ZOOLOGICAL SCIENCE 6: 915–926 (1989)

The Deep-Sea Tube Worm Hemoglobin: Subunit Structure and Phylogenetic Relationship with Annelid Hemoglobin

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ABSTRACT—The deep-sea giant tube worm *Lamellibrachia* (the phylum Vestimentifera) contains two extracellular hemoglobins, a 3,000 kDa hemoglobin consisting of six chains (AI-VI) and a 440 kDa hemoglobin consisting of four chains (BI-IV) (Suzuki *et al.*, 1988, Biochem. J., 255, 541–545). The subunit structures of the hemoglobins were investigated by polyacrylamide gel electrophoresis (PAGE). In sodium dodecyl sulfate (SDS), the unreduced 440 kDa hemoglobin dissociated into three subunits; two "myoglobin-like" monomers (BIII and BIV) and a disulfide-bonded dimer of chains BI and BII, while the 3,000 kDa hemoglobin dissociated into five subunits; two monomers (AIII and AIV), a disulfide-bonded dimer of chains AI and AII, and two 32–36 kDa linker subunits (AV and AVI). The dissociation pattern of 3,000 kDa hemoglobin resembles that of leech giant hemoglobin. A gel filtration study on the hemoglobins exposed to alkaline pH or 4 M urea showed that *Lamellibrachia* 3,000 kDa hemoglobin is much more susceptible to dissociation than 440 kDa hemoglobin. Furthermore, the 3,000 kDa hemoglobin is autoxidized about ten times faster than 440 kDa hemoglobin. These results suggest that the subunit assembly of 3,000 kDa hemoglobin is rather unstable. A molecular model, $[(BI, BII, BIII)_2BIV]_4$, for the subunit assembly of stable 440 kDa hemoglobin is proposed. Amino acid analyses of the isolated *Lamellibrachia* chains and structural comparison with annelid chains showed that chains AI, AII, AIII, BII and BIII have an additional free cysteine residue. This residue appears to be one of the most probable candidates for the sulfide binding site of the tube worm hemoglobin. Such a cysteine may be acquired by a molecular adaptation of hemoglobin, in order to transport sulfide to internal sulfide-oxidizing bacteria.

A phylogenetic tree was constructed from N-terminal partial sequences of 9 *Lamellibrachia* chains and 10 annelid chains. The tree showed that there are two distinct strains for the heme-containing globin chains of the phyla Vestimentifera and Annelida, consistent with our previous proposal that the vestimentiferan tube worms should be placed in the phylum Annelida (Suzuki *et al.*, 1988, Biochem. J. 255, 541–545).

INTRODUCTION

One of the recent, most exciting findings in biological fields was a discovery of the deep-sea hydrothermal or cold seep communities at a depth of 600–2500 m [1, 2], where the most conspicuous animals are the giant tube worms *Riftia* and *Lamellibrachia*, and the heterodont clam *Calyptogena*. Both animals are sustained by the mutual symbiosis with sulfide-oxidizing bacteria [3]. Although most of the animals in such communities were new to biologists, the tube worms, with an unique outward appearance such as the very long trunk region and the absence of a mouth, gut and anus, present a special interest in its taxonomic position. Very recently, Jones [4, 5] established a new phylum Vestimentifera for the deep-sea tube worms, *Rifia, Lamellibrachia, Escarpia, Tevnia, Oasisia* and *Ridgeia*.

The tube worms contain abundant extracellular hemoglobin, which is compatible with their high oxygen demand [6]. The hemoglobin also has a

Accepted January 4, 1989
Received November 17, 1988
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special ability to bind sulfide, and transports it to internal bacterial symbionts [3, 7]. Interestingly, several biochemical analyses of this hemoglobin suggest that the tube worms are closely related to annelids [8–10].

In the previous report [8], we showed that the tube worm *Lamellibrachia* contains two extracellular hemoglobins, a 3,000 kDa hemoglobin and a 440 kDa hemoglobin, and that all the constituent chains are highly homologous with those of annelid giant hemoglobin. Here we report the electron microscopic appearance, subunit structure, dissociation property and autoxidation of the two *Lamellibrachia* hemoglobins. As a first step to understand the sulfide binding ability of this hemoglobin, we also determined the number of half-cystine residues of the isolated chains. Furthermore, a phylogenetic tree was constructed from 19 partial sequences of the tube worm and annelid hemoglobins, to elucidate the evolutional position of the tube worms.

MATERIALS AND METHODS

Lamellibrachia sp. (undescribed) was collected from the cold-seep area located off Sagami Bay at a depth of 1,160 m, southeast of Hatsushima, Japan, by a Japanese submersible SHINKAI 2000 during November of 1987 [11].

Lamellibrachia hemoglobin was prepared according to the previous method [8].

Electron microscopy was carried out on the 3,000 kDa hemoglobin with a JEOL JEM 100U electron microscope. A solution of 1.5% potassium phosphotungstate was used for negative staining [9].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 15% acrylamide gel containing 0.087% bisacrylamide, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The samples were incubated in 0.75% SDS at 100°C for 5 min in the presence or absence of 2-mercaptoethanol, before electrophoresis.

Lamellibrachia hemoglobin, which had been exposed to an alkaline pH (in 0.4 $NaHCO_3$, pH 9.4, containing 4 mM EDTA) or 4 M urea (in 0.15 M phosphate buffer, pH 7.2) for 12 hr, was applied to a gel filtration column (Superose 12, 1×30 cm, Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.2, containing 150 mM NaCl. The column was operated at a flow rate of 0.5 ml/min with a Hitachi 655 high-performance liquid chromatography (HPLC).

