Crystal structures of two hemoglobin components from the midge larva Prosilicercus akamusi (Orthocladiinae, Diptera)

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Abstract

The polymorphic components of hemoglobin (Hb) of the midge larva Prosilverocerus akamusi were classified into two distinct types dependent on their spectroscopic properties, normal absorption (N) and low absorption (L). Analyses of the amino acid sequences of component VII (N-type Hb) and component V (L-type Hb) from P. akamusi indicated that one remarkable difference is the replacement of the distal histidine (His) with isoleucine (Ile) in component V. To clarify the structural differences between the two Hb components, we determined the crystal structures of components V and VII at resolutions of 1.64 Å and 1.50 Å, respectively. These crystal structures indicated a short additional helix comprising three amino acid residues at the C-terminal region in component V, and a typical globin fold including eight helices in component VII. Comparison of the heme regions of the Hb components suggests that the structural changes of the heme region in component V on ligation differ from that of usual Hb.

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Keywords: X-ray crystallography; Insect hemoglobin; Additional helix; Distal histidine; Amino acid replacement

1. Introduction

Both the biochemical nature and protein structure of invertebrate hemoglobins (Hbs) are unique because they are present in the coelomic fluid or hemolymph as well as in the cellular components (Vinogradov, 1985; Riggs, 1998). Crystallographic analyses of invertebrate Hbs have revealed the characteristics of their tertiary and quaternary structures and structural changes dependent on ligand binding (Bolognesi et al., 1997). The crystal structures of Hb from three species of Diptera, Chironomus thummi thummi (Chironomidae), Gasterophilus intestinalis (Oestradae), and Drosophila melagonaster (Drosophilidae), were determined and the structural features of each Hb component were characterized (Steingemann and Weber, 1979; Pesce et al., 2005; de Sanctis et al., 2005, 2006). However, within one insect species, the structural differences between polymorphic Hb components have not yet been analyzed by X-ray crystallography techniques.

Prosilverocerus akamusi (old species name: Tokunagayusurika akamusi) is a common species of midge found in eutrophic lakes in Japan that belongs to a different subfamily Orthocladiinae from C. thummi thummi in Chironominae. The coelomic fluid of the 4th-instar midge larva contains polymorphic hemoglobin with as many as eleven separable hemoglobin components, as discriminated on DEAE-cellulose column chromatography (Fukuda et al., 1993). All the components are of low molecular mass,
2. Materials and methods

2.1. Crystallization

The two distinct types of Hbs, components V and VII, from the 4th-instar midge larvae of *P. akamusi* were purified as described by Fukuda et al. (1993), and crystallization was carried out separately by hanging drop vapor diffusion techniques at 20 °C. Crystals of component V were grown in a drop containing the protein solution (2.0 μl of 24 mg/ml) mixed with an equal amount of a reservoir solution (25% PEG 3350, 200 mM ammonium sulfate, and 100 mM sodium acetate, pH 4.6). The dimensions of the crystal of component V reached 0.7 × 0.4 × 0.05 mm in about 1 week.

At the same time, crystals of component VII were grown as clusters of thin plates in the drop mixed with equal volumes of the protein solution (2.0 μl of 15 mg/ml) and a reservoir solution (30% PEG 3350, 200 mM MgCl₂, and 100 mM Tris–HCl, pH 8.5) within 1 week. Because these crystals were unsuitable for X-ray diffraction analysis, we employed a seeding experiment. The clusters of component VII thin plates were crushed and used as microseeds. Using a fine hair, the seeds of fine crystals were transferred into drops of a protein solution mixed with the reservoir solution (2.0 μl of 24 mg/ml). At the same time, crystals of component VII were grown as clusters of thin plates in the drop mixed with equal volumes of the protein solution (2.0 μl of 15 mg/ml) and a reservoir solution (30% PEG 3350, 200 mM MgCl₂, and 100 mM Tris–HCl, pH 8.5) within 1 week. Because these crystals were unsuitable for X-ray diffraction analysis, we employed a seeding experiment. The clusters of component VII thin plates were crushed and used as microseeds. Using a fine hair, the seeds of fine crystals were transferred into drops of a protein solution mixed with the reservoir solution containing 25% PEG 3350, 150 mM MgCl₂, and 100 mM Tris–HCl, pH 8.5. Finally, component VII crystals grew to about 0.2 × 0.2 × 0.05 mm within a day.

