Molecular and structural biology on the giant hemoglobin from pogonophoran Oligobrachia mashikoi(有鬚動物マシコヒゲムシの巨大ヘモグロビンに関する分子および構造生物学的研究)

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学位論文要旨

Introduction

Oligobrachia mashikoi belonging to the phylum Pogonophora possesses ca. 400 kDa hemoglobin in blood. In 1996, Yuasa *et al.* reported that the *Oligobrachia mashikoi* hemoglobin is composed of eight globin chains (a1~a5, b, c, d) on the basis of electrospray ionization mass spectrometry. They determined the partial amino acid sequences and cDNA sequences of four globin chains, a5 (A2), b (A1), c (B2), and d (B1) (Yuasa *et al.* 1996). However, these four globin genes were not clarified in full length, and furthermore, no biochemical evidences indicating that the *Oligobrachia mashikoi* giant hemoglobin is really composed of the eight kinds of globin chains, have been shown.

In the present study, the stable purification procedure of the *Oligobrachia mashikoi* giant hemoglobin was developed and it is found that the pure hemoglobin is composed of only four kinds of the globin chains, not eight globin chains, on the improved SDS polyacrylamide gel electrophoresis (SDS-PAGE). The cloning and sequencing of the four full-length cDNAs encoding each globin chain were successful. And the putative signal peptides in all of them and the interesting characters of the d (B1) globin chain were found. To characterize each of the globin chains and reconstitute the giant hemoglobin *in vitro*, three globin genes were expressed in *Escherichia coli* at first. Finally, the crystals diffracting up to 3.0 Å could be prepared with collaboration with K. Miki and N. Numoto (Department of Chemistry, Graduate School of Science, Kyoto University) to determine the three-dimensional structure of *Oligobrachia mashikoi* giant hemoglobin at SPring-8 (Japan Synchrotron Radiation Research Institute).

Amino acid sequences and alignment of the four globin chains

N-terminal amino acid sequences of the four globin chains which were prepared by SDS-PAGE of the purified hemoglobin were determined by a protein sequencer. These amino acid sequences were

consistent with the registered partial sequences of a5 (A2), b (A1), c (B2) and d (B1) globin chains in the database. Four complete cDNA sequences encoding a5 (A2), b (A1), c (B2) and d (B1) globin chains were determined with 5' RACE and RT-PCR. The a5 (A2) globin chain consists of 142 amino acid residues with the molecular weight and the isoelectric point (pI) of 15312 and 4.87, the b (A1) globin consists of 140 amino acid residues with the molecular weight of 15174 and pI of 5.6, the c (B2) globin consists of 147 amino acid residues with the molecular weight of 15606 and pI of 5.0, and the d (B1) globin consists of 145 amino acid residues with the molecular weight of 14778 and the pI of 4.3, respectively.

An alignment of the four globin chains was investigated with the amino acid sequences. Gaps have been inserted where necessary to provide maximum alignment of the sequences. The identity and homology of the four globin chains were calculated to be 13.42% and 45.64%, respectively, and these were much lower than those of human hemoglobin, alpha and beta chains, 42% and 78.4%, respectively. However, the amino acid residues participating in the globin folding and near the heme pocket were highly conserved between the four globin chains and human hemoglobin chains. Therefore, the structure surrounding heme of the *Oligobrachia mashikoi* giant hemoglobin may be similar to that of human hemoglobin.

The four globin chains of *Oligobrachia mashikoi* hemoglobin have 3~5 cystein (Cys) residues in the molecule. In Figure 15 (pp.65), the Cys (7) and Cys (152) of the four globin chains were predicted to form an intra-molecular disulfide bond. The Cys (6) of both c (B2) and d (B1) globin chain, and the Cys (143) of both b (A1) and c (B2) globin chain were predicted to form inter-molecular disulfide bonds, as a hetero-dimer and trimer subunit. However, only one Cys is free in each of the globin chains, such as the Cys (78) of b (A1) and c (B2), the Cys (88) of a5 (A2), and the Cys (98) of d (B1) globin chain. These cysteine residues might be the sulfide-binding sites. Therefore, it seems that the free Cys of the pogonophoran giant hemoglobin can bind to the sulfide *via* thiol-disulfide exchange, and it is transported to endosymbionts in the trophosome of the host.