The constituent polypeptide chains of *Lamellibrachia* hemoglobin were isolated by reverse-phase HPLC as described previously [8]. The isolated chains were carboxymethylated [12] and subjected to amino acid analyzer (Hitachi 835).

Lamellibrachia chain AVI, which could not be recovered by HPLC, was prepared by extraction from SDS-PAGE according to the method of Tsugita [13]. A 500 pmoles of chain AVI was applied to an automated protein sequencer (Applied BioSystems 477A sequencer).

Autoxidation rate of *Lamellibrachia* oxyhemoglobin was measured in 0.1 M phosphate buffer, pH 7.4, at 37°C and under air saturated conditions [14]. An absorption change at 578 nm was monitored with a Hitachi 220A spectrophotometer.

A phylogenetic tree was constructed from 19 partial sequences by an unweighted pair-group clustering method [15]. Calculation was carried out with a NEC PC-9801 personal computer.

RESULTS AND DISCUSSION

Subunit structure of Lamellibrachia *hemoglobin.*

Lamellibrachia contains two extracellular hemoglobins, a 3,000 kDa hemoglobin and a 440 kDa hemoglobin, which can be separated easily by gel filtration on a column of Sepharose CL-4B[8]. Electron micrographs of negatively stained 3,000 kDa hemoglobin are shown in Figure 1. Like the hemoglobins of annelids and other tube worms, a hexagonal bilayer structure was observed. The dimensions of *Lamellibrachia* 3,000 kDa hemoglobin were determined to be about 30×20 nm, which are very similar to those of annelid hemoglobin [22, 19]. However, the appearance of many incomplete structures such as those shown in Figure 1C suggests that this hemoglobin is unstable upon treatment with negative staining.

Figure 2 shows the SDS-PAGEs of *Lamellibrachia* 3,000 kDa hemoglobin (lanes 1 and 2) and 440 kDa hemoglobin (lanes 3 and 4) in the pre-

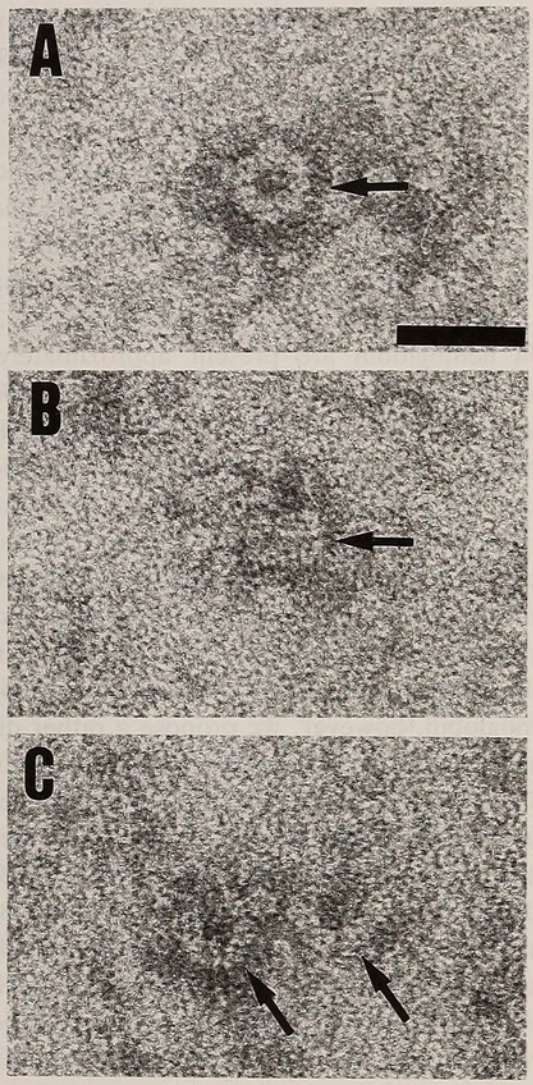

FIG. 1. Electron micrographs of *Lamellibrachia* 3,000 kDa hemoglobin. A, top view; B, side view; C, incomplete molecule. Scale bar, 50 nm.

sence or absence of a reducing agent. The unreduced 3,000 kDa hemoglobin dissociated in SDS into five subunits (lane 1): subunits A1 and A2 with an apparent molecular weight (*Mr*) of 16–17 kDa, subunit A3 with *Mr* 29 kDa, subunit A4 with *Mr* 30kDa and subunit A5 with *Mr* 36 kDa. The reduced 3,000 kDa hemoglobin (lane 2) dissociated into chains AI-VI. The chain AV, which was recognized as a single band in the previous report [8], was separated further into two chains at this time. This is not surprising, because it is already known that there is a hetero-type of chain AV by reverse-phase HPLC, whose amino acid sequence differs slightly from that of chain AV [8]. Re-electrophoresis of unreduced subunits A1-5 in the presence of a reducing agent showed that the subunits A1, A2, A4 and A5 corresponded to chains AIII, AIV, AV and AVI, respectively, and that the subunit A3 dissociated further into chains AI and AII. Therefore, it can be concluded that the 3,000 kDa hemoglobin consists of two "myoglobin-like" monomers (chains AIII and AIV), a disulfide-bonded dimer of chains AI and AII, and two 32–36 kDa chains (AV and AVI) which may act as "linker proteins" in the assembly of the

