5 min at 94 °C (denaturation), 0.5 min at 55 °C (annealing) and 1.5 min at 72 °C (primer extension). Gene *Taq* DNA polymerase (Wako) was used as the amplifying enzyme. The primers used were the oligo-dT adaptor and the redundant oligomer C.nHb-M1 (23-mer) designed on the basis of the internal amino acid sequence KINGMCAT for Hb III, and the oligo-dT adaptor and the redundant oligomer Csp2HbF (23-mer) based on the N-terminal amino acid sequence MVTAEEKA for Hb IV. The amplified products were sequenced directly with a PRISM dye terminator cycle FS ready reaction kit using a Model 373-18 DNA sequencer (Applied BioSystems).

The 5′ regions of the cDNAs were amplified as follows. The poly-G tail was added to the 3′ end of the single-stranded cDNA, which had been synthesized using the oligo-dT adaptor as a primer, with a terminal deoxynucleotidyl transferase (Promega) in 100 mM cacodylate buffer (pH 6.8) containing 1 mM $CoCl_2$, 0.1 mM DTT, 0.1 mg/ml bovine serum albumin and 0.5 mM dGTP. The 5′ region of Hb III cDNA was amplified by PCR, using the oligo-dC adaptor and the specific primer CsHb1R1 (5′-AGTTTCAACGTATTACACATCG). To improve the specificity, the amplified products were reamplified using the oligo-dC adaptor and the specific primer CnHb1R2 (5′-ACCTCGAATAGATCGACGTTTG). The 5′ region of Hb IV cDNA was also amplified by a similar method. The amplified products were sequenced directly.

Sequence determination of *C. tsubasa* Hbs

mRNA preparation and cDNA synthesis for *C. tsubasa* Hb was the same as for *C. nautilei*. The 3′ regions of Hbs I and II were amplified using the oligo-dT primer and several specific primers used in the PCR amplification of *C. soyoae* Hb cDNA [10] (Cs.F1 or Cs.F3 for Hb I amplification, and CsHb2.F2 or CsHb2.F5 for Hb II amplification). The 5′ regions were amplified as for *C. nautilei*.

Estimation of subunit structure by gel filtration chromatography

The molecular mass of native Hb was estimated on a Superdex 75 (1 × 30 cm; Pharmacia) gel filtration column, equilibrated with 50 mM sodium phosphate buffer (pH 7.2). The column was eluted with the same buffer at a flow rate of 0.5 ml/min and calibrated with *C. soyoae* dimeric Hb (32 kDa) and sperm whale myoglobin (18 kDa).

Results

Subunit structure and sequences of *C. nautilei* Hbs

Using Superdex 75 gel filtration chromatography, *C. nautilei* Hb was eluted as a peak corresponding to an apparent mass of 18,000, equivalent to the monomeric structure. The Hb was separated on a DEAE-5PW column into two components, Hbs III and IV in almost equimolar concentrations, as shown in figure 1. The N-terminal

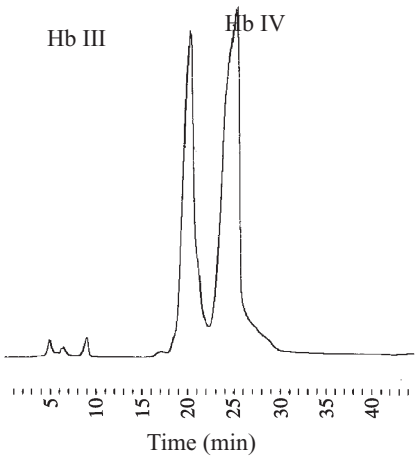

Figure 1. DEAE-5PW chromatography of *C. nautilei* Hb. The Hb solution in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT was applied to a column of TSK-Gel DEAE-5PW (7.5 × 75 mm; Tosoh) and eluted with a linear gradient of 0–0.5 M NaCl in the same buffer over 60 min at a flow rate of 1 ml/min.

sequence of *C. nautilei* Hb IV was determined to be MVTAEEKALVQQTW, but no sequence was detected for Hb III, suggesting that the N terminus was blocked. To obtain internal amino acid sequences of *C. nautilei* Hb III, the protein was digested with lysyl endopeptidase, and a peptide was isolated and sequenced as INGMCATHRT.

