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The Amino Acid Sequences of the α - and β -Globin Chains of Hemoglobin from the Aldabra Giant Tortoises, *Geochelone gigantea*

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ABSTRACT—Tetrameric hemoglobins (Hbs) A and D were isolated from red blood cells of the Aldabra giant tortoises, *Geochelone gigantea*, by a hydrophobic interaction chromatography. After reduction and S-pyridylethylation, two sets of two types of α -chains (α -1 and α -2) and one β -chain were purified from the major Hb A and minor Hb D in molar ratios of about 1:1:2, respectively, by a reversed-phase column chromatography. The complete amino acid sequences of the three globin-chains from Hb A were determined: 141 amino acid residues for the two α -chains and 146 amino acid residues for the β -chain. Using computer analysis (amino acid maximum homology), the two α -chains shared a 96.5% sequence identity and had low sequence identities (37.8% for α -1 and 35.8% for α -2) with the β -chain of the same species, *G. gigantea*.

We constructed a phylogenetic tree of 28 primary globin structures from *Reptilia* (7 species of squamates, 4 species of turtles, 3 species of crocodiles and 1 species of sphenodontids), including the three globins of *G. gigantea* Hb A. The following results were obtained: (1) The two terrestrial species of *Geochelone* (*G. gigantea* and *G. carbonaria*) were closely related: 139 amino acid residues (95.2%) of the two β -globin chains were conserved; (2) Based on the divergence patterns of globin-chains, the sea turtle *Caretta caretta* was shown to be unusual relatedness from the groups of terrestrial and freshwater species in turtles. The molecular relationships appearing on the phylogenetic tree also support the traditional classification of reptiles and partly confirm previous molecular studies of reptilian hemoglobin evolution.

INTRODUCTION

Hemoglobin, the major respiratory protein, has been extensively investigated in animals, plants, protozoans, fungi and bacteria (Keilin, 1956; Kleinschmidt and Sgouros, 1987). At the molecular level the protein has provided much information in both functional and evolutionary aspects (Bunn and Forget, 1986; Goodman *et al.*, 1988; Vinogradov *et al.*, 1993). In reptiles, to our knowledge, 35 globin-chains from 19 species have been sequenced (Gorr *et al.*, 1998; Kleinschmidt and Sgouros, 1987; Fushitani *et al.*, 1996). Among these studies, there are four investigations on turtle hemoglobin: one for a land tortoise, *Geochelone carbonaria* (Bordin *et al.*, 1997), one for a sea turtle, *Caretta caretta* (Petruzzelli *et al.*, 1996), and two for freshwater turtles, *Chrysemys picta bellii* (Rücknagel *et al.*, 1984) and *Phrynops hilarii* (Rücknagel *et al.*, 1984).

This study aimed to establish the complete primary structures of both types of globins (α and β) from the land tortoises

Geochelone gigantea, and analyze phylogenetic relationships among reptiles including two species of *Geochelone*, *G. gigantea* and *G. carbonaria*. First, the two hemoglobin components (Hb A and Hb D) were separated from *G. gigantea* hemoglobin under native conditions and purified constitutive polypeptide globin-chains from each of the two hemoglobin components for use in analyzing their primary structures. This study provides complete primary structures of the three globins from Hb A of *G. gigantea*. Here, we also describe a phylogenetic tree constructed for 14 complete amino acid sequences of both α -type globins and β -type globins of reptilian hemoglobins, including the two α -globins and one β -globin of *G. gigantea* Hb A. The phylogenetic tree supports previous studies on the classification, phylogeny and molecular evolution of reptiles (Benton, 1990, Fushitani *et al.*, 1996; Gorr *et al.*, 1998).

MATERIALS AND METHODS

Materials

Blood from a male Aldabra giant tortoise, *G. gigantea*, weighing approximately 36 kg, was collected in heparin-Tris-HCl buffer, pH 8.0, at Osaka Municipal Tennoji Zoo where the animal died just before

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bleeding. The animal came from the Aldabra Atoll located only about 350 km from the northern coast of Madagascar and about 600 km away from East Africa's coast. Acetonitrile, ammonium sulfate, ammonium bicarbonate, *tri-n*-butyl phosphine, 4-vinyl pyridine and V8 protease (from *Staphylococcus aureus* strain V8) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Trifluoroacetic acid (TFA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lysyl endopeptidase (*Achromobacter* protease I) was purchased from Wako Pure Chemicals Co. (Tokyo, Japan). Separation columns, Alkyl Superose column HR5/5 and Resource column (3 ml prepackaged with source 15 RPC gel matrix), were purchased from Pharmacia Biotech (Uppsala, Sweden), and placed in a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). All other chemicals and solvents used were of the most purified grade commercially available.

Preparation of hemoglobin solution

Red blood cells were washed three times in 10 vol. physiological saline, lysed with 1 mM Tris-HCl, pH 8.0, and centrifuged at 3000 × g for 15 minutes to remove cell debris. All procedures were done at 4°C. The cell lysate, hemoglobin solution, was aliquoted and stored at -80°C until use.

Separation of hemoglobin components

The hemoglobin solution which had been saturated at 40% by adding 60% saturated ammonium sulfate was subjected to an Alkyl Superose column equilibrated with 60% saturated ammonium sulfate (183 g/ 500 ml) in 50 mM ammonium bicarbonate, pH 8.0. Elution was carried out with a gradient of 60–0% saturated ammonium sulfate in the 50 mM ammonium bicarbonate buffer. The flow rate was maintained at 0.5 ml/min and fractions of protein peaks were collected. The fractions were monitored at 415 and 280 nm by spectrophotometers (Model 115, Gilson and UV-1, Pharmacia Biotech).

Protein modification

Reduction and *S*-pyridylethylation of globins were performed by the method described previously (Friedman *et al.*, 1970). After each reaction, the modified protein was dialyzed against 0.1M ammonium bicarbonate and lyophilized. Finally, the remaining reagents were completely removed from the sample by reversed-phase column chromatography on Resource from water containing 0.1% TFA to 80% acetonitrile containing 0.08% TFA. Flow rates were maintained at 0.5 ml/min. The fractions were monitored at 214 and 280 nm by a spectrophotometer (Model 116, Gilson).

Separation and purification of globin-chains

To separate α -type and β -type globin-chains, the *S*-pyridylethylated hemoglobin was subjected to a Resource column and eluted with a 0.1% TFA buffered gradient to 60% acetonitrile in 0.08% TFA. All fractions were monitored at 214 and 280 nm by a spectrophotometer (Model 116, Gilson). 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).

Enzymatic digestion

Lysyl endopeptidase digestion was performed essentially as described (Jekel *et al.*, 1983). Briefly, samples (15–20 nmoles) of the *S*-pyridylethylated protein were first dissolved in 8 M urea and incubated at 37°C for 30 min, followed by the addition of 0.5 M ammonium bicarbonate at a final concentration of 4 M urea in 0.1 M ammonium bicarbonate. Lysyl endopeptidase digestion of the sample was performed at an enzyme/substrate ratio of 1:30 (mol/mol) for 4 hr at 37°C in 0.1 M ammonium bicarbonate solution, pH 8.2 containing 4 M urea. To obtain overlapping peptides, the sample (20 nmoles) was digested with the V8 protease at a ratio of 1:100 (w/w, enzyme/substrate) for 48 hr at 37°C in a 0.1M Tris-HCl solution, pH 8.5 containing 1 M urea.

Peptide separation

All peptides derived from their parent molecules were separated using a reversed-phase column, Resource, in a 0.1% TFA buffered gradient to 60% acetonitrile in 0.08% TFA. Flow rates were maintained at 0.5 ml/min. All fractions were monitored at 214 nm and 280 nm by a spectrophotometer (Model 116, Gilson). Re-chromatography of selected peptides, when necessary, was performed as previously described (Shishikura *et al.*, 1987).