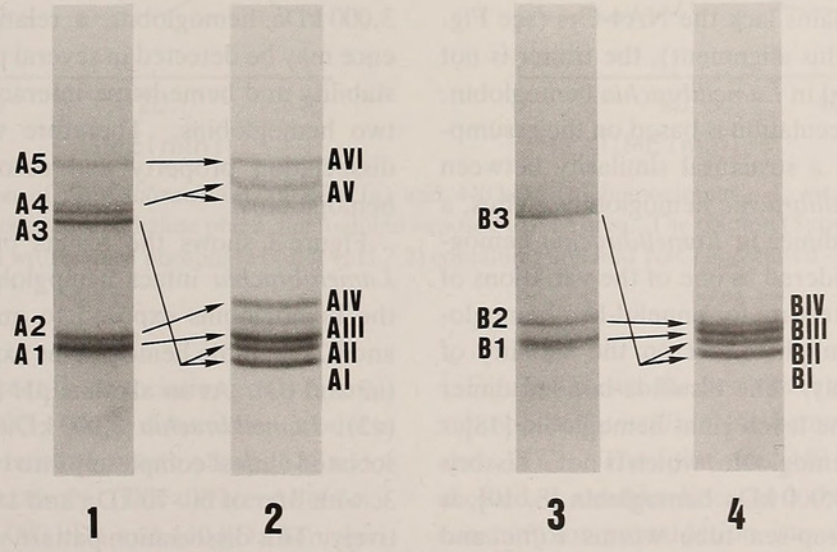

FIG. 2. SDS-PAGEs of *Lamellibrachia* 3,000 kDa (lanes 1 and 2) and 440 kDa (lanes 3 and 4) hemoglobins. Lanes 1 and 3, unreduced hemoglobin; lanes 2 and 4, reduced hemoglobin.

heme-containing chains [16, 17].

On the other hand, the unreduced 440 kDa hemoglobin dissociated in SDS into three subunits (lane 3): subunits B1 and B2 with *Mr* 16–18 kDa, and subunit B3 with *Mr* 29 kDa. The reduced 440 kDa hemoglobin dissociated into four chains BI-IV as reported previously [8]. Reverse-phase HPLC analyses and re-electrophoresis of unreduced subunits B1-3 in the presence of a reducing agent showed that the subunits B1 and B2 corresponded to chains BIII and BIV, respectively, and that the subunit B3 dissociated further into chains BI and BII. Therefore, it can be concluded that *Lamellibrachia* 440 kDa hemoglobin consists of two monomers (chains BIII and BIV) and a disulfide-bonded dimer of chains BI and BII.

It is of great interest to compare the subunit structure of *Lamellibrachia* hemoglobin with those of annelid hemoglobin. In most cases, annelid giant 3,000–4,000 kDa hemoglobin is composed of four subunits: a "myoglobin-like" monomer, a disulfide-bonded trimer and two 32–36 kDa chains [18]. This subunit structure appears to be very similar to that of *Lamellibrachia* 3,000 kDa hemoglobin, exept for a difference in either "trimer" or "dimer". Recent structural analyses of the hemoglobins from the polychaete *Tylorrhynchus* [19] and the oligochaete *Lumbricus* [20] showed that the trimeric subunit is linked by two interchain disulfide bridges, using four half-cystine residues located at NA4 and GH4 (see Fig. 5). Since *Lamellibrachia* chains lack the NA4-Cys (see Fig. 6, position 12 in this alignment), the trimer is not likely to be formed in *Lamellibrachia* hemoglobin. Of course, this speculation is based on the assumption that there is a structural similarity between annelid and *Lamellibrachia* hemoglobins. Thus, a disulfide-bonded dimer in *Lamellibrachia* hemoglobin can be considered as one of the variations of a molecular architecture for annelid-like hemoglobin, although it might affect on the stability of molecular assembly. The disulfide-bonded dimer is also found in the leech giant hemoglobin [18].

The 440 kDa hemoglobin, which is not a dissociated product of 3,000 kDa hemoglobin [8, 10], is unique to the deep-sea tube worms *Riftia* and *Lamellibrachia*. However, the subunit composition of the four heme-containing chains of *Lamellibrachia* 440 kDa hemoglobin was the same as that of 3,000 kDa hemoglobin. This is consistent with the idea that the 440 kDa hemoglobin is a prototype of 3,000 kDa hemoglobin [8].

It is also true that there is a slight, but significant difference in the ratio of heme-containing chains between the two tube worm hemoglobins. Reverse-phase HPLC analyses [8] showed that chains AI, AII, AIII and AIV of *Lamellibrachia* 3,000 kDa hemoglobin are present approximately in equimolar proportions, as in the case of *Tylorrhynchus* hemoglobin [21], while chains BI, BII, BIII and BIV of 440 kDa hemoglobin occur in a ratio of about 1:1:1:0.5. In *Tylorrhynchus* hemoglobin [21], a tetramer consisting of four chains in equimolar proportions is supposed to be a minimum structural entity. It is likely to take such a structure also in *Lamellibrachia* 3,000 kDa hemoglobin. For the subunit assembly of 440 kDa hemoglobin, however, we propose a rather different structure, in consideration of its chain ratio, as follows; $[(BI, BII, BIII)_2BIV]_4$. In this model, a trimer is composed of a disulfide-bonded dimer of chains BI and BII and a monomeric chain BIII, and two of the trimer are linked by chain BIV. Finally, the whole molecule is formed by four times of this structure. The calculated molecular mass for this model is 480 kDa, which is in good agreement with the observed value of 440 kDa.

As stated above, if the subunit assembly of 440 kDa hemoglobin is rather different from that of 3,000 kDa hemoglobin, a relatively large difference may be detected in several properties, such as stability and heme-heme interaction, between the two hemoglobins. Therefore we compared the dissociation property and autoxidation of both hemoglobins.

