Structure of an extracellular giant hemoglobin of the gutless beard worm *Oligobrachia mashikoi*

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Mouthless and gutless marine animals, pogonophorans and vestimentiferans, obtain their nutrition solely from their symbiotic chemoautotrophic sulfur-oxidizing bacteria. These animals have sulfide-binding 400-kDa and/or 3,500-kDa Hb, which transports oxygen and sulfide simultaneously. The symbiotic bacteria are supplied with sulfide by Hb of the host animal and use it to provide carbon compounds. Here, we report the crystal structure of a 400-kDa Hb from pogonophoran *Oligobrachia mashikoi* at 2.85-Å resolution. The structure is hollow-spherical, composed of a total of 24 globins as a dimer of dodecamer. This dodecameric assemblage would be a fundamental structural unit of both 400-kDa and 3,500-kDa Hbs. The structure of the mercury derivative used for phasing provides insights into the sulfide-binding mechanism. The mercury compounds bound to all free Cys residues that have been expected as sulfide-binding sites. Some of the free Cys residues are surrounded by Phe aromatic rings, and mercury atoms come into contact with these residues in the derivative structure. It is strongly suggested that sulfur atoms bound to these sites could be stabilized by aromatic-electrostatic interactions by the surrounding Phe residues.

crystal structure | Pogonophora | sulfide binding | supramolecular assembly

The Hb superfamily is widely found in all kingdoms of organisms. Within this superfamily, mammalian Hb as an oxygen transporter composed of the $\alpha_2\beta_2$ heterotetramer has been the most widely studied in terms of its function, structure, and allosteric effect. In contrast, primitive vertebrates and invertebrates have various types of Hb that appear in forms from monomer to oligomer and show remarkably different quaternary structures and various properties for oxygen affinities (1–3). A number of crystal structures of invertebrate monomeric or oligomeric Hbs are available, and all these oligomeric Hbs show quite different assemblage from mammalian Hb.

The Hb of the marine beard worm *Oligobrachia mashikoi* (Pogonophora) has the ability to bind oxygen and sulfide (4, 5), and a similar deep sea animal *Riftia pachyptila* (Vestimentifera) also has Hbs that can transport oxygen and sulfide simultaneously (6). These animals have no mouth or gut (7, 8), and their nutrition is provided by endosymbiotic bacteria living inside the trophosome (9–13). In the case of vestimentiferans, it has been reported that their endosymbionts are chemoautotrophic sulfur-oxidizing bacteria (11, 12). The extracellular Hb provides its host with oxygen, and the endosymbionts with sulfide that is expected to form either H_2S or HS^- ($\text{pK}_a = 7.04$ in water) in the animal body. Morphological and embryological studies (14–16) and genetic studies (17, 18) of pogonophorans, vestimentiferans, and annelids suggest that these groups are closely related. All three groups have giant (400-kDa and/or 3,500-kDa) extracellular Hbs, but Hbs of annelids living in sulfide-free environments have no physiological function of sulfide binding. *R. pachyptila* has three types of extracellular Hb, vascular V1 and V2 and coelomic C1. Mass spectrometry analyses suggest that “hexagonal bilayer”

V1 is composed of 144 globins and 36 linker peptides like annelid Hbs, V2 is composed of 24 globins and no linker peptide, and C1 is also composed of 24 globins (19). V1, V2, and C1 all have the ability to bind sulfide (20), and it has been proposed that free cysteine residues of these Hbs are involved with the sulfide-binding mechanism (20, 21). On the other hand, pogonophorans have only an ≈ 400 -kDa Hb that is composed of ≈ 24 globins. Sequence analyses of pogonophoran and vestimentiferan Hbs have shown that their putative sulfide-binding Cys residues are well conserved (4, 5), and their molecular mass suggests that pogonophoran Hbs are homologous to the vestimentiferan V2 Hbs.