Sequence determination

Sequence analysis was performed using a Shimadzu gas phase protein sequencer, PPSQ-10, equipped with a PTH-10 amino acid analyzer (Shimadzu Co., Kyoto, Japan). Phenylthiohydantoin (PTH)-derivatives from the sequencer were separated and quantified. PTH-cysteine was detected as pyridylethylated-PTH-cysteine, the elution point of which was determined as described in the manufacturer's manual.

Computer analysis

A multiple alignment program, Clustal W (Thompson *et al.*, 1994), was used in the alignment of 28 primary structures of globins from *Reptilia*. Pair-wise distances among the 28 globin sequences were analyzed using a computer program PROTDIST stored in the PHYLIP package (v. 3.51c; Felsenstein, 1993) under the Kimura-formula option. Based on the pair-wise distances, Neighbor-Joining/UPGMA in NEIGHBOR (Felsenstein, 1993) was used to construct the phylogenetic tree of hemoglobins from *Reptilia*.

RESULTS AND DISCUSSION

Two components of hemoglobins

The red blood cells of the Aldabra giant tortoises, *G. gigantea*, contain two main hemoglobin components, major and minor, which were successfully separated under native conditions (50 mM ammonium bicarbonate, pH 8.0) by use of an Alkyl Superose column HR 5/5 (Fig. 1). Two peaks were detected at 280 nm and 415 nm. They exist at a ratio of about 5:1 based on chromatogram area calculation. This value may vary from 5:1 to 5:3 depending on sample preparation. Braunitzer and coworkers have succeeded in separating two main components, Hb A and Hb D, from the adult Western Painted Turtle (*Chrysemys picta bellii*) by use of polyacrylamide gel electrophoresis under alkaline (pH 8.3) and dissociating conditions (Rücknagel and Braunitzer, 1988). Also other investigators successfully separated two components from sphenodontid hemoglobin on DEAE at pH 8.5, but in this case they added 0.1% mercaptoethanol and 0.1% dithiothreitol to the elution buffer (Abbasi *et al.*, 1988) and lysis buffer of red blood cells (Weber *et al.*, 1989), respectively. Brittain (1988) determined the existence of three carbomonoxy-form hemoglobin components, T1, T2 and T3, from *Sphenodon punctatus* by using DEAE-Sephadex eluted by a gradient of Tris-HCl buffer (pH 8.5) versus Bistris-HCl buffer (pH 5.5). Bonilla *et al.* (1994) used preparative isoelectric focusing and agarose gels with ampholines for separation of intact hemoglobin components. They purified two hemoglobins from the South American snake Riotropical Racer, *Mastigodryas bifossatus*, however, whose protein bands were closely separated with isoelectric points of 8.02 and 8.07, respectively. Hence, the Alkyl Superose column, a kind of hydrophobic interaction col-

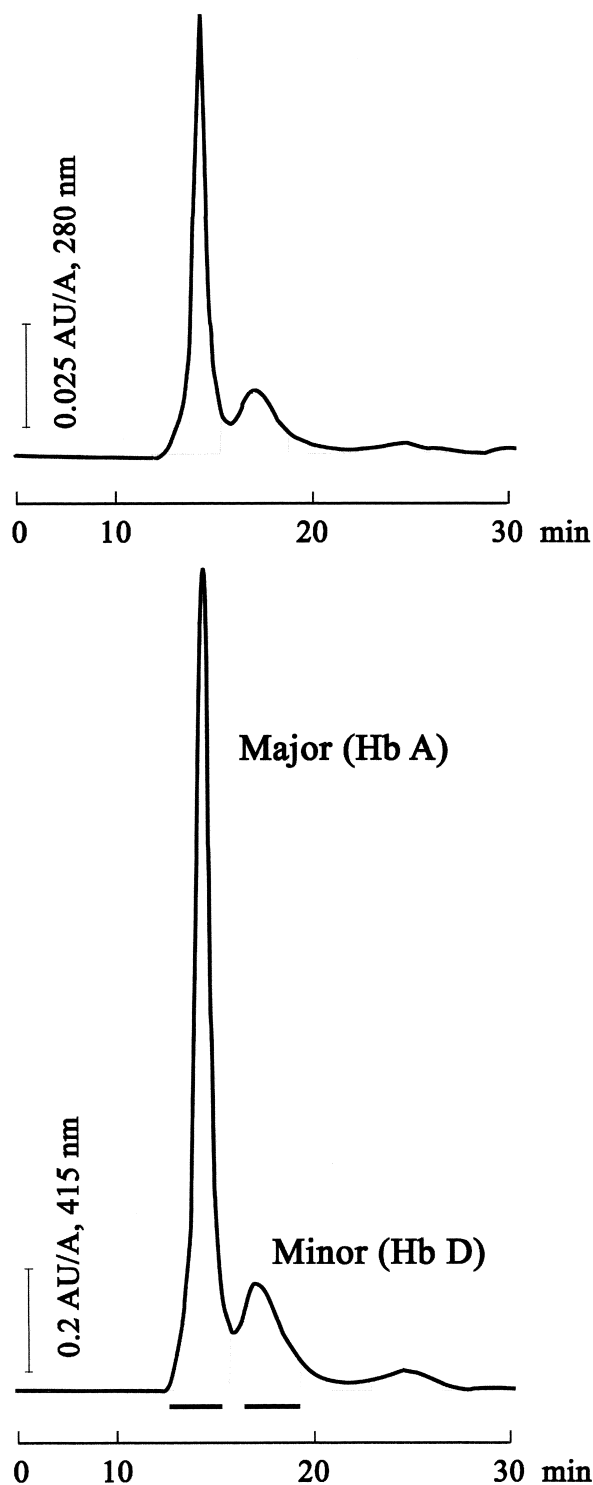


Fig. 1. Alkyl Superose HR5/5 column chromatography of the Aldabra giant tortoises, *G. gigantea*, intact hemoglobin. The hemoglobin solution was saturated at 40% by adding 60% saturated ammonium sulfate and applied to an Alkyl Superose HR5/5 column equilibrated with 60% saturated ammonium sulfate buffer (buffer A). After washing with buffer A, the adsorbed proteins were eluted with linearly decreasing ammonium sulfate concentration from 60% to 0% in 50 mM ammonium bicarbonate, pH 8.0. Elution was monitored at 280 nm (top) and 415 nm (bottom). Flow rate was maintained at 0.5 ml/min. Bars indicate the pooled fractions of major and minor peak, Hb A and Hb D, respectively.

umn, should provide excellent resolution in separation and purification of intact hemoglobin components of *G. gigantea*. Successful separation of intact hemoglobin components enables the manufacture of crystals from individual hemoglobin components for future study of the relationships between physiological functions and crystal structures of hemoglobin components.

Chain separation

After separation of individual components of *Geochelone* hemoglobin, the constituents of the major and minor hemo-

