The Amino Acid Sequences of Two \( \alpha \) Chains of Hemoglobins from Komodo Dragon \textit{Mu-anus komodoensis} and Phylogenetic Relationships of Amniotes

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To elucidate phylogenetic relationships among amniotes and the evolution of \( \alpha \) globins, hemoglobins were analyzed from the Komodo dragon (Komodo monitor lizard) \textit{Varanus komodoensis}, the world’s largest extant lizard, inhabiting Komodo Islands, Indonesia. Four unique globin chains (\( \alpha^A, \alpha^D, \beta^A, \) and \( \beta^D \)) were isolated in an equal molar ratio by high performance liquid chromatography from the hemolysate. The amino acid sequences of two \( \alpha \) chains were determined. The \( \alpha^D \) chain has a glutamine at E7 as does an \( \alpha \) chain of a snake, \textit{Lioptis miltioris}, but the \( \alpha^A \) chain has a histidine at E7 like the majority of hemoglobins. Phylogenetic analyses of 19 globins including two \( \alpha \) chains of Komodo dragon and one from representative amniotes showed the following results: (1) The \( \alpha \) chains of squamates (snakes and lizards), which have a glutamine at E7, are clustered with the embryonic \( \alpha \) globin family, which typically includes the \( \alpha^D \) chain from birds; (2) birds form a sister group with other reptiles but not with mammals; (3) the genes for embryonic and adult types of \( \alpha \) globins were possibly produced by duplication of the ancestral \( \alpha \) gene before ancestral amniotes diverged, indicating that each of the present amniotes might carry descendants of the two types of \( \alpha \) globin genes; (4) squamates first split off from the ancestor of other reptiles and birds.

Introduction

Knowledge about the origin and relationships of amniotes (mammals, squamates, turtles, sphenodon, crocodilians, and birds) has been provided by paleontological and morphological data. Recently, vast amounts of protein and nucleic acid sequence data have given us new insights (Bishop and Friday 1987, 1988; Donoghue et al. 1989). Hemoglobin has been sequenced from many vertebrates and nonvertebrates to give evolutionary relationships among species (Goodman, Moore, and Matsuda 1975; Dickerson and Geis 1983). However, because only a few reptilian species have been available, the gene-duplication events of a chains and the divergence order among amniotes have not been elucidated sufficiently. Squamates (snakes and lizards) showed multiple \( \alpha \) globins and \( \beta \) globins, but not all of these have been sequenced (Duguet, Chauvet, and Acher 1974; Naqvi et al. 1983; Matsura, Fushitani, and Rigs 1989; Abbasi and Braunitzer 1991). Here we report the amino acid sequences of both \( \alpha \) chains of hemoglobin from the Komodo dragon \textit{Varanus komodoensis} and discuss the phylogenetic relationships among amniotes and the evolution of \( \alpha \) globin genes.

Methods and Materials

Globin Preparation, Cleavage, and Purification of Peptides

Blood sample was legally collected from a Komodo dragon in Kebun Binatang Surabaya, Indonesia. Either carboxyamidomethylated or pyridylethylated hemoglobin was applied to high performance liquid chro-

matography (HPLC) (Waters Model 625LC system) to isolate constituent globins with a reverse phase semimicrobore column. Isolated globins were digested enzymatically with lysylendopeptidase C, endoproteinase Asp-N, and chymotrypsin or cleaved chemically at Asp-Pro site according to the procedures described previously (Fushitani, Matsuura, and Riggs 1988). The derived peptides were isolated by the same HPLC system.

Amino Acid Analysis and Amino Acid Sequence Determination

Amino acid compositions of the globins and the peptides were determined using the PICO-TAG system (Waters). Sequence analyses were performed using a pulse-liquid sequencer (model 477A, Applied Biosystems) equipped with an on-line phenylthiohydantoin-(PTH) amino acid analyzer (model 120A). The peptides derived from each of the \( \alpha \) chains (\( \alpha^A, \alpha^D \)) by cleavage with lysylendopeptidase C were first sequenced and then overlapped with peptides derived by endoproteinase Asp-N, chymotrypsin, or Asp-Pro cleavage to completion. Details of sequence strategies and amino acid compositions of the two \( \alpha \) globins and their peptides will be sent upon request.