2.2. X-ray diffraction analyses

X-ray diffraction data of Hb components were collected from flash-cooled crystals at 100 K using a Rigaku R-AXIS VII image plate detector with CuKα radiation from rotating anode generators: Rigaku FR-E,SuperBright for component V and Rigaku RA-Macro7 for component VII (Rigaku Co., Tokyo, Japan). A crystal of component V was immersed in cryoprotectant solution containing 20% glycerol in the reservoir solution for 10–15 s, picked up with a loop, and rapidly transferred to a cold stream. Following that, diffraction data were collected at 1.64 Å resolution. A total of 140,766 observed intensities were measured, 20,177 of which were unique reflections resulting in an *R*_merge of 0.027 and completeness of 97.3%. One crystal of component VII was transferred to a reservoir solution for 24 h before flash-freezing and data collection. A total of 317,903 observed intensities were measured, 23,622 of which were the unique reflections. The resulting dataset was of 100.0% completeness with an *R*_merge of 0.041. Each set of diffraction data was processed using the software program CrystalClear (version 1.3.5; Rigaku Co., Tokyo, Japan). Crystal data and data collection statistics of components V and VII are summarized in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Data collection statistics, crystal data and structure refinement statistics</th>
<th>Component V</th>
<th>Component VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data collection statistics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>49.14–1.64</td>
<td>33.77–1.50</td>
</tr>
<tr>
<td></td>
<td>(1.70–1.64)</td>
<td>(1.55–1.50)</td>
</tr>
<tr>
<td>No. of recorded observations</td>
<td>140,766</td>
<td>317,903</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>20,177</td>
<td>23,622</td>
</tr>
<tr>
<td><em>R</em>_merge</td>
<td>0.027 (0.068)</td>
<td>0.041 (0.244)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.3 (94.2)</td>
<td>100.0 (100.0)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>6.98 (6.91)</td>
<td>13.46 (12.55)</td>
</tr>
<tr>
<td></td>
<td>48.6 (15.8)</td>
<td>31.0 (8.5)</td>
</tr>
<tr>
<td>Crystal parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td><em>P</em>2₁<em>2</em>₁<em>2</em>₁</td>
<td><em>C</em>222₁</td>
</tr>
<tr>
<td><em>a</em> (Å)</td>
<td>65.14</td>
<td>42.01</td>
</tr>
<tr>
<td><em>b</em> (Å)</td>
<td>74.85</td>
<td>69.11</td>
</tr>
<tr>
<td><em>c</em> (Å)</td>
<td>33.42</td>
<td>99.65</td>
</tr>
<tr>
<td>Model statistics</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R</em>-factor/<em>R</em>_free</td>
<td>0.194 / 0.2140</td>
<td>0.194 / 0.227</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>1213</td>
<td>1169</td>
</tr>
<tr>
<td>No. of heme atoms</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>No. of water atoms</td>
<td>226</td>
<td>262</td>
</tr>
<tr>
<td>r.m.d bond lengths</td>
<td>0.006</td>
<td>0.015</td>
</tr>
<tr>
<td>r.m.d bond angles</td>
<td>1.024</td>
<td>1.470</td>
</tr>
<tr>
<td>Ramachandran plot (%)</td>
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<td></td>
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<tr>
<td>Most favored</td>
<td>96.2</td>
<td>97.0</td>
</tr>
<tr>
<td>Additional allowed</td>
<td>3.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are for the highest resolution shell.
program (QUANTA, MSI Inc.), guided by a simulated annealing omit map and/or 2Fo–Fc map. The model fitted to the electron density map was refined by CNX functions: it included simulated annealing, energy minimization, and B-factor refinements. Following several rounds of manual intervention and CNX refinement, the heme and water molecules were added to the model. The water molecules were added using the X-SOLVENT program (QUANTA, MSI Inc.), guided by Fo–Fc maps contoured at 3σ. The model including water molecules was refined by energy minimization and B-factor refinements. On the other hand, the SAD phases of component VII were determined using the anomalous signal from heme irons. With the SAD phases, the initial model of component VII was built using the program ARP/wARP (Morris et al., 2003), it included most residues except for one N-terminal and one C-terminal. The subsequent refinement of component VII model was conducted as described above.

The qualities of the final models were assessed using the program PROCHECK (Laskowski et al., 1993). The atomic coordinates and structure factors of \textit{P. akamusi} Hb components have been deposited with the RCSB Protein Data Bank with the PDB codes of 1X3K for component V and 1X46 for component VII.

3. Results and discussion

3.1. Refined models of Hb components from \textit{P. akamusi}

The crystal of component V (L-type Hb) from \textit{P. akamusi} belongs to the orthorhombic space group \textit{P}2\textsubscript{1}2121 with unit cell dimensions of \(a = 65.14\,\text{Å}, \quad b = 74.85\,\text{Å}, \quad c = 33.42\,\text{Å}\). The \(V_M\) value was calculated to 2.37 \(\text{Å}^3/\text{Da}\), suggesting that there is one monomeric molecule in an asymmetric unit. The model of

\begin{center}
\includegraphics[width=\textwidth]{Fig2}
\end{center}

\textbf{Fig. 2.} The overall structure of Hb component VII from \textit{P. akamusi}. The eight helices are labeled A–H. The structural figure was drawn with Pymol (http://pymol.sourceforge.net/).

\begin{center}
\includegraphics[width=\textwidth]{Fig3}
\end{center}

\textbf{Fig. 3.} The overall structure of Hb component V from \textit{P. akamusi}. The eight helices are labeled A–H. An arrow indicates the C-terminal additional helix (post-H helix). The structural figure was drawn with Pymol (http://pymol.sourceforge.net/).
component V was solved by the SAD method and refined at 1.64 Å resolution to an $R$-factor of 19.4% and an $R_{\text{free}}$ of 21.4%. The final model of component V consisted of a complete polypeptide chain (152 amino acid residues), one heme group, and 226 water molecules.

The crystal of component VII (N-type Hb) belongs to the orthorhombic space group $C222_1$ with unit cell dimensions of $a = 42.01$ Å, $b = 69.11$ Å, and $c = 99.65$ Å. The asymmetric unit contains one monomeric molecule corresponding to a $V_M$ of 2.19 Å$^3$/Da. The refined model of component VII solved by the SAD method contained all 150 amino acid residues of the native protein, one heme group, and 262 water molecules, yielding an $R$-factor of 19.4% and an $R_{\text{free}}$ of 22.7% at 1.50 Å resolution. Detailed refinement statistics and geometric properties of the final models of $P. akamusi$ Hb components are summarized in Table 1, showing that each was well refined with excellent stereochemistry.

### 3.2. Overall structures of $P. akamusi$ Hb components

In general, hemoglobin components comprise eight helices (named A–H from N to C terminus) and corner regions connecting helices, and insect Hbs also possess this typical globin fold (Steingemann and Weber, 1979; Pesce et al., 2005; de Santis et al., 2005) (Fig. 1). Fig. 2 shows the crystal structure of component VII. It has the typical globin fold based on eight $\alpha$ helices except for residues 82–84 in the F helix and residues 147–149 in the H helix which display a $3_{10}$ helix conformation. Fukuda et al. (1993) reported that component VII showed higher sequence similarity to $Chironomus$ Hb components than component V. When the secondary structures were compared, component VII resembled component III of $Chironomus$ Hb in the A, B, and C helices. The three helices, A, B, and C in both component VII of $P. akamusi$ and component III of $C. thummi thummi$, differ from Hbs in other insects in that they are 3, 4, and 2 residues shorter (Fig. 1).