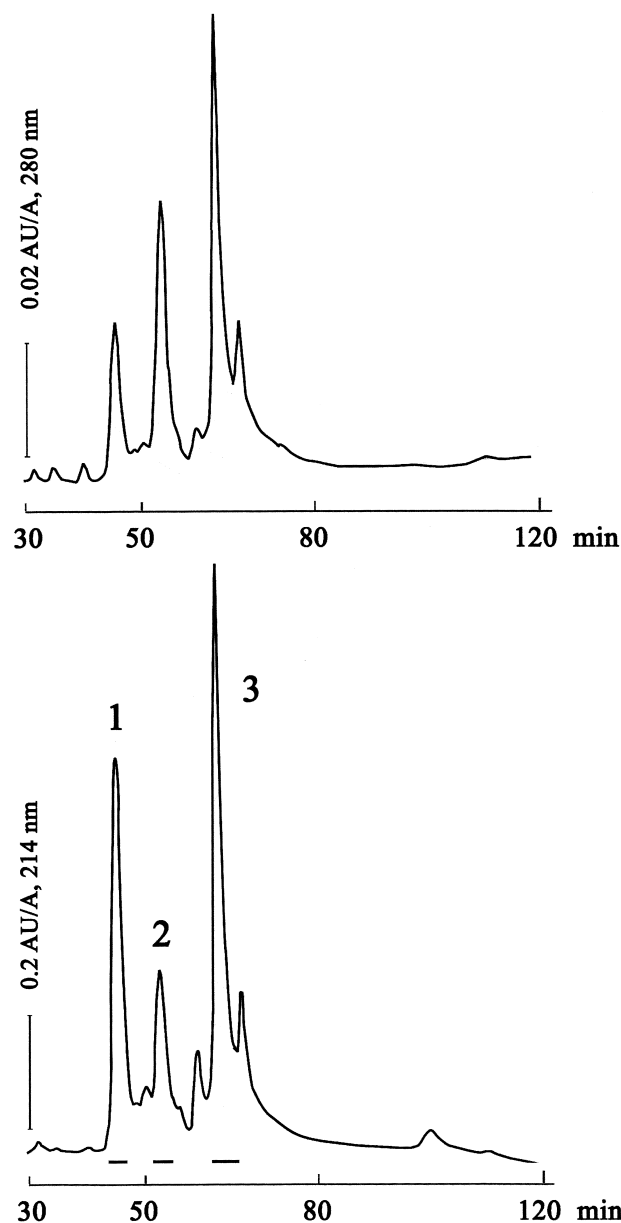


Fig. 2. Separation of globin-chains from reduced and S-pyridylethylated Hb A on Resource column. A linear gradient was used between 0.1% TFA in water and 0.08% TFA in 60% acetonitrile at a flow rate of 0.5 ml/min. Elution was monitored at 280 (top) and 214 nm (bottom). Globins from peaks 1, 2, 3 are α -1, α -2 and β globin-chains, respectively. Bars indicate fractions used for sequencing.

globins could have been further separated under intact conditions by a reversed-phase column using prepackaged Resource resins but their resolution proved to be inadequate. However, after reduction and S-pyridylethylation of the hemoglobin, the major hemoglobin yielded three more widely separated main peaks and several additional minor peaks on a chromatogram obtained by the same reversed-phase column described above. Many investigators have used reversed-phase semi-microbore type columns (Abbasi *et al.*, 1988; Matsuura *et al.*, 1989; Fushitani *et al.*, 1996; Petruzzelli *et al.*, 1996) or CM-columns (Liu, 1975; Leclercq *et al.*, 1982; Rücknagel and Braunitzer, 1988; Rücknagel *et al.*, 1988; Islam *et al.*, 1990; Naqvi *et al.*, 1994) for separation of globin-chains. Our method described here has a similar efficiency and gives excellent resolution in separation at high flow rates with low backpressure (*versus* flow rates). Figure 2 shows a typical separation profile of three main peaks of globin-chains from the major hemoglobin. The advantages to modify the protein by reduction and S-pyridylethylation were also true for separation of globin-chains from the minor hemoglobin (data not shown). As the results, we prepared six individual globin-chains in total from the two hemoglobin components of *G. gigantea*.

Table 1 shows the results of the first 20 amino-terminal amino acid sequences of the six globin-chains. The nomenclature of α - and β -globin chains depends on amino acid sequence similarities to those of the known sequences of reptilian hemoglobins (Kleinschmidt and Sgouros, 1987). Consequently, the major hemoglobin is a kind of Hb A and the minor is Hb D. Two α -types of globins in Hb A or Hb D have identical sequences each other so far sequenced (Table 1), while their patterns on reversed-phase column chromatography are shown distinctly different (Fig. 2, data not shown for those of Hb D). Figure 2 also shows that two kinds of α -types of globin-chains (α -1 and α -2) and one β -type globin-chain are separated at molar ratio of about 1:1:2. This indicates that the Hb A is tetrameric hemoglobin comprised α -1/ α -2/ β ₂. The presence of two subtypes of α -globins is completely confirmed by their primary structures shown in Fig. 3.

In birds (Dolan *et al.*, 1997) and crocodiles (Kleinschmidt and Sgouros, 1987) only one type of β -globin chain has been demonstrated, while lizards and snakes express two types of β -globin chains (Fushitani *et al.*, 1996; Gorr *et al.*, 1998). It is still unknown yet whether the β -globin chains of Hb A and Hb D from *G. gigantea* are identical or not, but peptide maps

digested with lysyl endopeptidase (data not shown) and the first 20 amino-terminal amino acid residues (Table 1) suggest that they might be the same. It was also indicated that the blood of adult Western Painted Turtles, *Chrysemys picta bellii* (Rücknagel and Braunitzer, 1988) were sharing the same β -globin chains when compared with the two complete amino acid sequences of β -types of globin-chains from *Chrysemys* Hb A and Hb D. On the contrary, in frogs there have been reported to present two subtypes of β -globin chains (Knöchel *et al.*, 1983; Patient *et al.*, 1983; Oberthür *et al.*, 1983 and 1986). On the numbers of subtypes of β -type globin-chains among amphibians, reptiles, birds and mammals, thus, reinvestigations are needed, in particular, in view of evolution of Tetrapoda.

Sequencing and alignments

In general, Amniota (reptiles, birds, and mammals) has two or more hemoglobin components (Ikehara *et al.*, 1997; Gorr *et al.*, 1998) which are expressed under different physiological conditions. The presence of α -type (α^D) globin-chain in Hb D is, in particular, of interest in the study of the molecular phylogeny of Amniota because α^D -globin chain was first studied in birds such as chickens (Hagopian and Ingram, 1971; Brown and Ingram, 1974; Kleinshmidt and Sgouros, 1987). The nomenclature of Hb A and Hb D was adopted in Ingram's laboratory (Hagopian and Ingram, 1971; Brown and Ingram, 1974) to describe the various domestic fowl hemoglobins: The embryonic and adult definitive erythrocytes contain the major adult (A) hemoglobin and the minor definitive (D) hemoglobin.

Hb D was also reported in the tuatara *Sphenodon* (Abbasi *et al.*, 1988). As for the presence of Hb D in turtles, it was first found in the adult Western Painted Turtle, *Chrysemys picta bellii* (Rücknagel *et al.*, 1984) and the Hilaire's Sideneck Turtle, *Phrynops hilarii* (Rücknagel *et al.*, 1984). This study describes the presence of Hb D in the Aldabra giant tortoises, *G. gigantea*, and also demonstrates the presence of two subtypes of α -type globin-chains. To ascertain the presence of α^D globin-chains in the Hb D of *G. gigantea*, a study on the primary structures of the α^D globin-chains is in progress (the primary structure of α^D -1 globin chain has been submitted to the JIPID with an accession number PC7116). To date, all reptiles sequenced (Abbasi *et al.*, 1988; Matsuura *et al.*, 1989; Islam *et al.*, 1990; Rücknagel *et al.*, 1988; Abbasi and Braunitzer, 1991; Fushitani *et al.*, 1996) except crocodiles (Leclercq *et al.*, 1981) have been clarified to possess two hemoglobin components, Hb A and Hb D.