Phylogenetic Analysis

Seventeen amino acid sequences used for phylogenetic analyses were retrieved from SWISS-PROT Protein Data Bank (release 30, 1994; see the legend of fig. 3 for accession numbers) and aligned manually with two sequences of Komodo dragon. The length of the aligned sequences including gaps is 149 amino acids. Phylogenetic trees were inferred with maximum parsimony (PAUP, version 3.1.1; Swofford and Olsen 1990; Swofford 1993) and neighbor-joining (Saitou and Nei 1987) methods. One hundred bootstrap replications of maximum parsimony were carried out with heuristic search, 10 random additions of sequences, the tree bisection-reconnection method for branch swapping, and the
Results and Discussion

Composition of Globin Chains

Chromatography of carboxyamidomethylated hemoglobins of Komodo dragon gave four peaks in about equal amount (fig. 1). N-terminal sequence analyses showed that Komodo dragon has two kinds of $\alpha$ chains (denoted $\alpha^A$, $\alpha^D$) and two kinds of $\beta$ chains ($\beta^B$, $\beta^C$). This characteristic globin composition, two kinds of both $\alpha$ and $\beta$ chains, has also been reported for lizards, Uromastix hardwickii (Naqvi et al. 1983), Varanus anthermaticus albicularis (Abbasi and Braunitzer 1991), and Iguana iguana (Rticknagel, Braunitzer, and Wiesner 1988), and for snakes, Liophis miliaris (Matsuura, Fushitani, and Riggs 1989). Although only one $\alpha$ chain from the viper Viper aspis (Duguet, Chauvet, and Acher 1974) and only one $\beta$ chain from the Indian cobra Naja naja (accession number in SWISS-PROT data bank: p22743) have been reported, hemoglobins from these snakes may also have two $\alpha$ and two $\beta$ chains. The general conclusion is that hemoglobins from squamates (lizards and snakes) have two kinds of $\alpha$ chains and two kinds of $\beta$ chains. Among the living reptiles, which include squamates, crocodilians, turtles, and sphenodon, the hemoglobins from sphenodon and turtles are also like those of squamates. Table 1 summarizes the major hemoglobin components found in adult amniotes and shows the evolutionary differences between groups.

The oxygen affinity of purified hemoglobin, though a mixture of multiple components, from Komodo dragon is higher by 2.8-fold than that of human hemoglobin in the absence of allosteric effectors. However, the affinity of red blood cells of Komodo dragon is lower by 1.7-fold at 37°C than that of human hemoglobin. This inversion of affinity is ascribed to the presence of abundant ATP in Komodo red blood cells (unpublished data). Detailed accounts for functional properties of Komodo dragon hemoglobin will be described elsewhere.

Amino Acid Sequences of $\alpha$ Globins

The sequences of two $\alpha$ chains of Komodo dragon are shown in figure 2 aligned with human $\alpha$ chain (Dick-

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Table 1
Numbers of Major Hemoglobin Components and Globin Compositions Found in Living Amniotes

<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>NUMBER OF Hb COMPONENTS</th>
<th>GLOBIN COMPOSITION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammals</td>
<td>1</td>
<td>$\alpha$, $\beta$</td>
<td>Leclercq et al. 1981</td>
</tr>
<tr>
<td>Crocodilians</td>
<td>1</td>
<td>$\alpha$, $\beta$</td>
<td>Takei et al. 1975</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Ricknagel, Reischl, and Braunitzer 1984</td>
</tr>
<tr>
<td>Turtles</td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Ricknagel and Braunitzer 1988</td>
</tr>
<tr>
<td>Sphenodon</td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Abbasi et al. 1988</td>
</tr>
<tr>
<td>Squamates</td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Duguet, Chauvet, and Acher 1974</td>
</tr>
<tr>
<td>Komodo dragon</td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Naqvi et al. 1983</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Ricknagel, Braunitzer, and Wiesner 1988</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$\alpha$, $\beta$</td>
<td>Abbasi and Braunitzer 1991</td>
</tr>
</tbody>
</table>

* These numbers should be considered minima.
Dawson and Geis 1983) and L. miliaris αD chain (which was termed α(C) by Matsuura, Fushitani, and Riggs [1989] but is called αD in the present paper). In Komodo dragon, the usually conservative threonine (T) at C4 in the heme pocket was replaced by serine (S) in αA, and the common distal histidine (H) at E7 was replaced by glutamine (Q) in αD. The substitution of His(E7) with glutamine has been shown to be a structurally and functionally conservative change in human hemoglobin (Na-gai et al. 1987). In fact, α chains of hemoglobins from several animals have a glutamine at E7, such as the opossum (Stenzel et al. 1979), the frog Ranacatesbeiana (Smith et al. 1993), and, as shown in figure 2, the αD chain from the snake L. miliaris. All the other α chains used in figure 3 have a histidine at E7. From those data, though limited, it is possible that one of two α chains from squamates has a glutamine instead of histidine at the E7 distal position, which implies that the substitution of histidine with glutamine at E7 might have appeared in the lineage of ancestral squamates before the divergence of snakes and lizards.