Fig. 3 shows the crystal structure of component V. It contains the same $\alpha$ helices, A to H in which the only differences occur in the C helix and residues 96–98 in the F helix which display a $3_{10}$ helix conformation. The presence of an additional helix at the C-terminal of component V is particularly remarkable. The helix is a one-turn long $3_{10}$ helix containing three residues (Ser149–Phe151). Fig. 4 shows the electron density in the refined model around the C-terminal region of component V. An additional helix is next to the F and H helices dependent on several noncovalent bonds and a disulfide bond between Cys143 and Cys148; this additional helix does not provide crystal contacts between neighbor molecules. The additional helix located at the N-terminal region has been observed in dimeric Hb (Hb I) from the blood clam, Scapharca inequivalvis, and was named the pre-A helix (Royer, 1994). We call an
3.3. Comparison of heme region

As shown in Fig. 5, electron density maps for the heme ligand are clearly evident in both components V and VII. Both ligands seem to be water molecules because of the shape and size of the electron density, and because there is no attention to ligand molecules on crystallization, which causes to form met-forms by autooxidation from oxy-forms (Ladner et al., 1977). Water molecules as ligand molecules are well fitted to electron density and display B-factors of 12.4 Å² and 12.2 Å² for components V and VII. The existence of these ligand molecules in *P. akamusi* Hb components suggests some interesting possibilities regarding the conformation of the heme region.

![Fig. 6. Overlapping of component V (pink) and component VII (blue) based on the F helix. Distal His (Ile for component V) (E7), proximal His (F8), and heme groups are shown by sticks. Structure superimpositions and figure preparation were performed with DeepView (Guex and Peitsch, 1997). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)

Additional helix of *P. akamusi* Hb the post-H helix, and we believe that it has no homologous relationships with any other known helix of Hb components. Its function cannot be confirmed at this stage, still the modification of the post-H helix associated with the heme state is remarkable because it contacts to the heme region.

### 3.3. Comparison of heme region

In vertebrate α2β2-type Hbs, the structural changes dependent on ligand binding are remarkable, thus the heme geometry of ligated Hb differs from that of unligated Hbs. Our findings indicate that both Hb components from *P. akamusi* are in the ligated form. However, overlapping the two components from *P. akamusi* reveals the differences between them. Fig. 6 indicates the narrow heme region of component V due to the close contact between the E and F helices: the distance between the Cα atom of E7 and the Cα atom of F8 is 13.58 Å, 0.69 Å shorter than the value in component VII (14.27 Å, nearly 14.5 Å (+0.2) in various Hb components (Riccio et al., 2002)); this is reminiscent of a scissors-like motion of the EF segment on hemichrome formation in the Antarctic fish Hb, where the distance between the Cα atoms of distal and proximal His is 12.5 Å (Riccio et al., 2002). Difference of the coordination geometry of the heme region between components V and VII is also significant.

The coordination geometry of the heme region in Table 2 is usual to illustrate the structural differences between ligated and unligated Hbs. Comparison of these parameters indicates that the heme geometry of component VII is similar to that of human ligated Hbs. In component V, the location of the iron atom in the heme plate is almost the same as in average ligated Hbs, and the distance between the heme iron and the ligand also is similar to those of ligated human Hbs. At the same time, component V closely resembles the unligated Hb components in the tilting of the imidazole of the proximal His. In many Hbs, the heme iron moves into the heme plate upon ligation, this brings about the movement of the imidazole of the proximal His. This structural feature of proximal His was not observed in ligated component V. It is possible that the structural changes in the heme on ligation do not trigger the associated movement of the imidazole of the proximal His in component V.

One of the most remarkable differences between component V and many other Hbs is related to the substitution of the usual distal His residue with Ile (Fukuda et al., 1993). Replacement of the distal His might cause unusual structural changes upon ligand binding. In fact, Hb components from a marine blood worm and a hagfish, in which each distal His is replaced, show small structural changes of heme region on ligation (Braden E7)

**Table 2**

<table>
<thead>
<tr>
<th>Distance (Å)</th>
<th>Ligated Hb</th>
<th>P. akamusi</th>
<th>Unligated (deoxy) Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxy-α</td>
<td>Oxy-β</td>
<td>CO-α</td>
</tr>
<tr>
<td>Fe-Ligand</td>
<td>1.82</td>
<td>1.78</td>
<td>1.74</td>
</tr>
<tr>
<td>Fe-Pn</td>
<td>0.05</td>
<td>0.04</td>
<td>−0.04</td>
</tr>
<tr>
<td>FeF8N2</td>
<td>2.07</td>
<td>2.06</td>
<td>2.11</td>
</tr>
<tr>
<td>Fe[C6-N3]-[Cε-N1]</td>
<td>0.14</td>
<td>−0.01</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Fe-Ligand, the distance between the heme iron and the ligand molecule. For calculation of this parameter in *P. akamusi* Hbs, a water molecule was treated as the ligand molecule. Fe-Pn, the distance between the heme iron and the center of the four porphyrin nitrogen atoms. This parameter represents the movement of the heme iron upon ligand binding. Fe-F8N2, the distance between the heme iron and the Nε atom of the proximal His. Fe[C6-N3]-[Cε-N1], the difference between two distances representing a tilting of the imidazole of the proximal His. One is between C6 of the proximal His and the nitrogen of pyrrole 1 of porphyrin, and the other is between Cε of the proximal His and the nitrogen of pyrrole 1. This tilting links the shift of the F helix toward the FG corner upon ligand binding (Mito et al., 2002). For comparison, the parameters from the structures of human Oxy-Hb (PDB code 2DN1), CO-Hb (2DN3), and Deoxy-Hb (2DN2) (Park et al., 2006) are also included.
et al., 1994; Mito et al., 2002). Our comparative studies of the crystal structure of \( P. \text{akamusi} \) Hb components revealed the characteristics of the tertiary structures of each component, and especially the interesting heme region of component V. It seems that the heme region of aquomet component V is in the intermediate state between a typical ligated (aquomet) and unligated or hemichrome form. Replacement of the distal His might play a part in the formation of the equivocal state of the heme region in component V. Crystallographic analyses of \( P. \text{akamusi} \) Hb in a variety of ligand state can prove helpful in understanding the details of the heme ligation and the associated structural changes.