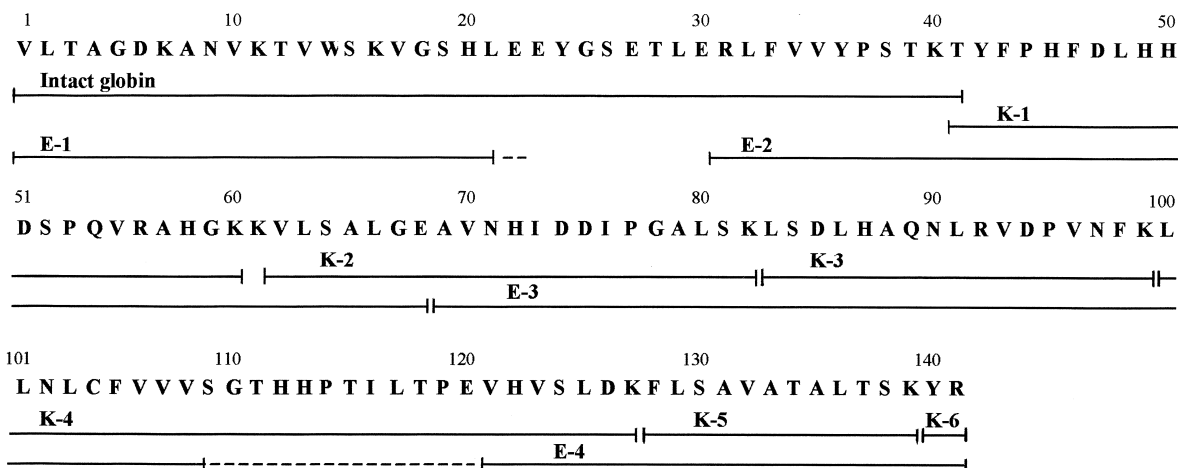
Reptilian phylogenetic tree

Geochelone is a unique group among turtles since it includes two big-size tortoises; *G. gigantea*, the Aldabra giant tortoises, and *G. elephantopus*, the Galapagos giant tortoises. These species may weigh up to 250 kg and measure 150 cm over the curve of their carapaces (Jackson, 1984). The Galapagos tortoises are, in general, known as the world's largest living tortoises. Recently, Bordin *et al.* (1997) have studied

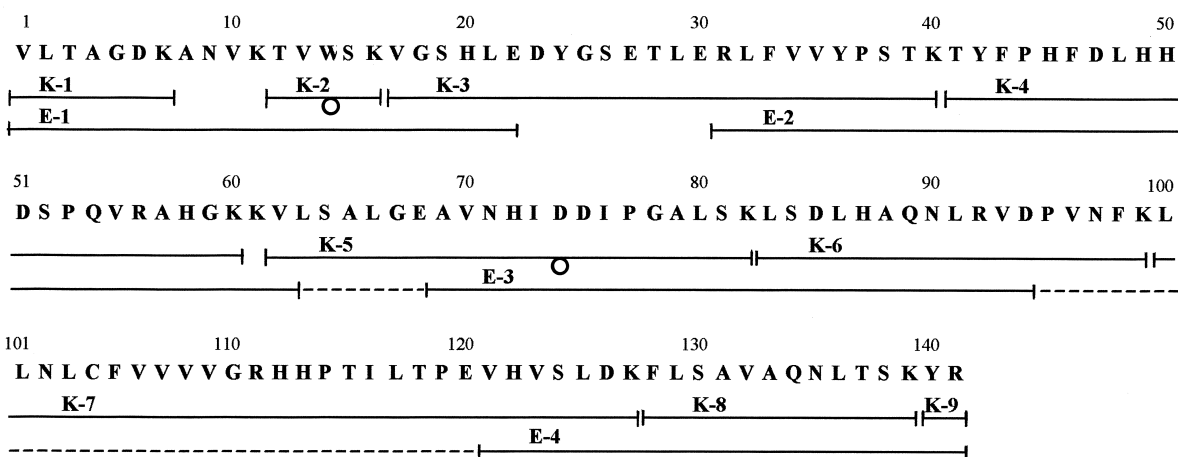
Table 1. Amino-terminal amino acid residues of six globin chains from the Aldabra giant tortoises, *Geochelone gigantea*

		1		10		20															
Hb A	α -1	V	L	T	A	G	D	K	A	N	V	K	T	V	W	S	K	V	G	S	H
	α -2	V	L	T	A	G	D	K	A	N	V	K	T	V	W	S	K	V	G	S	H
	β	V	H	W	T	S	E	E	K	Q	Y	I	T	A	L	Q	W	A	K	V	N
		1		10		20															
Hb D	α -1	M	L	T	E	D	D	K	Q	L	I	Q	H	V	W	E	K	V	L	E	H
	α -2	M	L	T	E	D	D	K	Q	L	I	Q	H	V	W	E	K	V	L	E	H
	β	V	H	W	T	S	E	E	K	Q	Y	I	T	A	L	Q	W	A	K	V	N

Hb A α -1



Hb A α -2



Hb A β

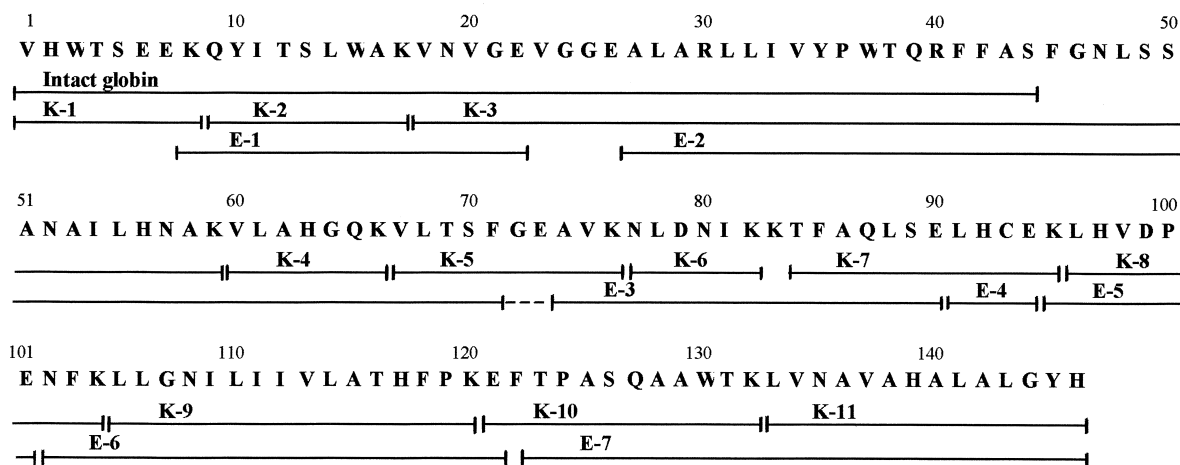


Fig. 3. Strategies and complete sequences of α -1 (top), α -2 (middle) and β (bottom) globin-chains of Hb A from the Aldabra giant tortoises, *G. gigantea*. The complete amino acid sequences of the α -1 (top), α -2 (middle) and β (bottom) globin-chains of *G. gigantea* Hb A are established from overlapping peptides and fragments. The residues marked with continuous lines are those identified by Edman degradation method. Vertical lines represent the beginning and the end of sequencing. Dashed lines indicate the residues not determined but which might be included in the fragment. Small open circles indicate residues incompletely identified using the fragment. Peptide nomenclatures are as follows: lysyl endopeptidase, K; V8 protease, E.

one of the *Geochelone* species, *G. carbonaria*, whose carapace size measures at most about 40 cm, and reported the primary structure of the β -globin deduced from its cDNA analysis. The distribution range of *G. carbonaria* stretches throughout mainland South America: Panama, Colombia, Venezuela, Brazil, Paraguay, and Argentina. Thus, the present habitats of the three *Geochelone* species are remote and isolated from one another. Many questions arise, such as "When did they diversify from their ancestor?", "Is there any correlation between diversity of morphological characteristics and evolution of protein structure?", "How do they differ in their primary structures?", and "How have they adapted protein functions to environmental and physical circumstances?" On these points, comparing the primary structures gives fairly important clues for understanding and elucidating the evolution and improvements of molecular structures of proteins as well as genes.

Figure 3 summarizes the strategies used to establish the complete amino acid sequences of the three globin-chains from Hb A. Appendix 1 provides the data supporting the sequences of Fig. 3. The two α -globin chains are composed of 141 amino acid residues and the β -chain is composed of 146 residues. All overlaps were quantitatively confirmed by duplicated analyses of amino acid residues, with the exceptions of residues 22 through 30 and 109 through 120 for α -1 globin-chain, 23 through 30, 61, 63 through 68, and 95 through 120 for α -2 globin chain, and 72, 73 and 122 for β -globin chain.