Neither the pogonophoran nor the vestimentiferan vascular Hb structure is available, although the crystal structures of the reconstructed Hb dodecamer core complex of *Lumbricus terrestris* (Annelida) hexagonal bilayer Hb (22) and *Riftia* coelomic C1 (23) have recently been reported. The structures of *Riftia* V1 and V2 Hbs, which function as oxygen-sulfide transporters, have not been reported, and detailed structural information on the sulfide-binding mechanism is still unclear. In the present study, we have determined the crystal structure of the vascular Hb (V2) from *O. mashikoi* as an intact oligomeric form. The structure shows a spherical 24-mer construction forming the double layer of dome-shaped dodecamers like that of *Riftia* coelomic C1. In addition, insights into the sulfide-binding mechanism were provided by the mercury atoms bound to free Cys residues in the structure of the derivative crystal used for phasing.

Materials and Methods

Protein Preparation and Crystallization. Extracellular Hb was purified from collected *O. mashikoi* dredged from Tsukumo Bay, Ishikawa Prefecture, Japan, at a depth of 20–25 m as described (5). The protein was desalted and concentrated by ultrafiltration in solutions of 50 mM Tris-HCl (pH 7.5). Crystals used in this work were obtained by the vapor diffusion method within 4 months by using 13% (vol/vol) polyethylene glycol (PEG) 10,000 and 100 mM Tris-HCl (pH 8.0) as a precipitant. Details of crystallization will be reported elsewhere (24). Heavy atom derivative crystals were prepared by soaking in reservoir solutions containing 1 mM methylmercury chloride for 21 h and 5 mM potassium tetrachloroplatinate for 29 h. These derivatives gave clear peaks in the difference Patterson maps.

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2D2M and 2D2N). The sequences have been deposited in the DNA Data Bank of Japan [accession nos. AB185392 (A1), AB185391 (A2), AB185394 (B1), and AB185393 (B2)].

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Table 1. Data collection and refinement statistics

Data collection	Native	CH ₃ HgCl	K ₂ PtCl ₄
Wave length, Å	1.0000	1.0000	1.0600
Space group	R32	R32	R32
Unit-cell parameters, Å			
a	111.50	111.75	111.91
c	276.84	276.70	277.14
Resolutions, Å	50–2.85 (2.95–2.85)	50–3.30 (3.42–3.30)	50–3.10 (3.21–3.10)
No. of observations	158,860	108,029	131,889
No. of unique reflections	15,821	10,332	12,518
Completeness, %	99.9 (100)	99.9 (100)	99.9 (100)
Average I/σ(I)	28.2 (4.7)	21.2 (6.2)	25.0 (5.0)
Redundancy	10.0 (9.6)	10.5 (10.9)	10.5 (10.9)
R _{sym} , %	7.4 (44.4)	8.2 (34.6)	7.5 (41.9)
R _{iso} , %		23.0	19.8
Phasing			
Resolution, Å		50–3.30 (3.55–3.30)	
Mean figure of merit		0.59 (0.42)	
Number of sites		4	3
Refinement			
R, %	23.6		
R _{free} , %	27.7		
No. of protein atoms	4,264		
No. of heme atoms	180		
No. of water molecules	13		
Average B factor, Å ²	58.2		
rms bonds, Å	0.005		
rms angles, °	1.0		

Values in parentheses are for the highest resolution shell.

* $R_{\text{sym}} = \sum_i |I(h) - \langle I(h) \rangle| / \sum_i I(h)$, where $\langle I(h) \rangle$ is the mean intensity after rejections.

† $R_{\text{iso}} = \sum |F_{\text{PH}} - F_{\text{P}}| / \sum |F_{\text{P}}|$, where F_{P} is the observed structure factor amplitude for the native data set and F_{PH} is the observed structure factor amplitude for the heavy-atom derivative.

‡ $R = \sum |F_{\text{O}} - F_{\text{C}}| / \sum |F_{\text{O}}|$, where F_{O} is the observed structure factor amplitude and F_{C} is the calculated structure factor amplitude.

§ R_{free} is as for R but calculated using a random set containing 5% of the data that were excluded during refinement.