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References


Axolotl hemoglobin: cDNA-derived amino acid sequences of two $\alpha$ globins and a $\beta$ globin from an adult *Ambystoma mexicanum*

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Abstract

Erythrocytes of the adult axolotl, *Ambystoma mexicanum*, have multiple hemoglobins. We separated and purified two kinds of hemoglobin, termed major hemoglobin (Hb M) and minor hemoglobin (Hb m), from a five-year-old male by hydrophobic interaction column chromatography on Alkyl Superose. The hemoglobins have two distinct $\alpha$ type globin polypeptides ($\alpha^M$ and $\alpha^m$) and a common $\beta$ globin polypeptide, all of which were purified in FPLC on a reversed-phase column after S-pyridylethylation. The complete amino acid sequences of the three globin chains were determined separately using nucleotide sequencing with the assistance of protein sequencing. The mature globin molecules were composed of 141 amino acid residues for $\alpha^M$ globin, 143 for $\alpha^m$ globin and 146 for $\beta$ globin. Comparing primary structures of the five kinds of axolotl globins, including two previously established $\alpha$ type globins from the same species, with other known globins of amphibians and representatives of other vertebrates, we constructed phylogenetic trees for amphibian hemoglobins and tetrapod hemoglobins. The molecular trees indicated that $\alpha^M$, $\alpha^m$, $\beta$ and the previously known $\alpha$ major globin were adult types of globins and the other known $\alpha$ globin was a larval type. The existence of two to four more globins in the axolotl erythrocyte is predicted.

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Keywords: Amphibia; Axolotl; *Ambystoma mexicanum*; Evolution; Globin; Hemoglobin; Nucleotide sequence; Primary structure

1. Introduction

Amphibian hemoglobins are of interest in the study of the evolution of vertebrate globin genes, gene expression and gene switching during metamorphosis, and functional properties of aquatic and terrestrial species. Although living amphibians comprise three major orders, Apoda, Urodela (Caudata) and Anura, most investigations have been carried out in anurans (frogs and toads): the bullfrog *Rana catesbeiana* (Hamada et al., 1966; Moss and Ingram, 1968; Aggarwal and Riggs, 1969; Chauvet and Acher, 1972; Baldwin and Riggs, 1974; Maruyama et al., 1980; Watt et al., 1980; Dorn and Broyles, 1982; Maples et al., 1983) and the South African clawed toad *Xenopus laevis* (Hentschel et al., 1979; Jeffreys et al., 1980; Hosbach et al., 1983; Kay et al., 1983; Banville and Williams, 1985; Knöchel et al., 1985; Weber et al., 1991). The Mexican axolotl *Ambystoma mexicanum*, on the other hand, belonging to order Urodela, is an important animal in the biological sciences because they become adult without metamorphosing, a phenomenon termed neoteny. Although axolotls receive much attention as a counterpart of metamorphosing species, little is known about hemoglobin components, population of globin subunits and primary structures of globins. In our references, there are only two reports on primary structures of axolotl globin polypeptides (Boissel et al., 1980;
GenBank accession no. AF308869); both were assigned to an adult α type of globin and a larval α type of globin by computer analysis described later.

We describe here the separation of two kinds of hemoglobin components, Hb M (major) and Hb m (minor), from a neotenous adult axolotl *A. mexicanum* and establish the complete nucleotide sequences of the three kinds of globin chains, two of adult α type globins and adult β type globin common to both hemoglobin components.

2. Materials and methods

2.1. Blood cell collection

Blood of an adult axolotl *A. mexicanum* (five-year-old male about 20 cm in body length and 600 g in mass) was drawn from the second branchial artery into physiological saline (0.9% NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0). The blood was then centrifuged at 1750 × g for 15 min, and the packed cells were divided: one half of the packed cells was lysed with 50 mM Tris–HCl, pH 8.0, containing 10 mM EDTA for preparation of hemoglobin solution, and the other half was used for extraction of total RNA by Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. All procedures were done at 4 °C and the samples were stored at −80 °C.

2.2. Separation of hemoglobin components

A hemoglobin solution that had been saturated at 40% by adding 60% saturated ammonium sulfate was subjected to an Alkyl Superose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 60% saturated ammonium sulfate (183 g/500 mL) in 50 mM ammonium bicarbonate, pH 8.0. The experimental procedures were conducted as previously described (Shishikura and Takami, 2001). Elution was carried out with a gradient of 60–0% saturated ammonium sulfate in the 50 mM ammonium bicarbonate buffer, pH 8.0. The flow rate was maintained at 0.5 mL/min, and fractions of the protein peaks were collected. The fractions were monitored at 415 and 280 nm by two spectrophotometers (Model Uvidec-340Q, Jasco, Tokyo, Japan, and Model 116, Gilson Medical Electronics, Inc., WI, USA), respectively.

2.3. Preparation of globin polypeptides

To separate α and β globin polypeptides, hemoglobin components were *S*-pyridylethylated separately by the method described previously (Friedman et al., 1970). Each of the *S*-pyridylethylated hemoglobin components was then subjected to a Resource column (Pharmacia Biotech) and eluted with a buffered gradient of 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, Inc., St. Louis, MO, USA) to 60% acetonitrile in 0.08% TFA. All fractions were monitored at 214 and 280 nm. For further purification, re-chromatography on the Resource column was conducted under shallower gradient conditions, as described in our previous report (Shishikura et al., 1987).

2.4. Protein sequencing

All three kinds of globin molecules modified by *S*-pyridylethylated were separately digested with lysyl endopeptidase (Achromobacter Protease I; Wako Pure Chemicals, Tokyo, Japan) at an enzyme/substrate ratio of 1:30 (mol:mol) for 4 h at 37 °C in 0.1 M ammonium bicarbonate, pH 8.2, containing 4 M urea. Peptide fragments derived from each of the parent molecules were separated using a reversed-phase column (Resource RPC, Pharmacia Biotech.) in a buffered gradient of 0.1% TFA to 60% acetonitrile in 0.08% TFA. Flow rates were maintained at 0.3 mL/min. All fractions were monitored at 214 and 280 nm. Re-chromatography of selected peptides was conducted as previously described (Shishikura et al., 1987). Sequencing analyses of these fragments as well as amino (N)-terminal amino acid residues of parent molecules were performed using a gas phase protein sequencer, PPSQ-10 (Shimadzu, Shiga, Japan), equipped with a class LC-10 amino acid analyzer (Shimadzu, Shiga, Japan). Phenylthiohydantoin (PTH)-derivatives from the sequencer were separated and quantified. The peptides were aligned with the assistance of sequence similarities toward the known globin structures of urodeles (Boissel et al., 1980; Kleinschmidt et al., 1988; AF308869).