The two α -globin chains and the β -globin chain of *G. gigantea* are aligned with those previously reported for reptilian hemoglobins (Appendix 2). When the globin sequences of the two α -chains and the β -chain of *G. gigantea* are compared with those of known sequences, there are 19 invariant amino acids among the 28 globins from reptilian hemoglobins. As for the invariant amino acid residues among the 14

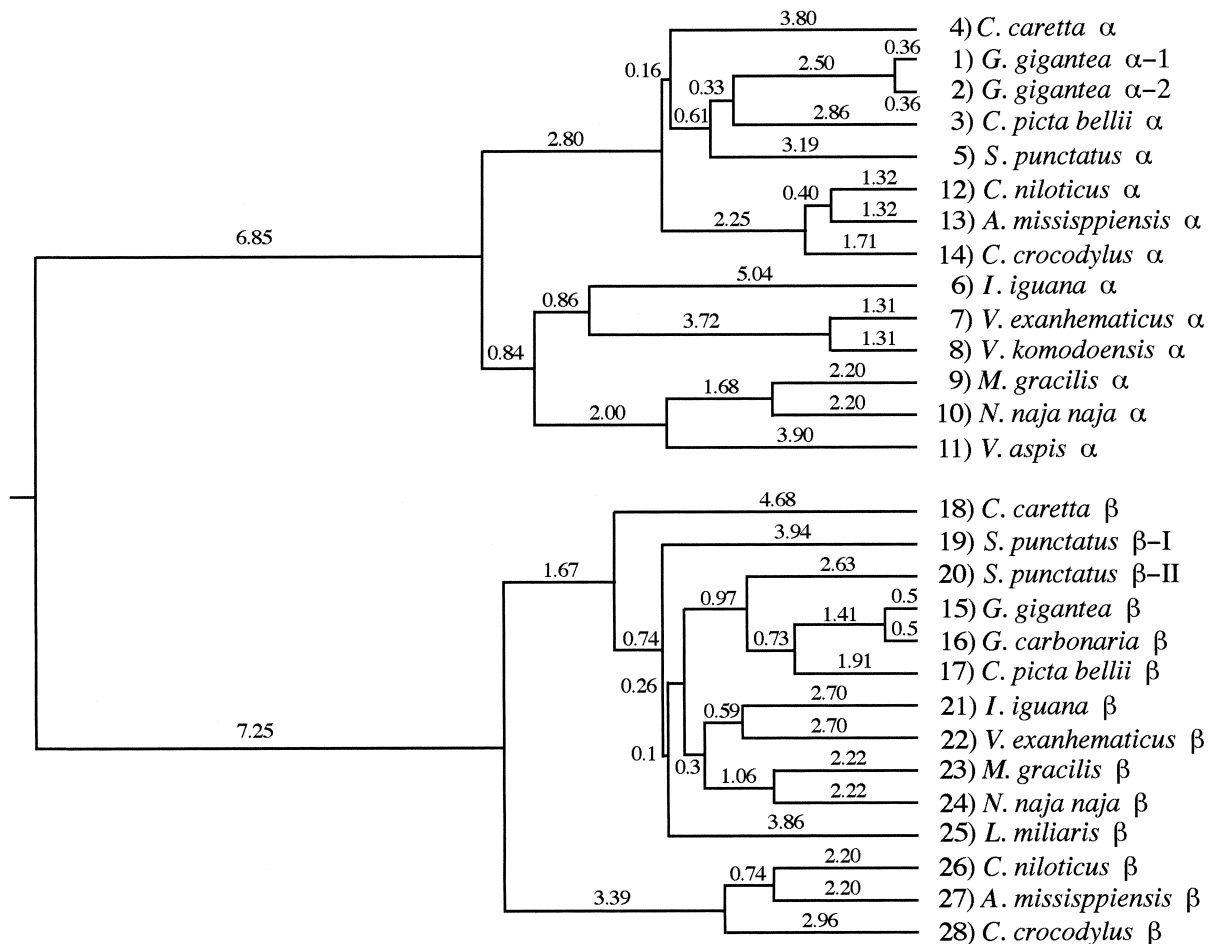


Fig. 4. The phylogenetic tree of 28 reptilian globins. Distance matrices estimated by PRTODIST (option: Kimura formula/data not shown) was used for construction of a rooted tree by NEIGHBOR under the UPGMA method in the package of PHYLIP (version 3.51c: Felsenstein, 1993). Branch lengths are proportional to protein distances and shown on the individual branches of the tree. The abscissa is a time scale in Myr (million years) ago based on the separations of the α - and β -globin chains described by Goodman *et al.*, 1975. The references of globin-chains used in the present analysis are as follows: 1) this study, 2) this study, 3) Rücknagel *et al.*, 1988, 4) Petruzzelli *et al.*, 1996, 5) Abbasi *et al.*, 1988, 6) Rücknagel *et al.*, 1988, 7) Abbasi *et al.*, 1988, 8) Fushitani *et al.*, 1996, 9) Islam *et al.*, 1990, 10) Naqvi *et al.*, 1994, 11) Duguet *et al.*, 1974, 12) Leclercq *et al.*, 1981, 13) Leclercq *et al.*, 1981, 14) Leclercq *et al.*, 1982, 15) this study, 16) Bordin *et al.*, 1997, 17) Rücknagel *et al.*, 1988, 18) Petruzzelli *et al.*, 1996, 19) Abbasi *et al.*, 1988, 20) Brittain, 1988, 21) Rücknagel *et al.*, 1988, 22) Abbasi *et al.*, 1991, 23) Islam *et al.*, 1990, 24) Naqvi *et al.*, 1994, 25) Matsuura *et al.*, 1989, 26) Leclercq *et al.*, 1981, 27) Leclercq *et al.*, 1981, 28) Leclercq *et al.*, 1982.

α -globins and 14 β -globins from reptiles, there are 44 invariant (31.2%) and 41 invariant (28.1%), respectively. When compared sequence similarities of globin chains within a species (*G. gigantea*), the sequence identities of 37.8% (α -1 versus β) and 35.8% (α -2 versus β) were obtained. These resemble the value (42.5% identity) obtained from the comparison with human α - and β -globin chains (Bunn and Forget, 1986). On the contrary, comparing the sequence of the β -globin chain of *G. gigantea* with that of *G. carbonaria*, there are 139 identical amino acid residues. This similarity (95.2%) coincides with the sequence similarity (96.5%) of the two α -globin chains of *G. gigantea*. This finding suggests that the two species are definitely very closely related to each other, and their protein structures, though they are limited, have somehow been conserved even when their morphological characteristics have greatly changed.

Reptilian phylogeny and diversity based on α - and β -globin chains

Phylogenetic analyses of 28 globin-chains including 4 species of Testudinata, 7 species of Squamata (snakes and lizards), 3 species of Crocodylia and 1 species of Rhynchocephalia were conducted by PROTDIST (Felsenstein, 1993). The rooted tree (Fig. 4) deduced by NEIGHBOR under the UPGMA method (PHYLIPS; Felsenstein, 1993) is highly correlated at the level of orders with the reptilian traditional phylogeny established mainly depending on morphological characteristics (Carroll, 1969; Benton, 1990). This partly supports the previous molecular studies on the evolution of reptilian hemoglobins (Goodman *et al.*, 1975; Fushitani *et al.*, 1996; Gorr *et al.*, 1998; Vinogradov *et al.*, 1993). The molecular relationships appearing on our phylogenetic tree are summarized as follows: (1) the two species of *Geochelone* have separated very recently (estimated to be about 17 million years ago): divergence dates are estimated 2.6–4.4 times later than those of the two species of Varanas and the two species of crocodiles (*Crocodylus niloticus* and *Alligator mississippiensis*); (2) the species Sphenodon is closely related to the group of tortoises; (3) the primary structures of β -globin chains from the sea turtle *Caretta* and the sea snake *Liophis miliaris* hemoglobins were, in particular, shown to be unusual relatedness from the group of terrestrial species in turtles and squamates, respectively; (4) in the branches of α -globin chains the squamates (snakes and lizards) diverged from the groups of turtles and crocodiles, but in the branches of β -globin chains the crocodiles first separated from the other groups of reptiles (turtles and squamates).