Structure Determination. The x-ray diffraction study was performed at the BL38B1, BL41XU, BL44XU, and BL45XU beamline at SPring-8. Before data collection, the crystals were soaked in mother liquor containing 20% (vol/vol) glycerol as a cryoprotectant and flash-frozen in a nitrogen gas stream at 90 K. All data were processed by using the HKL2000 package (25), and the CCP4 program suite (26). Determination and refinement of the heavy atom positions and initial phase calculation were performed with SOLVE (27) by using the MIRAS (multiple isomorphous replacement with anomalous scattering) method followed by density modification with RESOLVE (28). Three Pt positions and four Hg positions were successfully determined, and a clearly interpretable electron density map was obtained. Four globins (one sixth of the whole molecule) were contained in an asymmetric unit with the Matthews coefficient V_M value of 2.6 Å³·Da^{−1}, corresponding to 52.3% solvent content. Several cycles of manual model rebuilding and model refinement were performed by using the programs O (29) and CNS (30), respectively. For the further refinement, 5% of the reflections were set apart as a random test set to calculate R_{free} values. After each cycle, $2F_{\text{O}} - F_{\text{C}}$ and $F_{\text{O}} - F_{\text{C}}$ electron density maps were calculated to check the fit of the model to the map. A total of 573 residues, four hemes, four oxygen molecules, and 13 water molecules were included in the final model, the R and R_{free} values of which were 23.6% and 27.7%, respectively. The statistics for data collection and refinement are summarized in Table 1. Figures were prepared by using MOLSCRIPT (31), BOBSCRIPT (32), RASTER3D (33), and PYMOL (www.pymol.org).

Results and Discussion

Overall Structure. The structure of *Oligobranchia* Hb is composed of 24 globin chains, generated by a 32 symmetry along the crystallographic axes (Fig. 1A); the asymmetric unit contains one copy of each of four individual globin chains, termed A1, A2, B1, and B2 (Fig. 1B). This highly symmetrical 24-meric assembly is the same as *Riftia* C1 (23). The results confirm earlier suggestions in regard to the construction of vestimentiferan V2 and C1, deduced by the viewpoint of the molecular mass of each chain (19) and the construction of pogonophoran Hb, which is thought to be homologous to V2 because of their molecular mass. The entire structure is hollow-spherical, with outer and inner diameters of ≈120 Å and 50 Å, respectively. Difference Fourier maps in which ligand molecules were omitted around the distal heme pockets show significantly strong electron density at A1 and B2, and rather weak density at A2 and B1 (Fig. 2). The following observations and properties indicate that the current structure of the *Oligobranchia* Hb is in an oxygenated form: the oxygen affinity is high ($P_{50} = 0.82$ Torr; ref. 5), the absorption spectra of the purified Hb are identical with those of typical oxygenated Hbs (data not shown), and the color of the obtained crystals was bright red.

Dodecameric Assembly as Fundamental Unit of Giant Hbs. One half of the *Oligobranchia* Hb molecule (Hb 12-mer) is composed of the 3-fold “trimer of the tetramer,” having one intra-tetramer (A1-B2) disulfide bond and one inter-tetramer (B1-B2) disulfide bond per tetramer (Fig. 1B). The earthworm *Lumbricus* eryth-

Fig. 1. Oligomeric structure of *Oligobranchia* Hb. (A) Space-filling models of *Oligobranchia* Hb 24-mer viewed along with the crystallographic 3-fold axis (Upper) and 2-fold axis (Lower). Four individual globin subunits A1, A2, B1, and B2 are shown in red, green, yellow, and blue, respectively. (B) Stereo view of the Hb tetramer composed of one copy of each of four globin subunits colored as in A. Each helix is labeled according to the myoglobin helical designations. There is no D helix in any of the four globins and no C helix in B1. All four globin subunits form intramolecular disulfide bonds between their N terminus and the middle of their H-helix; these bonds are shown as cyan balls. Intermolecular disulfide bonds are also shown as light yellow balls formed between the A1 GH corner and B2 GH corner, and as magenta balls formed between the B1 N terminus and B2 N terminus of the neighboring tetramer (not shown).