2.5. Isolation of total RNA and purification of mRNAs

Total RNA was extracted from the blood cells by Purescript Total RNA Isolation Kits (Gentra Systems), and the mRNA fraction that included the three kinds of globin mRNAs was isolated with a Takara Oligoex™ -dT30<Super>mRNA Purification Kit (Takara Bio, Shiga, Japan).

2.6. Primer design

Degenerate primers were designed based on the amino acid sequences of lysyl endopeptidase-digested fragments of parent molecules as listed in Appendix 1. In order to sequence the PCR amplified fragments with a BigDye Terminator v. 1.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA), the degenerate oligo-nucleotide primers were tailed with the pUC/M13 forward or pUC/ M13 reverse sequencing primer tail (forward 17-mer: 5′-GTAAACGCGCCAGGT-3′, Sigma-Aldrich Japan, Tokyo, Japan, and reverse 17-mer: 5′-CAGGAAACAGC- TATGAC-3′, Promega, Tokyo, Japan).
2.7. Nucleotide sequencing

Single-strand cDNAs were synthesized with a Takara RNA PCR Kit (v. 2.1) using the Oligo dT-Adaptor Primer (M13 primer M4, 17-mer: 5'-GTTTCCCAGTCACGCTT-3'), according to the manufacturer’s instructions (Takara Bio).

For PCR amplification of the 3’ region of the cDNAs, the primers used were the adaptor and a redundant oligomer based on the N-terminal amino acid sequence of each globin (see Appendix 1). The second PCR-amplification was conducted with a nested PCR primer (a redundant oligomer) and the adaptor (Appendix 1). One major fragment was detected on agarose gel electrophoresis in each PCR. Then, the amplified fragment was extracted by a GenElute™ Agarose Spin Column (Sigma-Aldrich), and sequenced directly with a BigDye Terminator v. 1.1 Cycle Sequencing Kit. The rest of the unknown sequence of the 3’ end was afterwards confirmed by 3’ RACE (Frohman et al., 1988), with the adaptor and a gene-specific primer (Appendix 1).

For PCR amplification of the 5’ region of cDNAs, gene-specific primers with or without 5’ monophosphate as listed in Appendix 1, were designed in order to extend the sequences in the 5’ ends using a Takara 5’-Full RACE Core Set (Takara Bio) according to the manufacturer’s instructions. All forward and reverse primers, except for the Oligo dT, listed in Appendix 1, were tagged with the pUC/M13 sequencing primers.

2.8. Computer analysis

A multiple alignment program, Clustal X v. 1.81 (Jeanmougin and Thompson, 1998), was used to align the three kinds of adult axolotl globins, previously established known sequences of amphibians and those from representative species of tetrapods. Two phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), and the molecular trees were drawn by the NJ-prot program stored in Clustal X.

3. Results and discussion

3.1. Two hemoglobin components of adult axolotl

Separation of an intact component from multiple hemoglobins is a prerequisite to investigation of the properties of individual hemoglobin component from sources with such complicated life cycles as amphibians. Several investigators have succeeded: in adult anurans (toads and frogs), four hemoglobin components have been separated and characterized (Elli et al., 1970; MacLean and Jurd, 1971; Tam et al., 1993). In adult urodeles, four and eight intact hemoglobin components were separated from the newt Triturus cristatus (Salamandroidea) by anion-exchange column chromatography on DEAE-Sepha- dex (Grasso et al., 1979) and by analytical isoelectrofo- cusing (Koussoulakos et al., 1986), respectively. However, how many hemoglobin components are present in erythrocytes of the axolotl A. mexicanum, which belongs to the Ambystomoidea, in the embryo or larval stages, or even in the neotenous adult stage, has not been investigated.

Fig. 1 shows two well-delimited peaks corresponding to a major (Hb M) and a minor hemoglobin (Hb m) from erythrocytes of an adult axolotl. The two peaks, Hb M and Hb m, were detected at 280 and 415 nm. The peaks were separately pooled as shown by bars, and then, each pooled fraction was re-chromatographed on the same column under the same conditions (insets of Fig. 1). They exist in a ratio of about 5 (Hb M):2 (Hb m) based on chromatogram area calculation. This value varied from 5:1 to 5:3 depending on sample preparation. Several small distinct peaks (indicated by two arrows in Fig. 1) were still
detected in both purified and unpurified fractions, but no further purification was carried out because the other hemoglobin peaks obtained were very poor and it was impossible to characterize them further. Similar two peaks (Hb M and Hb m) were also detected in the case of hemoglobin solution prepared from a five-year-old female (about 18 cm in body length and 500 g in mass: data not shown here).

The Alkyl Superose HR 5/5 column that we used first in preparation of intact reptilian hemoglobin components (Shishikura, 2002; Kuwada et al., 2003) was used for preparation of hemoglobin components from axolotl blood. The separation pattern is superior to that obtained by isoelectric focusing (Hattingh and Bartels, 1973). The chemistry of the separation is based on hydrophilic interactions with the gel matrix and biological macromolecules. With the fraction, we are currently generating crystals of the intact hemoglobin components, Hb M and Hb m, of the axolotl hemoglobin.

3.2. Three globin chains, sequences, and alignment

Like birds, reptiles and mammals, amphibians produce different globin polypeptides during their life cycles (McCutcheon, 1936; McCutcheon and Hall, 1937; Gratzer and Allison, 1960; Bunn and Forget, 1986). Among them, the amphibians metamorphose dramatically, where the pattern of globin polypeptides of anurans (frogs and toads) is replaced completely by that of adult globin polypeptides (Hentschel et al., 1979; Maples et al., 1988). Some urodeles, on the other hand, coexpress larval and adult globin mRNAs in a single erythroid cell (Yamaguchi et al., 2000).