Molecular phylogenetic analyses of the *Oligobrachia mashikoi* giant hemoglobin

A maximum likelihood consensus tree was constructed with the complete amino acid sequences of the extracellular giant hemoglobin chains from Pogonophora, Vestimentifera, Annelida. The rooting as outgroup was made with the intracellular polymeric hemoglobin of Annelida, and the monomeric and homotetrameric hemoglobin of Mollusca. The character of these hemoglobin genes has three exons and two introns, and the second exon domain codes the entire heme-binding region. According to the molecular phylogenetic analyses of the hemoglobins, the first gene duplication event of the extracellular giant hemoglobins might have occurred in a common ancestor of Pogonophora, Vestimentifera and Annelida, resulting in division into strain A and B, as proposed by Goto *et al.* (Goto *et al.*, 1987). Furthermore, Negrisolo *et al.* have proposed that strain A and B can also be divided to the A1/A2 and

B1/B2 subclusters, respectively (Negrisolo *et al.*, 2001). Our constructed phylogenetic tree of the giant hemoglobins also supported the hypothesis that the strain A and B were mainly separated to A1/A2 and B1 /B2, respectively.

In the present study, the phylogenetic tree including d (B1) globin chain of *Oligobrachia mashikoi* hemoglobin was constructed using a new amino acid substitution model of WAG matrix, valid for soluble proteins. As shown in Figure 16 (pp.66), the clade of *Oligobrachia mashikoi* d (B1) globin chain was first branched from the strain B with relatively high bootstrap values, suggesting that d (B1) globin chain of *Oligobrachia mashikoi* might have the oldest properties in the strain B. Furthermore, B1 globin chains of ca.350~450 kDa hemoglobins of Pogonophora *Oligobrachia mashikoi* and Vestimentifera *Lamellibrachia* sp. were clearly separated into the cluster different from the another B1 globin cluster of ca. 3000~3600 kDa hemoglobins of Annelida *Lumbricus terrestris* and *Macrobdella decora*. Recently, based on the phylogenetic analyses of the elongation factor-1α genes and 18S rRNA sequences, some researchers have proposed that Pogonophora and Vestimentifera should be included in the class Polychaeta in Annelida and in Protostomia (Kojima *et al.*, 1993; McHugh, 1997; Halanych *et al.*, 1998). Furthermore, most of Annelida have only one kind of giant hemoglobin with the molecular mass of ca. 3000~3600 kDa. Therefore, before the annelid radiation about 600 MYA, the common ancestor of Annelida, Pogonophora and Vestimentifera may obtain the B1 globin chain which could construct the ca. 3000~3600 kDa hemoglobin after the first gene duplication. Only Pogonophora and Vestimentifera may independently and simultaneously obtain another B1 globin chain, which could construct the ca. 350~450 kDa hemoglobin without linker proteins, after the second gene duplication. However, it remains unanswered why Pogonophora *as Oligobrachia mashikoi* lost the ca. 3000~3600 kDa hemoglobin during evolution, and why Pogonophora can be alive under the sulfide-rich environment without the ca. 3000~3600 kDa giant hemoglobin. It may be one reason that the function of ca. 350~440 kDa hemoglobin can serve as well as, or more enough than, that of the ca. 3000~3600 kDa hemoglobin.

Expression of the three globin genes of *Oligobrachia mashikoi* hemoglobin in *Escherichia coli*

To investigate the mechanism of the assembly and reconstruction of the giant hemoglobin from the small globin chains and hemes *in vitro*, three globin genes were expressed in *Escherichia coli* at first. Each of a5(A2), b(A1), and c(B2) globin cDNAs were inserted into pET-15b vector contained the additional N-terminal His-Tag sequence. Each pET-15b/a5(A2), pET-15b/b(A1) and pET-15b/c(B2) vector obtained was transformed into *Escherichia coli* BL21(DE3). When these BL21(DE3) cells containing each expression vector were grown to $O.D_{600} = 0.6 \sim 0.8$ in LB medium containing 100 μg/ml ampicillin at 30℃, 1mM IPTG for induction and 20 μM hemin were added into the culture. After induction, the culture was collected with 1ml aliquot intervals of half or 1 hour till 6 hours incubated. As a result, the ca. 17 kDa recombinant globin chains of the a5(A2) and b(A1) were successfully overproduced in the BL21(DE3) cells. However, the recombinant c (B2) was not found in the cells.

The c(B2) globin chain has two Arg residues which are translated with the minor genetic codon. Therefore, using the pRARE for co-expression which provided with six minor tRNAs including the two minor Arg tRNAs, the recombinant c(B2) globin chain were successfully overproduced in the cells. However, unfortunately, each of the recombinant proteins was formed into the inclusion bodies. It is likely that soluble and correct folding-recombinant globin chains may require co-expression with another globin chains in *E. coli* at the same time.

Crystallization

Crystallization was performed with Crystal Screen™ Kit (50 sets of reservoir buffers and precipitant solutions, HAMPTON RESEARCH) by hanging drop vapor diffusion method. The hanging drops, which were mixture of the hemoglobin solution and the precipitant solution, were incubated at 4℃, 10℃, 15℃ and 25℃ in several months. After the microcrystals appeared in the drops, the reservoir buffer and the precipitant solution were changed to a new condition by the gradient of pH and concentration. Moreover crystallization conditions were changed to the desalting hemoglobin solution, the concentration of hemoglobin, temperature, and the drop volumes. Second crystallization was performed under at the same temperature for several months.