rocurin core subassembly is also trimer-of-tetramer composed of four individual chains (22); therefore, the quaternary structure of *Oligobranchia* Hb 12-mer is almost the same as *Lumbricus* erythrocrurin 12-mer, including all intra- and inter-subunit disulfide bonds (Fig. 5, which is published as supporting information on the PNAS web site). The two Hb 12-mers could be superimposed with rms deviations of 2.9 Å for 1,713 C α atoms. The *Riftia* C1 12-mer structure also has a trimer-of-tetramer composition (23) and could be superimposed to the *Oligobranchia* Hb 12-mer structure with rms deviations of 2.8 Å for 1,719 C α atoms. However, *Riftia* C1 12-mer lacks inter-tetramer (B1-B2) disulfide bonds (Fig. 6, which is published as supporting information on the PNAS web site). *Oligobranchia* Hb and *Riftia* C1 are considered to be a dimer of 12-mer, basically similar to *Lumbricus* Hb 12-mer. Interestingly, *Lumbricus* Hb 12-mer forms a “mouth opened” spherical 24-mer by the crystal packing. This form is mainly due to the large side chains of Arg-123 and Arg-127 of the globin b (A1), which prevent the formation of a “closed” complete 32 symmetry as in the case of *Oligobranchia* Hb. Closely related vestimentiferans have both a 3,500-kDa hexagonal bilayer Hb similar to annelid Hbs and 24-mer Hbs that are the same structure as pogonophoran Hbs. The dodecameric assembly of these Hbs could be a fundamental unit of Hbs from

pogonophorans, vestimentiferans, and annelids, not only in the 3,500-kDa hexagonal bilayer Hbs but also in the 400-kDa Hbs. Non-globin linker chains could be essential to gather 12 units of dodecameric assembly to a hexagonal bilayer Hb composed of 144 globins and 36 linkers.

Cooperativity. As in the case of *Lumbricus* Hb and *Riftia* C1, the tetrameric assembly of *Oligobranchia* Hb is essentially identical with that of clam *Scapharca inaequivalvis* HbII (34), and this Hb tetramer is therefore a dimer-of-dimer form (A1B1 dimer and A2B2 dimer in Fig. 1B), of which the interface is similar to those of *Scapharca* HbI (35) and echinoderm *Caudina arenicola* Hb-D (36), both of which are called “EF-dimer.” In addition, the key residues, Arg-E10, His-F3, and Gln-F7 (excluding A2 Arg-F3) at the EF-dimer interface are conserved and interact with the heme propionate group of the neighboring subunit in a manner quite similar to that of *Lumbricus* Hb and *Riftia* C1 (Fig. 3). Nevertheless, *Oligobranchia* Hb almost lacks the ability of cooperative oxygen binding. It has been reported that the Hill coefficient of *Oligobranchia* Hb is 1.1 (5). The sequence analysis indicates that only *Oligobranchia* Hb A2 has Arg-F3 and lacks cooperativity, whereas other vestimentiferan and *Lumbricus* Hbs have His-F3 without exception (Fig. 6) and show moderate (with the Hill

Fig. 2. The σ_A -weighted $2F_o - F_c$ map contoured at the 1σ level around the B2 heme site shown as a cyan mesh. The $F_o - F_c$ map calculated with a ligand-omitting model is also shown in magenta, contoured at the 3σ level. In the $2F_o - F_c$ map, the electron density of the second oxygen atom of O_2 is not clear, probably because of different orientations in the crystal. The position of the oxygen atom bonded to the Fe of heme was restrained during refinements.

coefficient of 2–3; ref. 37) or strong (with the Hill coefficient of 7–8; ref. 38) cooperativity, respectively. *Oligobranchia* A2 Arg-F3 might prevent the subunit reorientation proposed at *Lumbricus* Hb (22), but the detailed mechanism of this noncooperativity is still unclear. We need the structure of the deoxygenated form to examine lack of cooperativity.