Figure 3 shows the results of gel filtration of *Lamellibrachia* intact hemoglobins (a1 and b1), the hemoglobins exposed to an alkaline pH (a2 and b2), and the hemoglobins exposed to 4 M urea (a3 and b3). At an alkaline pH (a2) and 4 M urea (a3), *Lamellibrachia* 3,000 kDa hemoglobin dissociated almost completely into two fractions 2 and 3, with *Mrs* of 60–70 kDa and 14–16 kDa, respectively. This dissociation pattern was very similar to those of annelid giant hemoglobins [16, 22]. On the other hand, *Lamellibrachia* 440 kDa hemoglo-

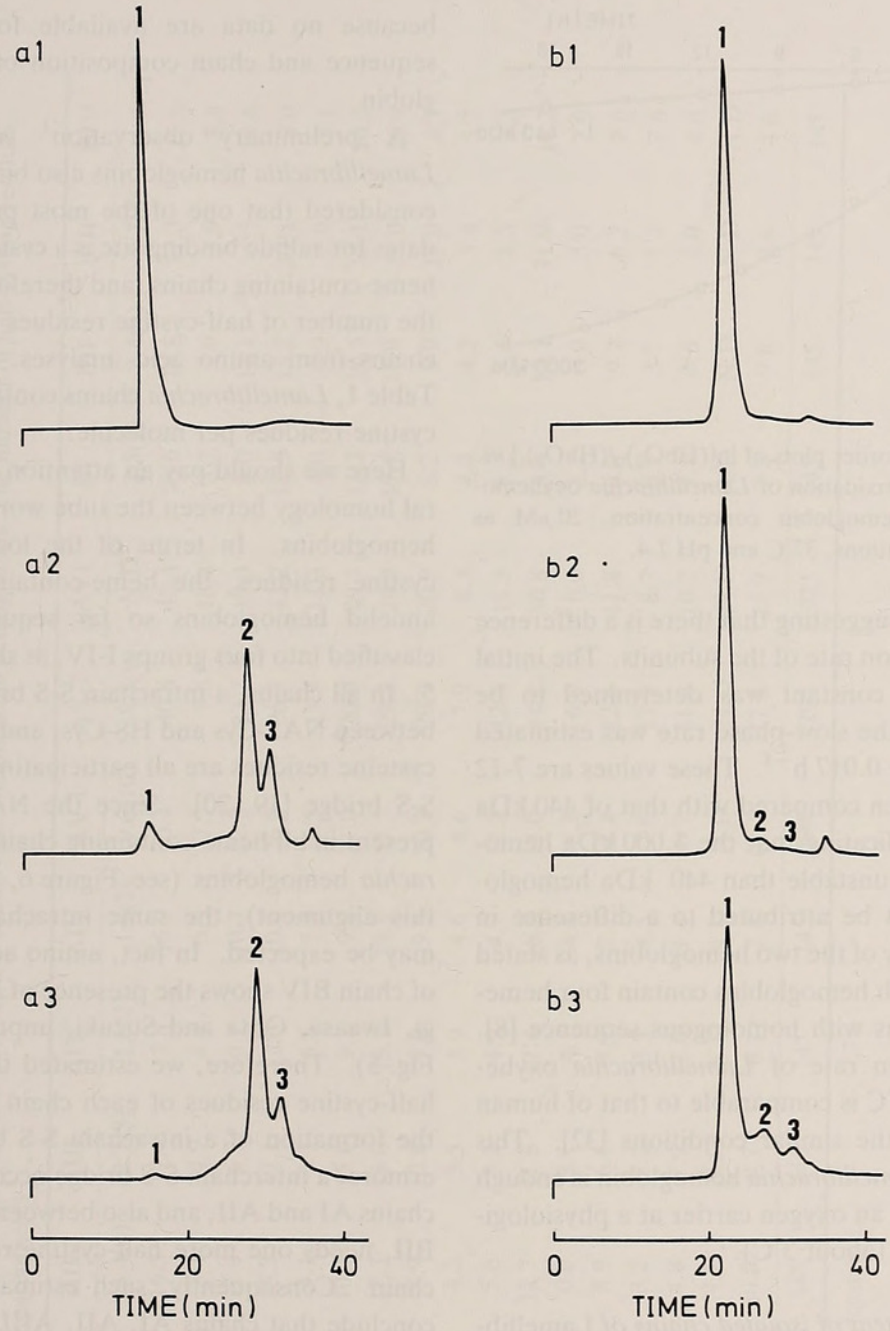

FIG. 3. Gel filtration of *Lamellibrachia* 3,000 kDa (a) and 440 kDa (b) hemoglobins. 1, intact hemoglobin; 2, hemoglobin exposed to an alkaline pH; 3, hemoglobin exposed to 4 M urea. The column (Superose 12, 1×30 cm) was equilibrated with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and eluted with the same buffer at a flow rate of 0.5 ml/min.

bin was very resistant to dissociation; only a small amount (at most 30%) of the dissociation products were observed by treatment with alkali (b2) and 4 M urea (b3), respectively. This different dissociation property of 3,000 kDa and 440 kDa hemoglobin is consistent with the idea that they have a different subunit assembly.