The cDNAs for *C. nautilei* Hbs III and IV were successfully amplified by PCR using redundant and specific primers. The complete nucleotide sequence of 944 bp of *C. nautilei* Hb III was determined; the open reading frame was 426 nucleotides in length and encoded a protein of 141 amino acid residues. The cDNA sequence of 951 bp for *C. nautilei* Hb IV was also determined; it encoded a protein of 134 residues. The initiation Met was not cleaved away in *C. nautilei* Hb IV. The nucleotide sequences were deposited in the DDBJ database.

cDNA sequences of *C. tsubasa* Hbs

The cDNAs for *C. tsubasa* Hbs III and IV were successfully amplified by PCR using primers designed for PCR amplification of Hbs from the congeneric clam *C. soyoae* [10]. The complete nucleotide sequence of 775 bp for *C. tsubasa* Hb I was determined; the open reading frame was 438 nucleotides in length and encoded a protein of 145 amino acid residues. The cDNA sequence of 891 bp for *C. tsubasa* Hb II was also determined; it encoded a protein with 137 residues. The nucleotide sequences were deposited in the DDBJ database.

Discussion

The Hb amino acid sequences from *C. nautilei* and *C. tsubasa* were aligned with those from *C. soyoae* and *C. kaikoi* (fig. 2). In the eight chains, 34 residues, includ-

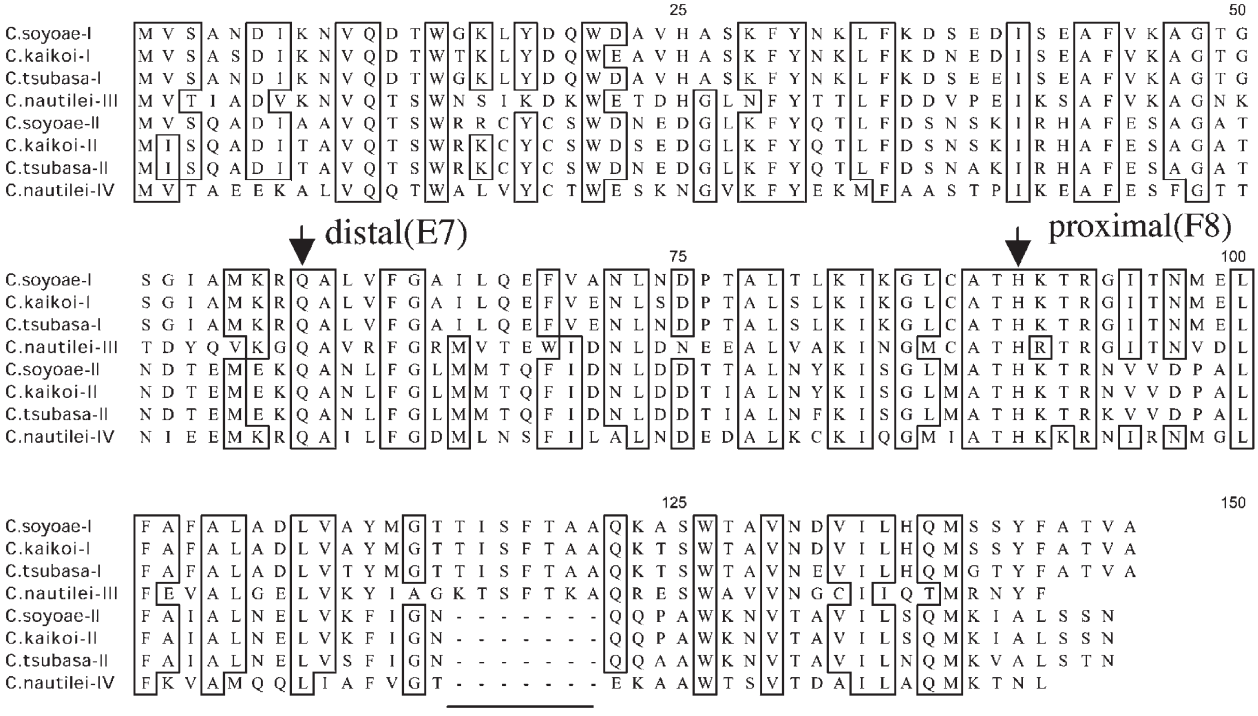

Figure 2. Amino acid sequence alignment of *Calyptogena* Hbs. Amino acids conserved in at least five chains are boxed. This alignment was obtained with the ClustalW method at the DDBJ home page (www.ddbj.nig.ac.jp/Welcome-j.html).