In contrast, Grasso et al. (1979) described at least 6–7 globin polypeptides in the adult urodele T. cristatus, of which Kleinschmidt et al. (1988) sequenced four chains including two α globin chains and two β globin chains. We purified one α and one β globin chain from the axolotl Hb M and Hb m, respectively, and then sequenced some of the peptide fragments derived from the parent molecules digested with lysyl endopeptidase (chromatograms of peptide maps by lysyl endopeptidase digestion are not shown here). Appendix 2 shows the results of amino acid sequence analyses, in which the two α globin chains were distinct, but the two β globin chains so far analyzed (only one sequence is shown here) had identical sequences. This reminds us that the two hemoglobin components, B and C, of the adult R. catesbeiana share a common β globin chain but have different α globin chains (Tam et al., 1986; Smith et al., 1993). The number of globin polypeptides we found in the adult axolotl erythrocytes confirmed the study of Ducibella (1974), who purified three globin subunits from the denatured hemoglobin solution of the neotenic adult, with apparent molecular weights of 15,000, 19,000 and 24,500 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Using the primers listed in Appendix 1, cDNA-fragments were amplified by PCR. Fig. 2 shows one major band and several very faint bands in each lane; each of the major fragments was extracted from the agarose gels and sequenced separately. Appendix 2 (A, B and C) shows the results of nucleotide sequencing of twelve cDNA-fragments providing enough information to determine complete nucleotide sequences of globin cDNAs. The entire coding regions of the three kinds of adult A.

Fig. 2. Agarose gel electrophoresis (1.5% gels) of the PCR products amplified from three kinds of cDNAs using primers as listed in Appendix 1. A: RT-PCR, B: Nested PCR, C: 3′ RACE, and D: 5′ cRACE. The major fragment in each lane, except for the M1 and M2 lanes, was extracted from the gel and sequenced. PCR conditions: 30 cycles each consisting of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, and 1 min at 72 °C for primer extension. M1 (100 bp ladder) and M2 (pHY) are DNA markers.
mexicanum globin genes have been deposited in the GenBank/DDBJ/EMBL (under DDBJ accession nos: AB185144 for αM globin, AB185145 for αm globin, AB185146 for β globin). The amino acid sequences deduced from the cDNA nucleotide sequences were identical with those determined by peptide sequencing analyses, whose complete primary structures are also shown in Appendix 2. Their sequences reinforce each other. The nucleotide sequences of mature proteins are composed of 141 amino acid residues for αM globin, 143 for αm globin, and 146 for β globin. Boissel et al. (1980) reported an extra residue for the α globin chain of the major component of A. mexicanum compared with that of ordinary α globin chains in tetrapods; however, this is not curious because one or two longer residues were recently found in newts T. cristatus α globin chain with 142 (Kleinschmidt et al., 1988) and Taricha granulosa α globin chain with 143 residues. (Coates et al., 1977), and αm of this study with 143 amino acid residues.

Fig. 3 shows an alignment in which two previously established globin sequences (Boissel et al., 1980; AF308869) and three other globin chains of the present study (αM, αm and β) are comparable to those of human α and β globin chains. Hereafter we would like to use the tentative nomenclature of globins of the axolotl: α-1 for the α globin chain named by Boissel et al. (1980) and α-2 for the α globin of AF308869. Compared with each other, four groups based on % homology can be detected: the first group is 61–70% homologous (αM versus α-1; αM versus αm; αM versus α-1), the second group is 51–57% homologous (αm versus human α; αM versus human α; α-1 versus human α), the third group is 47–49% homologous (αM versus α-2; αM versus α-2; α-1 versus α-2) and the fourth group is 28–38% homologous (α-2 versus human α; αm versus human α; α-1 versus human α; αM versus human α; α-2 versus human α).

Residue No. 1 31
αM globin VLAEDKAKYVKVAVKHDGKKEEFPGAELCNMFDSEFVTKYFPGK------DISEES
αm globin VFLSLGDKAKYVKVAVKHDGKKEEFPGAELCNMFDSEFVTKYFPGK------DINEGS
α-1 FLSGEDKAKYVKVAVKHDGKKEEFPGAELCNMFDSEFVTKYFPGK------DINEGS
α-2 MTLTAEDKALVYLGKAGHGTADALDGGEALDRLFACFGQSRTYFPGK------DLGSFS
β globin VHLAEERKVGIALGKV------------VDALGGQCLARKMVFYPSKRRYFPGFDSTMDCAIChN

Residue No. 61 91
αM globin SYLHSGKGVVAELNAVIDNECLKLDDTANELMDFFTNFFLGHNILVGH
αm globin AFHSGKGVVAELNAVIDNECLKLDDTANELMDFFTNFFLGHNILVLMH
α-1 FALSGKGVVAELNAVIDNECLKLDDTANELMDFFTNFFLGHNILVLMH
α-2 ADVYRSGKGVVAELNAVIDNECLKLDDTANELMDFFTNFFLGHNILVLMH
β globin ARVLAGHNVRSVECTAKHDLNQYFEADLSSHLKLVFDPWFQNFLQFRIVVCLQAT

Residue No. 121 149
αM globin MQPLLCATMCMLCKDFLQYAVLTSKRY (This study, AB185144/DDBJ)
αm globin LEQFTTPANDKFLKFLVTRMLCISKRY (This study, AB185145/DDBJ)
α-1 LPKFTTPAQCSDKFLKFLVTRMLCISKRY (P02015/Swiss-Prot)
α-2 FPADFTQAOAMDKFLAASVGLTSKRY (Q020A5/Swiss-Prot, AF308869/DDBJ)
β globin LTQETFHDKLAFKELHSVNLKSHOPKLSLLHVLAAH

Residue No. 1 258
αM globin MPQPLLCATMCMLCKDFLQYAVLTSKRY (This study, AB185144/DDBJ)
αm globin LEQFTTPANDKFLKFLVTRMLCISKRY (This study, AB185145/DDBJ)
α-1 LPKFTTPAQCSDKFLKFLVTRMLCISKRY (P02015/Swiss-Prot)
α-2 FPADFTQAOAMDKFLAASVGLTSKRY (Q020A5/Swiss-Prot, AF308869/DDBJ)
β globin LTQETFHDKLAFKELHSVNLKSHOPKLSLLHVLAAH