Figure 4 shows the first-order plots for the autoxidation of *Lamellibrachia* hemoglobins at pH 7.4 and 37°C. The plot for 440 kDa hemoglobin showed a straight line, and the first-order rate constant for autoxidation was determined to be 0.0026 h^{-1}, from its slope. On the other hand, the 3,000 kDa hemoglobin showed a biphasic auto-

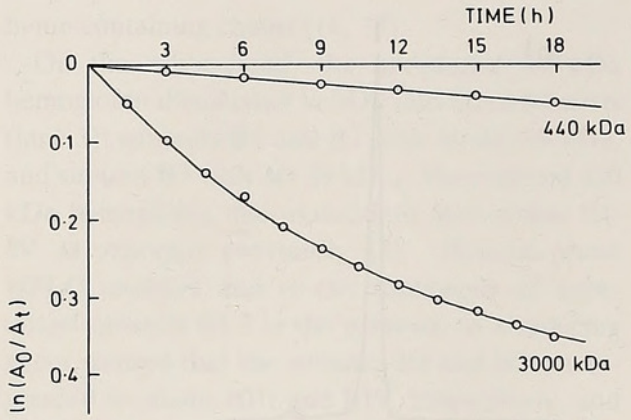

FIG. 4. The first-order plots of $\ln[(HbO_2)_0/(HbO_2)_t]$ vs. time t for autoxidation of *Lamellibrachia* oxyhemoglobins. Hemoglobin concentration, 20 μM as heme. Conditions, 37°C and pH 7.4.

xidation curve, suggesting that there is a difference in the autoxidation rate of the subunits. The initial fast-phase rate constant was determined to be 0.031 h^{-1}, and the slow-phase rate was estimated tentatively to be 0.017 h^{-1}. These values are 7-12 times larger when compared with that of 440 kDa hemoglobin, indicating that the 3,000 kDa hemoglobin is rather unstable than 440 kDa hemoglobin. This might be attributed to a difference in subunit assembly of the two hemoglobins, as stated above, since both hemoglobins contain four heme-containing chains with homologous sequence [8]. The autoxidation rate of *Lamellibrachia* oxyhemoglobins at 37°C is comparable to that of human hemoglobin at the similar conditions [32]. This suggests that *Lamellibrachia* hemoglobin is enough stable to play as an oxygen carrier at a physiological temperature (about 3°C).

Half-cystine content of isolated chains of Lamellibrachia *hemoglobins.*

Arp and Childress [7] showed that the hemoglobin of the deep-sea tube worm *Riftia,* a phylogenetically related species with *Lamellibrachia,* has a special ability to bind sulfide, which is transported to internal bacterial symbionts. This seems to be one of the most important physiological roles of the tube worm hemoglobins. Like *Lamellibrachia, Riftia* contains two extracellular hemoglobins, a 3,000 kDa hemoglobin and a 400 kDa hemoglobin, both of which can bind sulfide (H_2S) [23]. However the sulfide binding site is not known as yet, because no data are available for amino acid sequence and chain composition of *Riftia* hemoglobin.

A preliminary observation[1] suggested that *Lamellibrachia* hemoglobins also bind sulfide. We considered that one of the most probable candidates for sulfide binding site is a cysteine residue of heme-containing chains, and therefore determined the number of half-cystine residues of the isolated chains from amino acid analyses. As shown in Table 1, *Lamellibrachia* chains contained 2–4 half-cystine residues per molecule.

Here we should pay an attention to the structural homology between the tube worm and annelid hemoglobins. In terms of the location of half-cystine residues, the heme-containing chains of annelid hemoglobins so far sequenced can be classified into four groups I-IV, as shown in Figure 5. In all chains, a intrachain S-S bridge is formed between NA5-Cys and H8-Cys, and the remaining cysteine residues are all participating in interchain S-S bridge [19, 20]. Since the NA5-Cys is also present in all heme-containing chains of *Lamellibrachia* hemoglobins (see Figure 6, position 13 in this alignment), the same intrachain S-S bridge may be expected. In fact, amino acid sequencing of chain BIV shows the presence of H8-Cys (Takagi, Iwaasa, Ohta and Suzuki, unpubl. data) (see Fig. 5). Therefore, we estimated that two of the half-cystine residues of each chain were used for the formation of a intrachain S-S bridge. Furthermore, a interchain S-S bridge occurring between chains AI and AII, and also between chains BI and BII, needs one more half-cystine residue in each chain. Consequently, such estimation led us to conclude that chains AI, AII, AIII, BII and BIII have an additional free cysteine residue, which may be able to bind sulfide. The sequence analysis of chain BIII showed that it has cysteine residue at an unique position E18, where no cysteine residue is located in annelid hemoglobins (Suzuki, Takagi

[1] When we stored the purified *Lamellibrachia* hemoglobin at 4°C in a test tube with cap and opened it 6–7 days after, it smelled of mercaptan. The electrophoretic pattern (SDS-PAGE) of the stored hemoglobin was the same as that of freshly prepared sample, indicating that there is no change in the protein moiety during storage.

TABLE 1. Amino acid compositions of constituent polypeptide chains of *Lamellibrachia* hemoglobins. The number of half-cystine residues estimated is shown in parentheses