ing two essential heme contact residues, CD1-Phe (position 44) and proximal F8-His (position 90), appeared to be invariant. Another interesting feature of *Calyptogena* Hbs is the replacement of the distal (E7) histidine residue by glutamine (fig. 2). Most globins contain histidine at the distal position, which is capable of forming a hydrogen bond to the bound O_2, and stabilizing it [12]. This replacement by glutamine is found in several shark and elephant myoglobins and in opossum, hagfish, *Lucina*, *Urechis* and *Vitreosilla* Hbs [13]. Studies using an E7-Gln mutant human Hb have shown that it can form a hydrogen bond with the bound oxygen [14], like E7-His.

The amino acid sequence alignment in figure 2 clearly shows that all eight chains can be separated into two types by the characteristic deletion at position 115–121 (underlined); type I (globin I from *C. soyoae, C. kaikoi* and *C. tsubasa* and globin III from *C. nautilei*) and type II (globin II from *C. soyoae, C. kaikoi* and *C. tsubasa* and globin IV from *C. nautilei*).

In type I, the length of globin I is strictly conserved at 145 residues, but that of globin III from *C. nautilei* is 141 residues. In addition, globins I show 93–94% amino acid sequence identity with each other, but globin III shows only 45–46% identity with the three examples of globin I. In type II, the length of globin II is conserved at 137 residues, but globin IV from *C. nautilei* is shorter by 3 amino acids. Globins II show 91–96% identity with each other, but globin IV shows rather lower homology (43–44% identity) with globin II. Amino acid sequence identity between type I and type II globin is 33–42%. This value is less than the identity (44%) between alpha and beta chains of human Hb, which are estimated to have diverged 450 million years before the present [15]. These results indicate that each of the *Calyptogena* species expresses two types of intracellular Hbs, belonging to types I and II. However, the expression pattern is quite different in *C. nautilei* (Hbs III and IV) compared with those of other *Calyptogena* species (Hbs I and II).

To make clear the relationship among *Calyptogena* Hb chains, we constructed phylogenetic trees using the amino acid sequence alignment in figure 2. Figure 3 shows a maximum-likelihood tree [16] for the *Calyptogena* globins. The same topology was obtained with the neighbor-joining and Fitch methods. The tree fully supports the above observations on the globin classification based on sequence alignment: there are two types (globin I and III, and globin II and IV) in the expressed globins of *Calyptogena* species.

However, we do not know why a quite different set of Hbs (III and IV) is expressed in *C. nautilei*, when the other three *Calyptogena* species express a conventional set of Hbs (I and II). Two reasons can be considered: (i) *C. nautilei* is very distantly related genetically to *C. soyoae, C. kaikoi* and *C. tsubasa*, or (ii) the Hb expression pattern in

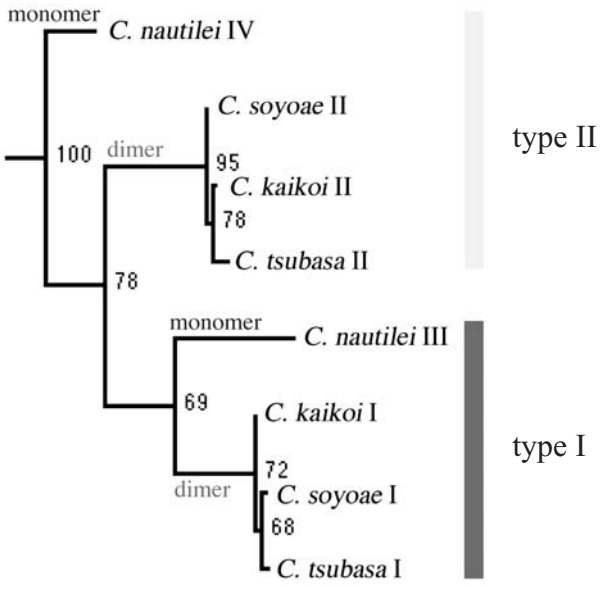

Figure 3. A phylogenetic tree for *Calyptogena* Hbs. This tree was constructed with the maximum likelihood method [16]. The numbers at each branching point show the quartet puzzling support value (%), which can be considered comparable to the bootstrap value. The sequences of Hbs from the blood clams *Barbatia* and *Anadara* were used as the out group.