Fig. 3. Alignment of the amino acid sequences of five kinds of globins from A. mexicanum. The 26 amino acid residues conserved in the five kinds of globins are shown by asterisks in the upper part of the alignment. The residue number is an arbitrary number with the numbering beginning from the N-terminal of the globins with the longest N-terminal extension. 'Helix position' refers to the helix position in human β globin (Nagel, 1995). The symbols refer to those residues functionally important in the human hemoglobin molecule (Fermi, 1975): #, residues involved in contact with the heme group; o, residues involved in contacts between subunits α1 and β1; §, residues involved in contacts between subunits α1 and β2.
αm versus β; αM versus β; α-1 versus β). Watt et al. (1980) reported that the tadpole β chain of *R. catesbeiana* differed greatly from that of the adult frog; only about 50% of the residues were identical. Based on the assumption of the overall rate of change in hemoglobin as about 1% per 3.3 million years (Wilson et al., 1977), they estimated a 50% difference corresponded to about 165 million years. Our findings, together with molecular relationships shown in Fig. 4A and B, suggest that the three α types of globins of the first group, αM, αm and α-1, which are homologous globin polypeptides in the same organism are caused by gene duplications of the α globin genes, and the second group, whose homology was estimated to be about 50%, may have occurred at least as long ago as the common ancestor to the axolotl and humans, dating about 165 million years ago.

Of considerable importance in hemoglobin function are the two contact regions between the subunits designated α1h1 and α2h2, and heme contact residues (Perutz et al., 1968; Fermi, 1975). In mammalian hemoglobin, oxygenation is accompanied by large changes at the α1h2 contact and relatively small changes at the α1h1 contact (Perutz, 1970). To gain information on axolotl hemoglobin, we...
compared the appropriate amino acid residues with those involved in subunit interactions (α1β1 and α1β2) as well as in heme contacts as compared with amino acid residues responsible for subunit interactions and heme contacts of human deoxymyoglobin described by Fermi (1975). As shown in Fig. 3, seven and eight out of 27 amino acid residues were variant at the α1β2 contacts of Hb M and Hb m, respectively, whereas many more differences (18 and 17 residues) were apparent at the α1β1 contacts of Hb M and Hb m. Such a marked difference in the α1β1 contacts compared to that at the α1β2 contacts has been also reported for X. laevis hemoglobin (Knöchel et al., 1985; Banville and Williams, 1985). The heme contact residues, on the contrary, were well conserved compared with those detected in human globins (Fermi, 1975): 13 residues (81.3%) and 12 residues well conserved compared with those detected in human globins (1985). The heme contact residues, on the contrary, were well conserved compared with those detected in human globins (Fermi, 1975): 13 residues (81.3%) and 12 residues well conserved compared with those detected in human globins (1985). The heme contact residues, on the contrary, were well conserved compared with those detected in human globins (Fermi, 1975): 13 residues (81.3%) and 12 residues well conserved compared with those detected in human globins (1985). The heme contact residues, on the contrary, were well conserved compared with those detected in human globins (Fermi, 1975): 13 residues (81.3%) and 12 residues well conserved compared with those detected in human globins (1985).

In general, the Bohr effect in human hemoglobin has been postulated to involve the breakage of at least four salt bridges on oxygenation (Perutz, 1970). On this basis, the two salt linkages between the protonated NH₂-terminal group of the valine of one α chain and the COOH-terminal of the other α chain in the deoxymyoglobin tetramer carboxyl group and between the guanidino group at HC3(141)ψ and the aspartic acid at position 9 of the H helix of α₂ globin chain, designated Asp H9(126)ω, are conserved. However, the other two salt linkages between the HC3(146)ψ imidazole group and the Asp (94)β in the same chain and between the carboxyl group of HC3(146)ψ and the ε-NH₂ of the lysine at C6(40)α are replaced with other amino acid residues. These findings may account in large part for the decreased Bohr effect on the axolotl hemoglobin compared with that of human hemoglobin (Amiconi et al., 1970; Hattingh and Bartels, 1973; Banville and Williams, 1985). It is worth noting that Lys C6(40) of the αM chain is still conserved, compared with other α types of axolotl globins. Although Boissel et al. (1980) reported the replacement of histidine at H5(122)α by glutamine in axolotl, we detected that the amino acid residue at H5(122) of the two α chains, αM and αm, are unchanged, as reported in humans. Hence, it is advantageous to present multiple globins in situations where living organisms need to express a potential globin for adaptation to changes in surroundings or changes of life cycles, in particular, in amphibians (Hutchison et al., 1976; Perutz, 1983).

In addition, Boissel et al. (1980) found a proline at G6(107)α in the axolotl. Maruyama et al. (1980) noted that the prolyl residue at this position causes distortion of the G helix of hemoglobin of Glycera dibranchiata, an annelid (Padlan and Love, 1974). We also detected a proline at G6 of αM globin rather than αm globin of the axolotl. To confirm the above estimation of changes in the tertiary structures, a crystallographic investigation is required.

3.3. Molecular Trees

The gene family of X. laevis globin consists of twelve genes in two clusters, each containing larval and adult α and β genes in a symmetrical arrangement (Hosbach et al., 1983). In the urodeles, we sequenced only three globin cDNAs from an adult axolotl, A. mexicanum, and analyzed their phylogenetic positions and relationships on molecular trees including representatives of tetrapodes (Fig. 4A) and, in particular, amphibia (Fig. 4B).

Fig. 4A shows that α types of axolotl globins (αM, αm and α-1, but not α-2) make a group, which is more closely related to the adult α type globins of amniotes (tortoises, birds and mammals) than the αD type globins of some reptiles and birds or embryonic globins of birds and humans, π’ and ξ (Chapman et al., 1980; Aschauer et al., 1981). The αD globin is one of the globin subunits of the hemoglobin D tetramer (αDβ2), which was first found in birds as a minor component of the embryonic and adult definitive erythrocytes (Hagopian and Ingram, 1971), and also found in some reptilians (Abbasi et al., 1988; Ruchnagel and Braunitzer, 1988; Gorr et al., 1998; Shishikura and Takami, 2001). On the contrary, the α-2 globin (AF308869) was assigned to an ancestral type of embryonic globins since human ξ and bird π’ globins split off.