A.A.	AI-1	AI-2	AII	AIII	AIV′	AIV	AV-1	AV-2	AVI	BI	BII	BIII	BIV
Cys*	3.4(4)	3.6(4)	3.4(4)	2.7(3)	1.6(2)	1.9(2)	9.8(10)	8.7(9)	N.D.	2.5(3)	3.4(4)	2.7(3)	1.7(2)
Asp	16.6	16.6	16.5	17.4	17.8	16.9	40.4	40.8	42.4	18.6	16.8	17.3	17.3
Thr	3.3	2.5	8.1	4.9	4.4	5.9	7.5	7.6	18.4	3.7	7.9	4.9	8.8
Ser	7.7	8.0	11.4	6.7	10.1	10.7	22.8	23.2	19.3	8.0	11.1	6.5	10.8
Glu	13.4	13.4	15.3	17.1	12.0	10.6	26.2	26.7	42.2	12.5	15.2	17.1	8.0
Pro	5.4	5.2	4.1	3.1	5.5	5.4	13.5	13.9	11.3	4.0	3.9	2.9	5.1
Gly	12.2	12.1	10.3	15.0	9.2	9.8	21.0	20.0	29.1	12.3	10.5	15.1	10.7
Ala	20.3	20.3	17.3	15.0	15.3	13.1	27.7	29.3	29.9	17.7	17.4	15.0	16.9
Val	8.4	8.2	8.9	10.8	9.9	9.5	20.2	20.3	20.9	8.2	9.0	11.0	8.7
Met	2.3	2.4	4.3	3.5	3.9	4.6	8.6	7.9	8.3	4.2	4.3	3.4	5.2
Ile	8.9	9.5	5.6	5.2	4.9	4.7	12.2	12.2	11.3	5.2	5.6	5.3	7.3
Leu	12.4	12.3	12.7	11.8	17.4	16.2	14.0	14.2	30.8	13.8	12.7	11.9	18.2
Tyr	2.6	2.9	0.0	3.1	4.1	4.8	8.3	6.9	6.0	2.2	0.0	3.0	2.9
Phe	6.3	6.4	8.9	6.1	7.7	7.7	12.4	12.6	11.8	10.8	9.2	6.2	7.0
Lys	6.2	6.1	3.2	5.2	3.3	4.5	17.6	17.9	8.7	10.5	3.7	5.2	7.8
His	5.9	6.0	4.8	7.9	11.6	11.4	10.7	11.0	16.2	3.5	4.6	8.0	4.0
Trp	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Arg	9.7	9.6	10.0	9.7	6.1	7.3	17.4	17.8	15.9	7.2	9.8	9.6	4.6
Total	145	145	145	145	145	145	290	290	323	145	145	145	145

*Determined as carboxymethylcysteine.

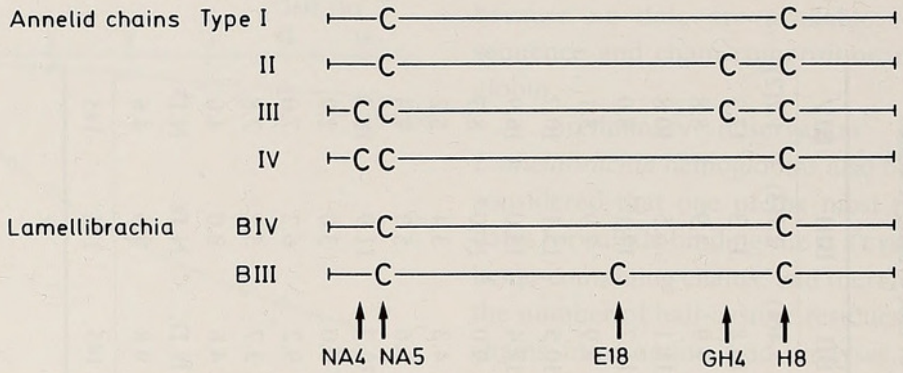

FIG. 5. Location of half-cysine residues of heme-containing chains of annelid and tube worm hemoglobins.

and Ohta, unpubl. data) (see Fig. 5). It is likely that such a cysteine was acquired by a molecular adaptation of hemoglobin, in order to transport the sulfide to internal sulfide-oxidizing bacteria.

Amino acid sequence comparison and construction of a phylogenetic tree

In the previous report [8], we succeeded in isolating most of the chains of *Lamellibrachia* hemoglobins by reverse-phase HPLC and sequenced the N-terminal 20–40 residues. Chain AVI, which was not recovered by HPLC, was extracted from SDS-PAGE and the N-terminal sequence was determined to be Phe-Ser-Thr-His-Leu-Asp-Thr-X-X-Val-X-Val-Gln-Asp-X-X-Phe by an automated protein sequencer.

N-Terminal amino acid sequences of all chains of *Lamellibrachia* hemoglobins are compared in Figure 6, with those of annelid giant hemoglobins. It has been already pointed out that the heme-containing chains of annelid hemoglobin can be separated two distinct groups [12, 24, 20]. Extend-

		1	10	20	30	34
Strain A						
Lam.	AI	(ref.8)	T D C G M L Q R I K V K Q Q W A S V Y - - S S G			
	AIII & BIII	(8)	Y E C G P L Q R L K V K R Q W A E A Y - - G S G			
	BI	(8)	D C N I L Q R L K V K M Q W A K A Y - - G F G			
Tyl.	I	(25)	T D C G I L Q R I K V K Q Q W A Q V Y - - S V G			
	IIA	(12)	S S D H C G P L Q R L K V K Q Q W A K A Y - - G V G			
Are.	I	(26)	D C G P L Q R L K V K H Q W V Q V Y - - S G H			
Lum.	I	(27)	E C L V T E G L K V K L Q W A S A F - - G H A			
	II	(20)	K K Q C G V L E G L K V K S E W G R A Y - - G S G			
Phe.	I	(unpublished)	D C N T L K R F K V K H Q W Q Q V F - - S G E			
Strain B						
Lam.	AII & BII	(8)	S S N S C T T E D R R E M Q L M W A N V W S A Q F T			
	AIV	(8)	S G N V A E A P K H Y H C S Y E D A D I V M R E W Y H V W - - G S G			
	AIV'	(8)	S V A N A P K H N H C S Y E D A E I V M S E W Y H V W - - G S G			
	BIV	(8)	S K F C S E G D A R I V I K Q W N Q I Y - - N			
Tyl.	IIB	(28)	D D C C S A A D R H E V L D N W K G I W S A E F T			
	IIC	(29)	D T C C S I E D R R E V Q A L W R S I W S A E D T			
Lum.	III	(20)	D E H E H C C S E E D H R I V Q K Q W D I L W R D T E S			
	IV	(20)	A D D E D C C S Y E D R R E I R H I W D D V W S S S F T			
Strain C						
Lam.	AV	(8)	A A V Q P L S V S D A M G A R V D A Q - - A W R			
Lam.	AVI	(this work)	F S T H L D T X X V X V Q D X X F			

FIG. 6. Comparison of the N-terminal sequences of *Lamellibrachia* and annelid hemoglobins. The invariable residues are boxed. Lam., *Lamellibrachia*; Tyl., *Tylorrhynchus*; Are., *Arenicola*; Lum., *Lumbricus*; Phe., *Pheretima*.