Sulfide-Binding Mechanisms. The crystal structure of *Riftia* C1 shows the presence of tightly bound zinc ions, and it is suggested that these zinc ions play a key role in sulfide-binding (23), rather than conserved Cys residues, which have been expected to play a main role in the sulfide-binding (20, 21). However, the electron density map of *Oligobranchia* Hb does not show any significant peaks for potential metal ions. In addition, the environments of the zinc-binding sites mainly composed of His residues are not conserved in the *Oligobranchia* Hb structure (Fig. 7, which is published as supporting information on the PNAS web site). On the other hand, the environments around conserved Cys residues are quite similar between *Oligobranchia* Hb and *Riftia* C1 (discussed below). Moreover, these conserved Cys residues are found in several animals living in sulfide-rich environments (39). These facts indicate the possibility that these Cys residues are involved in the sulfide-binding by forming S-sulfohemoglobin (20). Although the obtained structure in this study is a sulfide-free form, the structure of the Hg derivative provides significant insights into the sulfide-binding mechanism because Hg atoms specifically bind to free Cys residues and the molecular size of methylmercury we used is rather closer to sulfide than other well known Cys specific compounds such as *p*-chloromercuribenzoic acid (PCMB). It has been suggested that conserved Cys residues among pogonophorans and vestimentiferans are included in the potential sulfide-binding sites located at the positions of the distal E7+1 or E7+11 (21) (Fig. 6). These residues are free for inter- and intramolecular disulfide bonds. In the *Oligobranchia* Hb, both A1 and B2 possess an E7+1 Cys, and A2 has an E7+11

Fig. 3. Interactions of the EF dimer interfaces. (A) *Oligobranchia* A1B1 dimer. (B) A2B2 dimer. (C) *Lumbricus* A1B1 dimer. E and F helices are represented as C_α traces, and the key residues coming into contact with the neighboring heme propionate group are drawn as sticks together with heme molecules. The C_α traces and heme molecules of *Oligobranchia* A1, A2, B1, and B2 and *Lumbricus* A1 and B1 are red, green, yellow, blue, and gold, respectively. The interactions of *Lumbricus* A2B2 dimer and *Riftia* A1B1 and A2B2 dimer interfaces appear almost the same manner as that of *Lumbricus* A1B1 dimer. Only in the case of *Oligobranchia* A2, the position F3 is Arg and forms a hydrogen bond with the other propionate group of which His-F3 of A1, B1, and B2 forms a hydrogen bond.

Fig. 4. The environments around free cysteine residues at the proposed sulfide-binding sites. The positions of the mercury atoms in the Hg derivative structure are represented as magenta balls, indicating the putative sulfide positions. (A) The conserved free Cys site (E7+1) of the A1 surrounded by Phe aromatic rings. Because the Cys residue is in a subsequent position to distal His, this site is located almost right above the heme distal pocket, with the atomic distance between the Fe of the heme and the S of the Cys being ≈ 10 Å. (B) Another conserved free Cys site (E7+11) of the A2. No striking interactions with the putative sulfide position were observed. (C) B1 Cys-E7+21 is not a conserved residue, but Tyr-136 is located at a suitable position for stabilizing bonded sulfide. (D) The E7+1 site of the B2, which is quite similar to the A1 shown in A.

Cys. There is neither an E7+1 Cys nor an E7+11 Cys in B1, but one free Cys that is not conserved, E7+21, is present. All four of these free Cys residues are bound by the Hg compound. To investigate the hypothesis, we performed sulfide-binding analysis on purified Hb (see *Supporting Text*, which is published as supporting information on the PNAS web site), using direct UV detection of dissolved HS^- in solutions (40). The results show that *Oligobranchia* Hb could bind 19.8 mol of sulfide per 1 mol of Hb. This finding is close to the number of conserved free Cys residues (a total of 18 residues per molecule) of *Oligobranchia* Hb and is consistent with our suggestion that, in the case of *Oligobranchia* Hb, the conserved free Cys residues play the principal role of sulfide binding. Excess sulfide (≈ 1.8 mol) probably is due to nonconserved Cys-E7+21 of B1 to which sulfide is partly binding.