Phylogenetic Analyses

Figure 3 shows a phylogenetic tree for 19 α-globins including two from V. komodoensis and from representative amniotes. Four groups (EM, SQ, BCST, and MA; see the legend of fig. 3) were strongly supported by both bootstrap analyses of neighbor-joining and maximum parsimony methods. EM group includes α chains from V. komodoensis (αD), L. miliaris D (αD), and the other αD chains, which were first found as an embryonic hemoglobin in birds and persist in adults. Distribution of αD type of globin was thought to be restricted only to birds and turtles (Rüegg and Braunitzer 1984), and it was suggested that gene duplication leading to αA and αD occurred before the divergence of birds and turtles (Rüegg and Braunitzer 1988). Table 1 and figure 3 clearly show that all reptiles except crocodilians have the embryonic type of α chain, αD. Figure 3 shows that αD chains in the group EM are distinct from the α chains in the other groups and form an orthologous family. These results suggest that the embryonic type of α-globin gene was acquired by gene duplication of the ancestral gene before the divergence of amniotes took place. The embryonic α chain of crocodilians may exist, but it has not yet been found, or, alternatively, it is possible that crocodilians have lost the corresponding embryonic α-globin gene.

The amino acid substitution rates of the αD chains from V. komodoensis and L. miliaris in the group EM are larger than those of the other α globins even within the group EM (fig. 3). Such large substitution rates were first described for the αD chain from L. miliaris and were suggested to be caused by a different genetic origin, that is, embryonic, which then was persistently expressed in the adult (Matsuura, Fushitani, and Riggs 1989). However, figure 3 clearly indicates that the reason for higher substitution rates is not that the αD chains are embryonic. It seems that during the evolution of squamates, selection constraints on the embryonic type of α chains became weaker and the rates were accelerated. Further molecular evolutionary analyses at DNA level would be required. A similar acceleration of amino acid substitution rates already has been known for the β chains from crocodilians (Leclercq et al. 1981).

Varanus komodoensis is the first squamate whose two α chains (embryonic and adult) were determined. Figure 3 shows clearly that the αA from V. komodoensis
is clustered with another adult type of $\alpha$ chain from the living squamates and forms the SQ group, whereas $\alpha^D$ chain of V. komodoensis is in the EM group. Therefore, when both of the two $\alpha$ chains from other squamates are sequenced, they will be clustered in the way that the two $\alpha$ chains from V. komodoensis are grouped into the separate groups EM and SQ.

**Phylogenetic Relationships of Amniotes**

The evolutionary relationships among amniotes have been controversial (for review, see Patterson et al. 1993). In particular, the evolutionary positions of mammals and birds have been debated (Dickerson and Geis 1983; Hedges, Moberg, and Maxson 1990; Hedges and Maxson 1991; Van de Peer et al. 1993; Seutin et al. 1994). Whether mammals or crocodilians are the closest to birds among amniotes has seemed to depend on the genes or proteins used (structural genes, rRNA, or mitochondrial DNA) and also on tree-making methods (maximum parsimony or distance). Paleontological and morphological data have suggested crocodilians and birds as sister groups (Donoghue et al. 1989; Patterson, Williams, and Humphries 1993). From the present analyses (fig. 3), phylogenetic relationships in the adult type of $\alpha$ chains showed that those of birds are closely related to those of crocodilians, turtles, and sphenodon. Although the evolutionary relationships among the BCST group are ambiguous, a high bootstrap value (96%) was obtained for this cluster by the neighbor-joining method. Among the embryonic $\alpha$ chains (EM group), the birds/turtle/sphenodon cluster is also well supported (98% for neighbor joining and 70% for maximum parsimony).