One of the most interesting objectives is, therefore, determining when and how the Galapagos giant tortoises, *G. elephantopus*, diversified from their sister species, the Aldabra giant tortoises *G. gigantea*. At the present time, the habitats of the two giant tortoises are remote oceanic islands and separated by two continents, Africa and South America, and the Atlantic Ocean. According to our β -globin data, the divergence time of *G. gigantea* and *G. carbonaria* was estimated as 17 myr (million years) ago. This coincides with the recent study

of Caccone *et al.* (1999) who have estimated that the colonization of Madagascar by tortoises occurred in 22–14 myr ago based on tortoises mtDNA sequences. It is not unreasonable to make a scenario that the two living giant species had diversified less than 17 myr ago from their common ancestor and reached oceanic islands, one is volacinc (the Galapagos) and the other is atoll (Aldabra), by rafting, the most reliable way of migration for terrestrial animals. Future studies on globin structures of *G. elephantopus* may well explain the divergence times and molecular relationships of hemoglobins among the three *Geochelone* species.

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Appendix 1. Sequence analyses of peptides obtained by cleavage with lysyl endopeptidase and V8 protease.

Step	<u>Hb A α-1</u>		<u>Hb A α-2</u>			<u>Hb A β</u>		
	Amino Acid (p moles)		Amino Acid (p moles)			Amino Acid (p moles)		
	Intact globin	E-1	K-1		E-1	Intact globin	K-1	
1	V (1650)	V (3281)	V (2099)		V (243)	V (576)	V (2689)	
2	L (1610)	L (2685)	L (2733)		L (272)	H (206)	H (991)	
3	T (666)	T (1189)	T (1174)		T (145)	W (264)	W (1952)	
4	A (1520)	A (2077)	A (2233)		A (252)	T (171)	T (814)	
5	G (1008)	G (1504)	G (1715)		G (160)	S (60)	S (241)	
6	D (1006)	D (2096)			D (116)	E (289)	E (1323)	
7	K (1690)	K (2744)			K (142)	E (349)	E (1460)	E-1
8	A (1185)	A (1690)			A (191)	K (231)	K-2 K (812)	K (1622)
9	N (1092)	N (1598)			N (169)	Q (213)	Q (1643)	Q (1375)
10	V (1048)	V (1414)			V (149)	Y (171)	Y (1231)	Y (1079)
11	K (1438)	K (1720)	K-2		K (189)	I (196)	I (1782)	I (1261)
12	T (464)	T (645)	T (1715)		T (100)	T (85)	T (770)	T (1537)
13	V (1006)	V (1092)	V (2333)		V (137)	S (31)	S (242)	S (218)
14	W (556)	W (693)	N.D.		W (60)	L (160)	L (1377)	L (1050)
15	S (147)	S (179)	S (303)		S (24)	W (50)	W (849)	W (516)
16	K (777)	K (961)	K (1431)	K-3	K (140)	A (179)	A (1208)	A (945)
17	V (665)	V (652)		V (2182)	V (114)	K (49)	K (1019)	K-3 K (780)
18	G (395)	G (437)		G (1589)	G (77)	V (142)		V (1625) V (660)
19	S (86)	S (98)		S (343)	S (18)	N (123)		N (1397) N (625)
20	H (183)	H (214)		H (786)	H (60)	V (148)		V (1738) V (549)
21	L (484)	L (397)		L (1634)	L (62)	G (93)		G (1015) G (332)
22	E (384)			E (1685)		E (103)		E (1387) E (258)
23	E (172)			D (1179)		V (126)		V (1146)
24	Y (266)			Y (1041)		G (83)		G (872)
25	G (211)			G (842)		G (104)		G (882)
26	S (48)			S (200)		E (82)		E (991)
27	E (217)			E (1029)		A (131)		A (1032) A (1119)
28	T (111)			T (412)		L (101)		L (1020) L (922)
29	L (304)			L (755)		A (130)		A (979) A (1476)
30	E (183)	E-2		E (763)	E-2	R (60)		R (944) R (565)
31	R (152)	R (768)		R (723)	N.D.	L (97)		L (852) L (780)
32	L (261)	L (2331)		L (567)	L (232)	L (114)		L (900) L (882)
33	F (157)	F (2060)		F (489)	F (208)	I (61)		I (747) I (569)
34	V (239)	V (2060)		V (475)	V (232)	V (76)		V (706) V (600)
35	V (264)	V (2004)		V (496)	V (206)	Y (38)		Y (558) Y (453)
36	Y (97)	Y (1510)		Y (334)	Y (153)	P (45)		P (505) P (473)
37	P (138)	P (1670)		P (349)	P (174)	W (11)		W (326) W (171)
38	S (27)	S (286)		S (56)	S (37)	T (13)		T (249) T (256)
39	T (57)	T (653)		T (126)	T (94)	Q (45)		Q (373) Q (383)
40	K (89)	K-1 K (1543)	K-4	K (231)	K (194)	R (29)		R (437) R (375)
41		T (763)	T (515)	T (1520)	T (98)	F (51)		F (405) F (395)
42		Y (1690)	Y (740)	Y (2342)	Y (87)	F (70)		F (466) F (398)
43		F (2475)	F (965)	F (2797)	F (103)	A (76)		A (343) A (444)
44		P (1501)	P (679)	P (1796)	P (99)			S (53) S (48)
45		H (460)	H (375)	H (850)	H (58)			F (270) F (319)

46		F (1739)	F (724)	F (1967)		F (74)		G (173)	G (164)
47		D (893)	D (619)	D (1810)		D (102)		N (214)	N (184)
48		L (1451)	L (577)	L (1788)		L (72)		L (177)	L (228)
49		H (451)	H (326)	H (851)		H (56)		S (37)	S (34)
50		H (658)	H (380)	H (1066)		H (61)		S (37)	S (29)
51		D (893)	D (411)	D (1283)		D (71)		A (162)	A (169)
52		S (171)	S (64)	S (211)		S (14)		N (110)	N (111)
53		P (674)	P (295)	P (1372)		P (72)		A (144)	A (163)
54		Q (636)	Q (226)	Q (824)		Q (54)		I (82)	I (82)
55		V (668)	V (268)	V (869)		V (59)		L (90)	L (110)
56		R (527)	R (240)	R (936)		N.D.		H (39)	H (43)
57		A (684)	A (258)	A (798)		A (62)		N (83)	N (85)
58		H (238)	H (122)	H (341)		H (36)		A (88)	A (129)
59		G (405)	G (131)	G (421)		G (46)	K-4	K (14)	K (90)
60		K (443)	K (167)	K (592)		K (57)	V (2182)		V (68)
61	K-2		K (214)		K-5	K (60)	L (2201)		L (81)
62	V (3256)		V (121)		V (2749)	V (45)	A (2052)		A (113)
63	L (3474)		L (105)		L (2873)		H (969)		H (31)
64	S (489)		S (19)		S (459)		G (1171)		G (42)
65	A (2602)		A (95)		A (2381)		Q (1220)		Q (65)
66	L (2452)		L (62)		L (1972)	K-5	K (965)		K (45)
67	G (1693)		G (38)		G (1269)	V (2425)			V (36)
68	E (2120)	E-3	E (22)		E (1948)	E-3	L (2591)		L (42)
69	A (2070)	A (1907)			A (1654)	A (1931)	T (1186)		T (33)
70	V (1820)	V (1171)			V (1453)	V (214)	S (312)		S (4)
71	N (1489)	N (1100)			N (1381)	N (193)	F (1730)		F (19)
72	H (618)	H (454)			H (606)	H (89)	G (1129)		
73	I (1521)	I (970)			I (1227)	I (105)	E (1487)	E-3	
74	D (911)	D (911)			N.D.	D (166)	A(1403)	A (4964)	
75	D (994)	D (968)			D (1046)	D (194)	V (1108)	V (4570)	
76	I (731)	I (791)			I (816)	I (86)	K (901)	K (5937)	
77	P (578)	P (838)			P (667)	P (119)	K-6	N (2636)	N (3659)
78	G (476)	G (628)			G (509)	G (101)	L (2788)	L (3700)	
79	A (635)	A (956)			A (686)	A (118)	D (2238)	D (2842)	
80	L (577)	L (1293)			L (620)	L (118)	N (2286)	N (2972)	
81	S (80)	S (144)			S (90)	S (26)	I (1890)	I (2807)	
82	K (301)	K (1044)	K-3	K-6	K (480)	K (115)	K (1684)	K (4045)	
83		L (956)	L (2787)	L (3202)		L (96)	K-7	K (4113)	
84		S (125)	S (405)	S (351)		S (17)	T (1096)	T (1122)	
85		D (598)	D (1876)	D (1593)		D (66)	F (2400)	F (2071)	
86		L (954)	L (1901)	L (1619)		L (73)	A (2179)	A (1994)	
87		H (262)	H (922)	H (912)		H (39)	Q (1670)	Q (1462)	
88		A (566)	A (1709)	A (1089)		A (70)	L (1911)	L (1253)	
89		Q (298)	Q (1358)	Q (1203)		Q (59)	S (250)	S (173)	
90		N (398)	N (1427)	N (1172)		N (53)	E (1924)	E (297)	E-4
91		L (671)	L (1407)	L (1087)		L (56)	L (1254)		L (1461)
92		R (357)	R (1561)	R (1200)		R 68)	H (578)		H (1110)
93		V (634)	V (1052)	V (1003)			C/pe-Cys		C/pe-Cys
94		D (322)	D (999)	D (1344)			E (1037)	E-5	E (291)
95		P (368)	P (640)	P (1372)			K (979)	K (672)	
96		V (532)	V (648)	V (553)			K-8	L (1064)	
							L (2331)		