First, the rhombohedral and trigonal prism crystals (Form I) were obtained with the mixture of 1 μl of 85 mg/ml hemoglobin solution containing 0.05M Tris-HCl (pH 8.0) and 0.2M NaCl, plus 1 μl of the reservoir solution containing 0.1M imidazole (pH 6.9) as buffer and 1.0M sodium acetate trihydrate as precipitant, at 25℃, for a month. And the well-shaped hexagonal crystals (Form II) were also obtained with the mixture of 1 μl of 85 mg/ml hemoglobin solution containing 0.05M Tris-HCl (pH 8.0) and 0.2M NaCl, plus 1 μl of the reservoir solution containing 0.1M imidazole (pH 7.0~8.1) as buffer and 1.0M sodium acetate trihydrate as precipitant, at 10℃, for a month. However, these crystals were very small and thin under about 0.05 mm length..

Secondly, to obtain the crystals being big, stable, and suitable for X-ray crystallographic analysis, the various crystal growth conditions were investigated by changing the protein concentration, pH, temperature and the concentration of the precipitant. The pH was varied from 6.0 to 8.1 at 4℃, 10℃, 15℃ and 25℃ with the same concentration of the precipitant. The most suitable concentration of hemoglobin solution was 50 mg/ml through the many experiments performed, and the pH of the hemoglobin solution from pH 8.0 to 7.5. Furthermore, an concentration of the precipitant, sodium acetate trihydrate, in the reservoir solution was changed to be 0.6M, 0.8M, 1.0M, 1.2M and 1.4M at pH 6.5~8.8. Among the various growth conditions investigated, the big crystals (Form II) were obtained at 10℃ in the drops of pH 7.3 to 8.7 with 0.6M precipitant for five months (Figure 7, pp.89). These crystals were very thick with about 0.5 mm length. However, it took long days (five months) for the crystals to grow up to 0.5 mm in size, and this crystal was liable to dissolve at even 4℃, unfortunately.

For getting more stable and big crystals in a few months, the crystal growth conditions were investigated with the desalting hemoglobin solution. Figure 8 (pp.90) shows the crystals obtained from

the desalting hemoglobin solution for three months in the drops of pH 8.0 to 8.8 with 1.0M precipitant at 10℃. These crystals (Form II) were very big, shape, and thick with about 0.2~0.3 mm, and more stable at 4℃. Recently, the X-ray diffraction data of these crystals were collected by N. Numoto and K. Miki, and the data were enough for determination of the crystal structure of the giant hemoglobin at 3.0 Å resolution, which will be reported in the near future.

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学位論文審査結果の要旨

有鬚動物は、口、消化管、肛門を持たない動物であり、生育に必要な有機物は、細胞内に共生する化学合成細菌から獲得していると考えられている。巨大ヘモグロビンは、共生細菌が必要とする硫化水素と宿主が必要とする酸素を貯蔵・輸送する特異なヘモグロビンである。本論文は、有鬚動物 Oligobrachia mashikoi から巨大ヘモグロビンを精製し、X 線結晶構造解析が可能な結晶調整方法を確立するとともに、巨大ヘモグロビンを構成する 4 種類のグロビン鎖の分子構造を解明し、さらに各グロビン鎖の大腸菌での大量発現系の構築及びアミノ酸配列に基づく分子系統解析からの有鬚動物と環形動物の系統関係を考察した論文である。

有鬚動物 O. mashikoi から分子量約 440,000 の巨大ヘモグロビンを簡便かつ高度に大量精製する方法を確立し、様々な結晶化条件を検討した。その結果、3 Å分解能の X 線回折像を観察できる結晶調整に成功し、巨大ヘモグロビンの立体構造の解明が飛躍的に進展した。また、巨大ヘモグロビンが分子量約 15,000 の 4 種類のグロビン鎖で構成されることを明らかにし、それらの完全長 cDNA の塩基配列を決定した。その結果、すべてのグロビン鎖は N 末端に細胞外シグナルペプチドを持つこと等の多くの分子的特徴を明らかするとともに、他の有鬚動物や環形動物のグロビン鎖のアミノ酸配列と比較することにより有鬚動物 O. mashikoi 巨大ヘモグロビンの分子系統を明らかにした。さらに、各グロビン鎖 cDNA の大腸菌での発現に成功し、巨大ヘモグロビンの構造形成機構の解明につながる実験系を確立した。

以上、本研究により、有鬚動物巨大ヘモグロビン関して、多くのことを明らかにし、それらの知見は当該分野の研究発展に大いに寄与するものと考えられる。従って、審査委員会は、本論文が博士論文として妥当であると判断した。