TABLE 2. Matrix for sequence homologies between the constituent chains of extracellular hemoglobins. The percent identity shown above the diagonal was obtained for 19 residues common to all the chains shown in Fig. 6. Values below the diagonal indicate Poisson corrected percent differences obtained by an equation; $-100\times\ln$(percent identity/100)

		Lam.									Tyl.				Are.	Lum.				Phe.
		AI	AII	AIII	AIV	AIV′	BI	BII	BIII	BIV	I	IIA	IIB	IIC	I	I	II	III	IV	I
Lam.	AI		26	63	21	21	63	26	63	26	89	68	21	26	74	42	42	21	26	58
	AII	134		21	32	32	21	100	21	21	26	21	32	47	21	21	11	37	47	26
	AIII	46.0	156		26	21	74	21	100	26	63	84	21	21	68	58	58	21	16	42
	AIV	156	115	134		89	21	32	26	37	21	26	32	37	21	21	26	42	42	21
	AIV′	156	115	156	11.1		21	32	21	37	21	26	32	37	21	21	32	42	42	21
	BI	46.0	156	30.5	156	156		21	74	26	68	79	21	26	63	53	53	21	16	53
	BII	134	0	156	115	115	156		21	21	26	21	32	47	21	21	11	37	47	26
	BIII	46.0	156	0	134	156	30.5	156		26	63	84	21	21	68	58	58	21	16	42
	BIV	134	156	134	99.9	99.9	134	156	134		32	26	32	37	32	21	21	53	26	26
Tyl.	I	11.1	134	46.0	156	156	37.9	134	46.0	115		68	21	26	79	37	42	21	26	63
	IIA	37.9	156	17.2	134	134	23.6	156	17.2	134	37.9		21	21	68	53	58	21	16	42
	IIB	156	115	156	115	115	156	115	156	115	156	156		58	21	16	16	37	42	21
	IIC	134	74.7	156	99.9	99.9	134	74.7	156	99.9	134	156	54.7		21	21	16	53	53	21
Are.	I	30.5	156	37.9	156	156	46.0	156	37.9	115	23.6	37.9	156	156		37	47	21	32	68
Lum.	I	86.5	156	54.7	156	156	64.2	156	54.7	156	99.9	64.2	185	156	99.9		58	21	11	37
	II	86.5	225	54.7	134	115	64.2	225	54.7	156	86.5	54.7	185	185	74.7	54.7		16	11	32
	III	156	99.9	156	86.5	86.5	156	99.9	156	64.2	156	156	99.9	64.2	156	156	185		47	21
	IV	134	74.7	185	86.5	86.5	185	74.7	185	134	134	185	86.5	64.2	115	225	225	74.7		32
Phe.	I	54.7	134	86.5	156	156	64.2	134	86.5	134	46.0	86.5	156	156	37.9	99.9	115	156	115	

ing this idea, we classified all the heme-containing chains of tube worm and annelid hemoglobins into two groups, strain A and strain B. Strain A contains the sequences of *Lamellibrachia* chains AI, AIII (BIII) and BI [8], of the polychaete *Tylorrhynchus* chains I and IIA [25, 12], of the polychaete *Arenicola* chain I [26], of the oligochaete *Lumbricus* chains I and II [27, 20] and of the oligochaete *Pheretima* chain I (Suzuki, unpubl. data). This group has five invariable residues, Cys-13, Lys-20, Val-21, Lys-22 and Trp-25. Strain B contains the sequences of *Lamellibrachia* chains AII (BII), AIV, AIV' (a hetero-type of AIV) and BIV [8], of *Tylorrhynchus* chains IIB and IIC [28, 29] and of *Lumbricus* chains III and IV [20]. This group has three invariable residues, Cys-13, Asp-17 and Trp-25.

In addition to the two strains, we propose a "third" strain (strain C) for the constituent polypeptide chains of giant 3,000–4,000 kDa hemoglobin. This strain C includes the "linker proteins" with an unusual *Mr* 32–36 kDa [16, 17]. At present, only two sequence data (*Lamellibrachia* chains AV and AVI) shown in Figure 5, are available for strain C, but we now learn that *Lumbricus* chain V, belonging to strain C, has been sequenced partially (Vinogradov *et al.*, unpubl. data). In all cases, the proteins of strain C show a rather different amino acid sequence, and may have evolved from a different gene, compared with that of other heme-containing chains.

Many taxonomists agree that the worm-like animals such as annelids, pogonophores and deep-sea tube worms are closely related [see 9]. But recently, Jones [4, 5] placed the tube worms in a new phylum Vestimentifera. Inconsistent with