Fig. 4B shows the molecular relationship of 43 globin sequences of amphibians, the members of which seem to be almost all the known sequences of amphibians stored in the Swiss-Prot data bank at the present time (release 45.5). It is not unreasonable to predict that the split of the adult types of globins from the larval types or vice versa might be significant for consideration of the evolution of vertebrate globin genes since each of the ancestor genes of α and β globins first duplicated itself into the four main clusters in total, and thereafter further divergences occurred, each branching into anuran species and urodele species.

Finally, to compare the distribution pattern of the five globin genes of the axolotl with that of X. laevis, R. catesbeiana or other urodele species (Fig 4B), we can predict that another two to four globin genes will be expressed in either larval stages or neotenous adult stages. However, it is necessary to investigate more currently unknown globin genes that will be equivalent to the unique gene arrangements reported in Xenopus and human globin gene families.

Acknowledgements

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### Appendix 1

#### Appendix 1A. Oligonucleotide primers used in the first PCR (RT-PCR) of globin cDNAs

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#### Appendix 1B. Oligonucleotide primers used in 5’ cRACE amplification of globin fragments

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|            | Nucleotide sequence | t | c | t | c | e | c | g | t | g | a | c | a | c | g | g | g | a | t | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a | c | a |c
### Appendix 2B. Primary structure and nucleotide sequence of α-globin of *Amphystoma mexicanum*

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>65</th>
<th>70</th>
<th>75</th>
<th>80</th>
<th>85</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>KVM C A L T N A V A H I D N I E A C L D K L S D T H A H E</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotide seq.</td>
<td>aag gtg atg tgc ggc ctg acc aat gcc gtc gcc cac ata gaa gac ttc gca gac aag gcc cac ggc cac gag</td>
<td></td>
<td></td>
<td></td>
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3' RACE stop

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>95</th>
<th>100</th>
<th>105</th>
<th>110</th>
<th>115</th>
<th>120</th>
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</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>L M V D P T N F P R L G H N I L L V I G I H M P Q L L T C A</td>
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<tr>
<td>Nucleotide seq.</td>
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3' RACE stop

<table>
<thead>
<tr>
<th>Amino acid residue</th>
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<th>130</th>
<th>135</th>
<th>140</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>M H C S L D K F L C Q V A E V L T S K Y R</td>
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<td></td>
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<tr>
<td>Nucleotide seq.</td>
<td>atg cac tgc ctc gct gac aag ttc ctc gtc gac gct gca gaa gtc gtt gcc gct gcc acc aac tgc gta</td>
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3' RACE stop

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<tr>
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<td>atg gtg ctc gtc gca ggg gac aag gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc ccg gcc</td>
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5' cRACE stop

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<td>Nucleotide seq.</td>
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5' cRACE stop

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<th>75</th>
<th>80</th>
<th>85</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>G K K V M S A L T N A V A H I D D L E A A L S K L I D K H A</td>
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<tr>
<td>Nucleotide seq.</td>
<td>gga aag gtt atg ctt gcc tta act aac gcc gtc gcc cat atc gac gac ctt ggt gct gcc ctc cag aag ctg atc gac gaa aac ctc ggc</td>
<td></td>
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3' RACE stop

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>95</th>
<th>100</th>
<th>105</th>
<th>110</th>
<th>115</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>H D L M V D P A N F V L L N H I L A V L A M H L P Q L L F T</td>
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<td></td>
</tr>
<tr>
<td>Nucleotide seq.</td>
<td>cat gac ctc atg gtt gat cct gca aac ttt gtt ctt ctt atc cat atc ctc gca gtc ctt ggc atg cac ctg ccc cag ctc ttc aca</td>
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3' RACE stop

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>125</th>
<th>130</th>
<th>135</th>
<th>140</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid seq.</td>
<td>P A N H R S L D K F L H T V M R C L I S K Y R</td>
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</tr>
<tr>
<td>Nucleotide seq.</td>
<td>cct gca aac cac cgc tct tgg gac aag ttc tgg cat aca gtt atg ctt tgt tgg att aca aaa tac cgt taa</td>
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</tbody>
</table>

3' RACE stop

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Appendix 2C. Primary structure and nucleotide sequence of H-α-globin of Ambystoma mexicanum

<table>
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<th># of a. a. residue</th>
<th>Amino acid seq.</th>
<th>N-terminal</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>V H L T A E E K</td>
<td>k-1</td>
</tr>
<tr>
<td>5</td>
<td>D V G A I L G K V N V D A L G G Q C L A R</td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide seq.: gtt cac ctc aca gcc gaa gaa cgc aag gac gtc gtt gcc att tta gta gac gct ctc gga ggt ctc aac tgc ctt gca aag

- RT-PCR

<table>
<thead>
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<th># of a. a. residue</th>
<th>Amino acid seq.</th>
<th>N-terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>L M C V Y P W S R R Y F P D F G D M S T C D A I C H N A R V</td>
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</tr>
</tbody>
</table>

Nucleotide seq.: cct atg tgt tgt tat ccc tgg tca cgg aag tac ttc cgg gat tgt ggt gac aag acc aag ctc gac aac ctc cta gaa tac ttc ggc gac ctc aag

- RT-PCR

<table>
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<th># of a. a. residue</th>
<th>Amino acid seq.</th>
<th>N-terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>T H C L K L F V D P Q N F K L F G R I V V V C L A Q T L Q T</td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide seq.: act cat tgc ctc aag ctc ttt gtt gac cgc aac ttc aag ctt cgg gac ctt ggc aag ctc aag ctc gac ctc gaa acc acc agg

- RT-PCR

<table>
<thead>
<tr>
<th># of a. a. residue</th>
<th>Amino acid seq.</th>
<th>N-terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>E F T W H K Q L A F E K L M R A V A H A L S H S Y Y H</td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide seq.: gaa ttt aca tgt cat aag cag ctc ggc ttc gaa aag tgt atg cgg gcc gtt gca cat gtg ctc aag ctc aac cac tac cag tga

- RT-PCR

References


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Weber, R., Blum, B., Muller, P.R., 1991. The switch from larval to adult globin gene expression in *Xenopus laevis* is mediated by erythroid cells from distinct compartments. Development 112, 1021–1029.