97		N (271)	N (585)	N (469)				H (850)	H (567)		
98		F (300)	F (494)	F (415)				V (2029)	V (1121)		
99	K-4	K (482)	K (433)	K (473)	K-7			D (1509)	D (710)		
100	L (2512)	L (472)			L (879)			P (1450)	P (441)		
101	L (2298)	L (597)			L (914)			E (1794)	E (597)	E-6	
102	N (1617)	N (216)			N (758)			N (1350)		N (153)	
103	L (1774)	L (498)			L (740)			F (1234)		F (462)	
104	C/pe-Cys	C/pe-Cys			C/pe-Cys			K (1129)	K-9	K (365)	
105	F (1569)	F (212)			F (606)				L (1695)	L (284)	
106	V (1478)	V (359)			V (585)				L (1550)	L (259)	
107	V (2016)	V (424)			V (692)				G (977)	G (104)	
108	V (2133)	V (451)			V (697)				N (999)	N (74)	
109	S (241)				V (694)				I (647)	I (80)	
110	G (943)				G (360)				L (392)	L (147)	
111	T (483)				R (502)				I (387)	I (54)	
112	H (417)				H (190)				I (563)	I (71)	
113	H (633)				H (257)				V (258)	V (150)	
114	P (834)				P (327)				L (252)	L (162)	
115	T (457)				T (166)				A (237)	A (121)	
116	I (695)				I (244)				T (94)	T (55)	
117	L (784)				L (426)				H (106)	H (37)	
118	T (337)				T (141)				F (137)	F (43)	
119	P (457)				P (176)				P (86)	P (39)	
120	E (541)		E-4		E (235)	E-4	K-10		K (63)	K (44)	
121	V (513)		V (401)		V (195)	V (242)	E (2396)		E-7	E (25)	
122	H (205)		H (144)		H (78)	H (70)	F (1684)		N.D.		
123	V (539)		V (371)		V (284)	V (149)	T (962)		T (1215)		
124	S (46)		S (62)		S (17)	S (24)	P (1487)		P (1081)		
125	L (289)		L (266)		L (95)	L (74)	A (1793)		A (1427)		
126	D (194)		D (338)		D (71)	D (105)	S (316)		S (87)		
127	K (206)	K-5	K (318)	K-8	K (63)	K (125)	Q (1099)		Q (440)		
128		F (842)	F (244)	F (1051)		F (58)	A (1265)		A (681)		
129		L (856)	L (247)	L (1116)		L (83)	A (1201)		A (575)		
130		S (140)	S (51)	S (176)		S (18)	W (797)		W (141)		
131		A (670)	A (326)	A (772)		A (104)	T (407)		T (143)		
132		V (588)	V (166)	V (723)		V (83)	K (490)	K-11	K (318)		
133		A (665)	A (208)	A (1088)		A (77)		L (2011)	L (309)		
134		T (255)	T (103)	Q (1203)		Q (61)		V (1763)	V (183)		
135		A (511)	A (153)	N (1172)		N (51)		N (1325)	N (37)		
136		L (1003)	L (216)	L (1087)		L (41)		A (1803)	A (260)		
137		T (191)	T (68)	T (216)		T (62)		V (1596)	V (159)		
138		S (61)	S (28)	S (69)		S (14)		A (1740)	A (281)		
139	K-6	K (230)	K (116)	K (377)	K-9	K (72)		H (485)	H (60)		
140	Y (2846)		Y (66)		Y (1715)	Y (33)		A (1530)	A (220)		
141	R (698)		R (61)		R (310)	R (39)		L (1522)	L (153)		
142									A (1459)	A (154)	
143	Microheterogeneity:								L (1347)	L (119)	
144	α -1; 104 (L)								G (901)	G (56)	
145	β ;115(L), 116(A), 119(F)								Y (873)	Y (50)	
146									H (96)	H (20)	

Appendix 2. Alignment of amino acid sequences of 28 reptilian globins.

Clustal W (Thompson *et al.* 1994), a multiple alignment program, was used. The invariant amino acid residues are indicated by asterisks. The nomenclatures of globin-chains with a numerical order are the same as Fig. 4.