The environments around the sites E7+1 of A1 and B2 are revealed to be more suitable for the sulfide-binding. These sites are quite similar to each other and are highly aromatic, contributed by Phe-B10, Phe-B14, and Phe-E4 (Fig. 4 A and D). These three Phe residues are completely conserved between pogonophorans Hb and vestimentiferan Hb. Phe-B10 and Phe-E4 come into contact with the Hg atom in the derivative structure, and it is strongly suggested that, in the native Hb, the sulfide bound to Cys-E7+1 could be stabilized by aromatic-electrostatic interac-

tions (41) with these two Phe side-chains. A similar stabilization mechanism by aromatic-electrostatic interactions is present in the sulfide-binding *Lucina pectinata* (clam) HbI (42). In the *Lucina* HbI, hydrogen sulfide is bound to the ferric heme iron, forming a hydrogen bond with Gln-E7 and stabilized by the "Phe-cage" composed of Phe-B10, Phe-CD1, and Phe-E11. The distances between Hg atoms located at the putative sulfur atom and the center of aromatic planes of Phe-B10 and Phe-E4 are 4.2 Å and 2.5 Å in A1, and 4.0 Å and 2.4 Å in B2, respectively. A small conformational change may need to occur to form a suitable geometry for the sulfide-binding. Such conformational change can be achieved only by changing the directions of the side chain and aromatic ring plane, and conformational change of the main chains is probably not necessary. Indeed, enough spaces for these Phe side-chain reorientations are available. The mercury derivative structure [refined at 3.2 Å resolution (see Table 2, which is published as supporting information on the PNAS web site)] can superimpose on the native structure, with rms deviations of 0.27 Å for all (573) C α atoms. This result supports the hypothesis that no large conformational changes are necessary when sulfide is bound to the Hb. The hydrophobic environment around the persulfide group has been reported in several proteins. The sulfur-accepting residue Cys-51 of the SufE protein, which participates in iron-sulfur cluster assembly and

repair, was buried in a hydrophobic cavity (43). In cystine C-S lyase from *Synechocystis*, the aromatic rings of Phe and Trp residues enclose a substrate disulfide bond within a binding pocket (44). In addition, molecular dynamics simulation and direct observation of the β -subunit of T-state hemoglobin show that the ligands move easily to a hydrophobic cavity contributed by Leu-B10, Val-E11, Leu-E12, and Leu-G8 (45, 46). This position is ≈ 8.5 Å away from the distal pocket, and the E7+1 sites of *Oligobranchia* Hb are located near this cavity (≈ 5.5 Å). Because the side chains of E7+1 Cys residues are completely isolated from a solvent in the present crystal structure, some transient conformational change may occur to bind sulfides. The hydrophobic environment around the E7+1 site also prevents bound sulfides from excessive solvent contacts to avoid undesired oxidation.

On the other hand, E7+11 of A2 (Fig. 4B) and E7+21 of B1 (Fig. 4C) might have less affinity for sulfide. The environment around E7+11 of A2 is contributed by Leu-87, Leu-90, and Ile-136, and these residues are also completely conserved between pogonophorans Hb and vestimentiferan Hb. But they

could not contribute to any stabilization mechanism by hydrogen bonds or aromatic-electrostatic interactions. In the Hg derivative structure, the Hg atom does not come into contact with these Leu and Ile. Because E7+11 of A2 is conserved at the putative sulfide-binding site, and the surrounding Leu and Ile are also conserved, it might have the ability to bind with sulfide, but its affinity for sulfide would be less than that of the E7+1 site of A1 and B2. In the case of E7+21 of B1, Tyr-136 comes into contact with the Hg compound in the Hg derivative structure, and it is suggested that this Tyr residue might contribute to the stabilization of sulfide. Because no more potential stabilization mechanisms are observed, it is likely that this site shows somewhat less affinity for sulfide than for A1 and B2 sites.

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