**Evolutionary Position of Squamates**

Phylogenetic relationships of the embryonic and adult types of $\alpha$ chains are consistent as to the position of squamates. Within the group EM, squamates, V. komodoensis, and L. miliaris form a cluster distinct from the turtle/sphenodon/birds cluster. For the adult type of $\alpha$ chains, all of squamates except the viper form the SQ group (bootstrap values are 94% and 97% for neighbor joining and maximum parsimony, respectively), whereas all other reptiles and birds form the BCST group. Most of other phylogenetic studies on molecular and paleontological data have suggested that turtles split off first from the ancestral amniotes. Seutin et al. (1994) described phylogenetic trees inferred from five tRNA gene sequences (only stem regions were used) with the neighbor-joining method. In their trees, squamates were located outside a cluster of mammals/crocodilians/turtles/sphenodon/birds. They pointed out the "peculiar nature of tRNA evolution" and concluded that those molecular data cannot resolve the phylogenetic relationships. For amino acid sequences of $\alpha$ chains, we have to consider quantitatively and qualitatively different selection constraints on these peptides in each lineage. Also, there may be parallel amino acid substitutions as we described for histidine (E7) in several different lineages. These could cause ambiguity in phylogenetic reconstruction. However, we would note that both embryonic and adult types of $\alpha$ chains and also two different tree-making methods showed similar relationships between squamates and other reptiles.

The phylogenetic position of the viper V. aspis is not clear. From both neighbor joining and maximum parsimony, the viper did not make any well-supported cluster with other $\alpha$ chains. The bootstrap values for the viper/human/horse cluster are 39% and less than 5% from neighbor-joining and maximum parsimony methods, respectively. On the other hand, the viper/SQ cluster was supported by 31% and 35% from those methods. Therefore, contrary to the consensus phylogenetic trees from both neighbor-joining and maximum parsimony methods, it is possible that the position of the viper is closer to the cluster of other squamates (SQ), but it may have diverged from them very early. Alternatively, the $\alpha$ chain of the viper could be different from other $\alpha$ chains.

**Phylogenetic Relationships Between Sphenodon and Squamates**

The position of sphenodon is also intriguing. Paleontological and morphological studies have shown that sphenodon is the closest relative of snakes and lizards and shares the immediate ancestor with them. However, as shown in figure 3, in both phylogenies of the embryonic and adult type $\alpha$ chains, sphenodon formed clusters with turtles, birds, and crocodilians but not with squamates. Only tRNA data (Seutin et al. 1994) showed similar relationships to our results.

**Evolution of Hemoglobin $\alpha$ Chains**

SQ, BCST, and MA groups in figure 3 represent the adult type of $\alpha$ globin. However, the phylogenetic relationships among these groups are ambiguous. All of the bootstrap values for the nodes between these groups are lower than 40%. The consensus tree from the maximum parsimony method even showed a different relationship among these groups, (Shark, Viper, (SQ, (MA, (BCST, EM))), for which bootstrap values of the nodes between these four groups were also lower than 40%. As mentioned, the sequence of the viper seems different from other squamates. We tried excluding the viper sequence (data not shown). The consensus tree by the neighbor-joining method with bootstrap replications was (Shark, (SQ, (EM, (MA, BCST))), and bootstrap supporting values were 80% for MA/BCST cluster and 37% for EM/MA/BCST cluster. By using the maximum parsimony method, the consensus tree was (Shark, (EM, (SQ, (MA, BCST)))) and all of the bootstrap supporting values between groups were lower than 50%. Again, we could not resolve the relationships between four major $\alpha$ globin groups from this analysis.

These ambiguous relationships among adult type $\alpha$ chains are contrasted with embryonic type $\alpha$ chains, which cluster into the EM group. One possible explanation is that the duplication of $\alpha$ globins into the embryonic and adult types occurred just before the divergence among amniotes. After the duplication, selection constraints on those $\alpha$ chains changed. In particular, adult type $\alpha$ chains have evolved differently among each lineage (MA, SQ, and BCST). Dickerson and Geis
(1983) showed the phylogenetic tree of the globin family based on augmented parsimony (fig. 3.13 in Dickerson and Geis 1983), where two gene duplications in the amniote lineage were described. The first gene duplication was postulated to produce the viper D chain group and the adult type of α chain group. Whether or not the viper sequence is different from other αglobin sequences is still ambiguous and we will have to wait for clarification.

Acknowledgments

We are greatly indebted to Jyunpei Igarashi, NHK Science Program Production, Shibuya-ku Jinnan 2-2-1, Tokyo, Japan 150-01, for providing the blood sample of Komodo dragon. We thank Keiko Oda and Akiko Ono for preparing the figures.

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Mitiko Go, reviewing editor

Accepted May 29, 1996