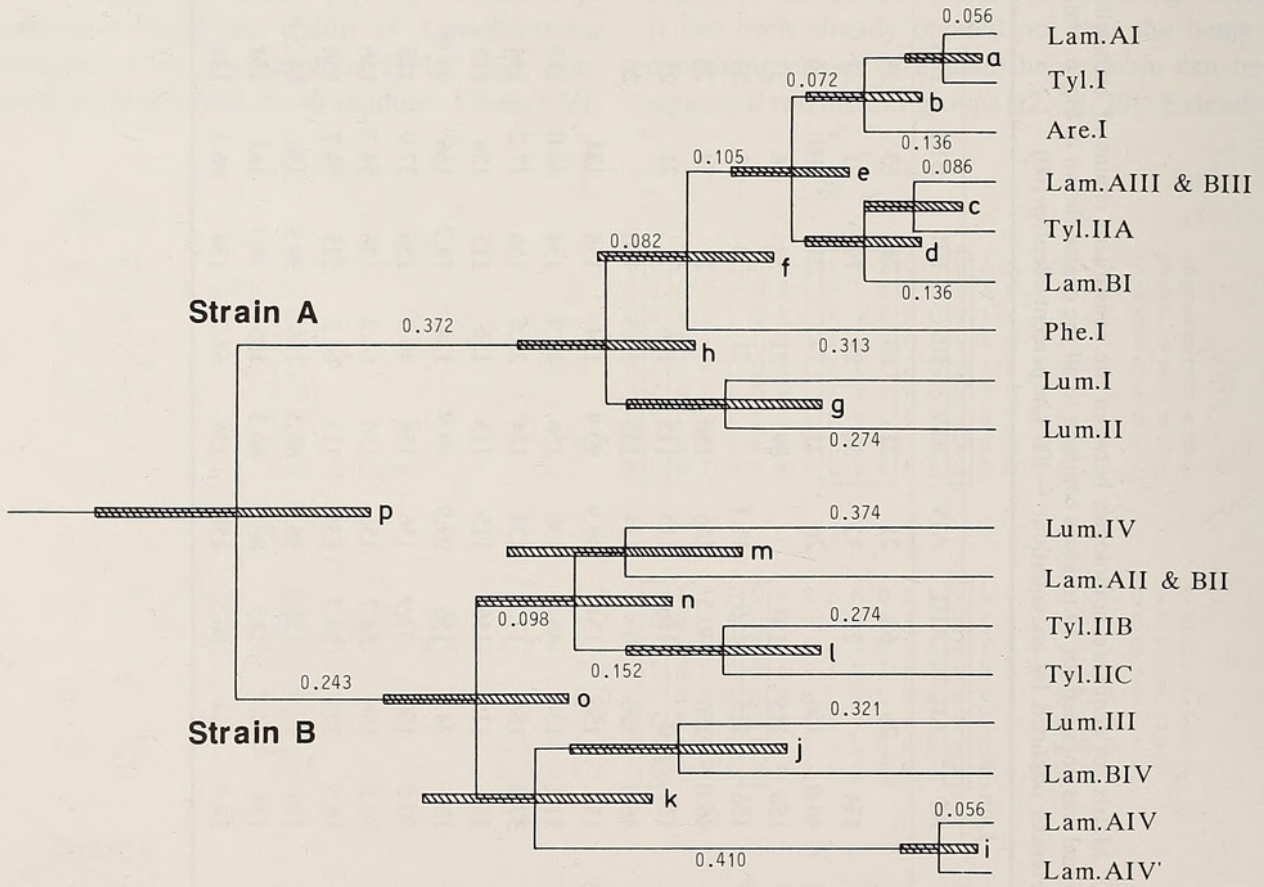

FIG. 7. A phylogenetic tree constructed from partial sequences of *Lamellibrachia* and annelid hemoglobins. The tree was constructed from Table II by an unweighted pair-group clustering method [15], using 19 amino acid residues common to all heme-containing chains (strains A and B) shown in Fig. 6. Standard errors at the branching points, a-p, are 0.039, 0.060, 0.050, 0.058, 0.063, 0.091, 0.098, 0.091, 0.039, 0.109, 0.122, 0.098, 0.121, 0.100, 0.094 and 0.104, respectively.

this, all of the biochemical data for the tube worm hemoglobin, such as electron microscopic appearance, subunit structure, chain composition and amino acid sequence, suggest that the tube worm is a member of annelids [8–10].

To make clear the taxonomical position of tube worms, we constructed a phylogenetic tree from the homology matrix of heme-containing chains shown in Table II. It is well known that the phylogenetic tree constructed from globin sequences shows a good correlation with that from classical taxonomy [30, 31]. The result is shown in Figure 7. The standard error is given at each branching point, to help evaluation of the tree. This pattern is essentially similar to that constructed from eight sequences of annelid hemoglobins [20], and suggests the following points. (i) There are two distinct globin strains A and B, which can be separated without any overlaps of standard errors, through the heme-containing chains of the phyla Vestimentifera and Annelida. Each strain may be produced by a gene duplication. (ii) A hemoglobin contains two chains in each of the two strains. Therefore, one more gene duplication is needed. After this process, the giant molecular architecture was constructed. (iii) The most closely related chains are *Lamellibrachia* AI and *Tylorrhynchus* I, *Lamellibrachia* AIV and AIV', and *Lamellibrachia* AIII(BIII) and *Tylorrhynchus* IIA. For example, 19 out of the N-terminal 22 residues are surprisingly identical between *Lamellibrachia* AI and *Tylorrhynchus* I. This indicates that there is a very close relationship between *Lamellibrachia* and *Tylorrhynchus*.

In conclusion, the phylogenetic tree shown in Figure 7 implies that the tube worm *Lamellibrachia* should be placed in the phylum Annelida, instead of the phylum Vestimentifera [4, 5]. The tube worms probably evolved in the deep-sea from other polychaetes, taking on an unique outward appearance, which is high adaptations for symbiosis with sulfide-binding bacteria.

ACKNOWLEDGMENTS

We thank Prof. S. N. Vinogradov of Wayne State University, Prof. T. Gotoh of Tokushima University, Prof. A. Kajita, Dr. K. Fushitani and Dr. Y. Igarashi of Dokkyo University and Dr. Y. Machida of Kochi University for their interest during this work.

This work was partly supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture of Japan (No. 63740417) to T. S.

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