C. nautilei is completely different from that found in other *Calyptogena* species.

An evolutionary relationship among the *Calyptogena* species, including *C. soyoae, C. kaikoi, C. tsubasa* and *C. nautilei,* has been inferred from the mitochondrial DNA sequence (partial sequences of cytochrome oxidase I and III) by Kojima et al. [17; and personal communication]. The results show that the four congeneric species are closely related (fig. 4). Among the four species, *C. tsubasa* and *C. soyoae* (subgenus *Archivesica*), and *C. kaikoi* and *C. nautilei* (subgenus *Ectenagena*) have closer relationships [11], suggesting that reason (i) is unlikely.

We assume that a Hb gene was duplicated at least three times in an immediate ancestor of *Calyptogena* (fig. 4) and, presumably depending on physiological conditions such as oxygen concentration in the deep sea, a different Hb set is being expressed: dimeric Hbs I and II in *C. soyoae*, *C. kaikoi* and *C. tsubasa*, and monomeric Hbs III and IV in *C. nautilei* (fig. 4). We have previously found a similar case where the blood clam, *Barbatia virescens* contains a heterodimeric Hb, while the congeneric clam *B. lima* has homodimeric and tetrameric Hbs, in addition to an unusual polymeric Hb consisting of a two-domain chain [18]. *B. virescens* and *B. lima* are live in significantly different circumstances, intertidal zone and sublittoral zone, respectively. Thus, hemoglobin expression in molluscan species appears to be strongly influenced by their physiological conditions.

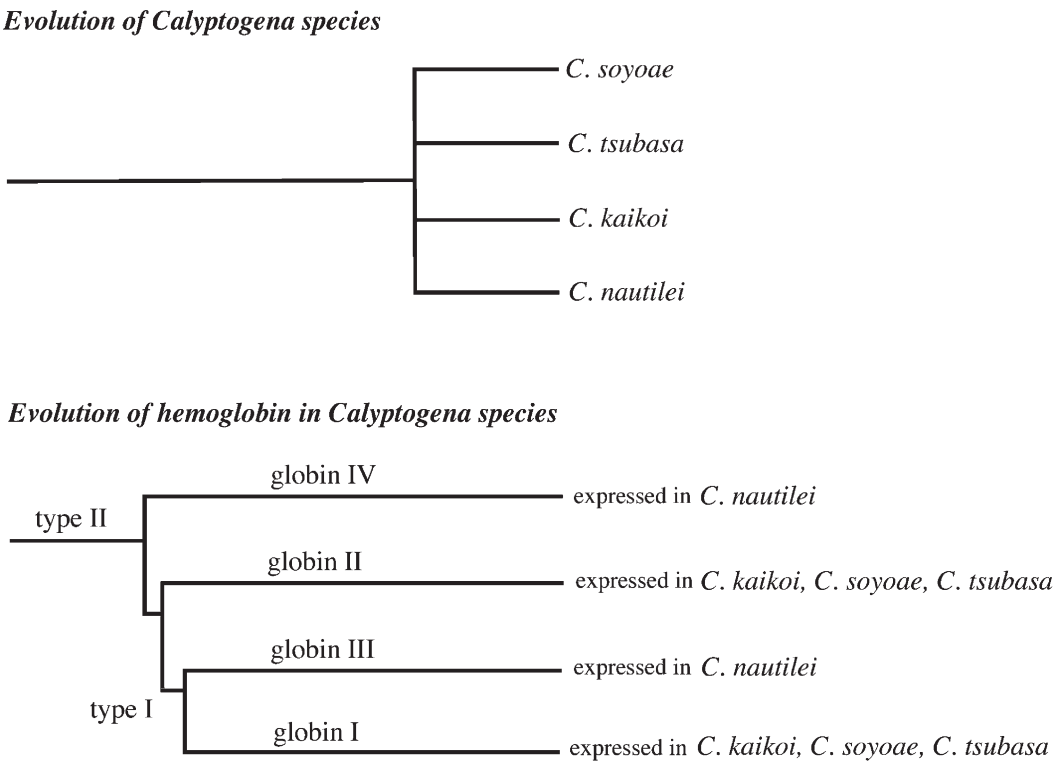

Figure 4. Schematic presentation of evolution and expression of *Calyptogena* Hbs.

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