	1	10	20	30	40	50	60
1) <i>G. gigantea</i> α -1	-VLTAGDKANVKT	VWSKVGSHLEE	---	YGSETLERLRFV	VYPSTKTYFPHFDLHH	-----	DSPQVR
2) <i>G. gigantea</i> α -2	-VLTAGDKANVKT	VWSKVGSHLED	---	YGSETLERLRFV	VYPSTKTYFPHFDLHH	-----	DSPQVR
3) <i>C. picta bellii</i> α	-VLNAGDKANVKAV	VNWVAAHVEE	---	YGAETLERMFTV	YPQTKTYFPHFDLHH	-----	GSAQIR
4) <i>C. caretta</i> α	-VLSGDKANVKS	VWSKVQGHLED	---	YGAETLDRMFTV	FPQTKTYFSHFVH	-----	GSTQIR
5) <i>S. punctatus</i> α	-MLSASDKANVKAI	WSKVCVHAE	---	YGAETLERMFTV	YPSTKTYFPHFDLTH	-----	GSAQVK
6) <i>I. iguana</i> α	-VLTEDDKNHIRAI	WGHVDNNEA	---	FGVEALTRLFL	AYPATKTYFAHFDLNP	-----	GSAQIK
7) <i>V. exanthematicus</i> α	-VLTEDDKNHV	KGLWAHVHIDE	---	IAADALTRMFL	AHPASKTYFAHFDLSP	-----	DNAQIK
8) <i>V. komodoensis</i> α	-VLTEDDKTHV	KTWLVGHVNHAE	---	IAADALTRMFL	AHPTSKTYFAHFDLSP	-----	NSANIK
9) <i>M. gracilis</i> α	-VLTEDDKARVR	VAVVPVSKNAEL	---	YGAETLTRLFA	AHPPTKTYFPHFDLSP	-----	GSNDLK
10) <i>N. naja naja</i> α	-VLTDEDKARVR	ASVWPVSKNAEL	---	YGSETLTRMFA	AHPPTKTYFPHFDLSP	-----	GSNNLR
11) <i>V. aspis</i> α	-VLSSEDDK	NRVRS---	VGKPELPGEY	GSETLTRMFA	AHPPTKTYFPHFDLSS	-----	GSPNLK
12) <i>C. niloticus</i> α	-VLSDDKCNV	KAVWSKVAGHLEE	---	YGAEALERMFC	AYPQTKTYFPHFDLSH	-----	GSAQIR
13) <i>A. mississippiensis</i> α	-VLSMEDKSNV	KAIWKGASGHLEE	---	YGAEALERMFC	AYPQTKTYFPHFDMSH	-----	NSAQIR
14) <i>C. crocodylus</i> α	-VLSSEEDK	SHVKAIWKGAVAGHLEE	---	YGAESLERMFC	AYPQTKTYFPHFDMSH	-----	NSAQIR
15) <i>G. gigantea</i> β	VHWTSEEEKQY	ITSLWAKVNVGEV	----	GEALARLLIV	PWTQRFASF	GNLSSANAILHN	AKVL
16) <i>G. carbonaria</i> β	VHWSCEEKQF	ITSLWAKVNVEEV	----	GEALARLLIV	PWTQRFSS	GNLSSPNAI	LHN
17) <i>C. picta bellii</i> β	VHWTADEKQ	LITSLWAKVNV	EECG----	SEALARLLIV	PWTQRFSS	TGNLSNAE	AILHN
18) <i>C. caretta</i> β	THWTAEE	RHYITSMWDKIN	VAEIG----	GESLARMLIV	PWTQKFFS	DFGNLSS	SSAIMHN
19) <i>S. punctatus</i> β -I	VHWTAE	EKKLLGSLWAKVD	VADIG----	GEALGRLLV	VYPWTQRF	FADGNLSS	ATAICGN
20) <i>S. punctatus</i> β -II	VHWTAE	EKKLVTS	LWTKVNVDECG----	GEALGRLLV	YPWTQRF	SSFGNLS	SSTAICGN
21) <i>I. iguana</i> β	VHWTAE	EKKLITQVW	KIDVAQIG----	GETLACLLV	YPWTQRF	PDFGNLS	NAAICGN
22) <i>V. exanthematicus</i> β	VHWTAE	EKKLICSLW	KIDVGLIG----	GETLAGLLV	YPWTQRF	SFGNLS	SPTAICGN
23) <i>M. gracilis</i> β	VHWSAE	EKKLITGLW	KVDVAEVG----	GATLGKLLV	VPWTQRF	FAHGNLSS	ANAI
24) <i>N. naja naja</i> β	VHWSAE	EKKLITSLW	KVDVPEV	-----	AATLGKMMV	YPWTQRF	FAHGNL
25) <i>L. miliaris</i> β	VHWTAE	EKSATTAIW	KVDVAAIG----	GEALCRL	LIVYPWTQRF	TSFGNLS	NAAIQSNA
26) <i>C. niloticus</i> β	ASFDPHE	KQLIGDLW	HKVDVAHCG----	GEALSRL	LIVYPWKRRY	FENFGD	ISNAQAIMHNEK
27) <i>A. mississippiensis</i> β	ASFDAHE	RKFIVDLW	KVDVACG	-----	ADALSRL	LIVYPWKRRY	FEHFGKMCNAHDILHNSK
28) <i>C. crocodylus</i> β	SPFSAHE	ESLIVDLW	KVDVASC	-----	GDALSRL	LIVYPWKRRY	FEHFGKLDQDVLHNEK
					*	*	*
	70	80	90	100	110	120	130
1)	AHGKKVLSALGEAVNH	IDDIPGALS	KLSDLHAQ	NLRVDPVNF	KLLNLCFVVV	SGTHHPTILT	PEVHVS
2)	AHGKKVLSALGEAVNH	IDDIPGALS	KLSDLHAQ	NLRVDPVNF	KLLNLCFVVV	VGRHPTILT	PEVHVS
3)	THGKKVLTALGEAVNH	IDDLASALS	KLSDLHAQ	TLRVDVNF	KFLNHCFLV	VVAIHQPS	VLTP
4)	SHGKKVMLALGDVAVNH	IDDIATALS	SKLSDLHAQ	ILRVDVNF	KLLSHCLLV	VVARHPTL	FTPDVH
5)	AHGKKVVNAMGEAVNH	LDDMAGALL	KLSDLHAQ	KLVDVNF	KLLAQCF	LVVVG	VHHPAAL
6)	AHGKKVVDALTAQAVNH	LDIPDALAK	LADLHAQ	KLVDVNF	FGLLGHC	ILVTIAAH	NHGPLKAD
7)	AHGKKVANALNQAVNH	LDIKGTL	SKLSDLHAQ	QLRVDVNF	GFRLH	CLEVSI	AAHLHD
8)	AHGKKVANALNQAVNH	LDIGGTL	SKLSDLHAQ	QLRVDVNF	GFRLH	CLEVSI	AAHLHD
9)	VHGKKVIDALTEAVNH	LDVAGALS	KLSDLHAQ	KLVDVNF	QFLGL	CLEVTIA	AHSGG
10)	AHGKKVIDAITEAVNH	LDVAGALS	KLSDLHAQ	KLVDVNF	KLLAH	CLLV	TIAAH
11)	AHGKKVIDALDNAV	EGLDDAVAT	LSKLSDLHAQ	KLVDVNF	KILSQ	LLSTLAN	HNRNPE
12)	AHGKKVFAALHEAVNH	LDLPGAL	CRLSELHA	SLRVDVNF	KFLAQ	CVLVVVAI	IHPGSL
13)	AHGKKVFSALHEAVNH	LDLPGAL	CRLSELHA	SLRVDVNF	KFLAQ	CVLVVVAI	IHPGSL
14)	GHGKKVFAALHDAVNH	LDLALGAL	CRLSDLHA	NLRVDPVNF	KFLSQ	ILVVF	GVHHP
15)	AHGQKVLTSFGEAVK	NLDNIKKTFA	QSELHCE	KLHVDPEN	FKLLGN	ILIVLATH	FPKEFTP
16)	AHGQKVLTSFGEAVK	NLDNIKKTFA	QSELHCE	KLHVDPEN	FKLLGN	ILIVLATH	FPKEFTP
17)	AHGQKVLTSFGEAVK	NLDHIKQTF	ATLSKLH	CEKLHVDPEN	FKLLGN	ILIVLASH	FTKEFTP
18)	EHGQKVLNSFGSAV	KNDHIKETF	ADLSKLH	CETLHVDPEN	FKLLGS	ILIVLAMH	FGKEFTPT
19)	AHGQKVF	TMFGEALKH	LDNLKETF	ASLHCE	DKLHVDPEN	FKLLGN	ILIVLA
20)	AHGQKVF	TMFGEAVK	NLDNIKATY	AKLSELH	CEKLHVDPEN	FKLLGN	ILIVLA
21)	AHGQKVLTSFGDAV	KNDNIKDTF	AKLSELH	CEKLHVDPEN	FKLLGN	ILIVLA	AHFGKDF
22)	AHGQKVLTSFGDAI	KNDNIKDTF	AKLSELH	CEKLHVDPEN	FKLLGN	ILIVLA	ADHFGKDF
23)	AHGQKVLTSFGDAI	KNDNIKDTF	AKLSELH	CEKLHVDPEN	FKLLGN	ILIVLA	AGHFGKDF
24)	AHGQKVF	TAFGDAV	KNPEGVKDTF	AKLSELH	CEKLHVDPEN	FKLLG	QILITV
25)	AHGQKVF	TAFGDAV	KNPEGVKDTF	AKLSELH	CEKLHVDPEN	FKLLG	QILITV
26)	AHGQKVLASFGEAV	CHLDGIRAH	FANLSKLH	CEKLHVDPEN	FKLLG	DIILIVLA	AHYPKDF
27)	EHGQKVLASFGEAV	KHLDNIKGF	FANLSKLH	CEKHFVDPEN	FKLLG	DIILIVLA	AHHPEDF
28)	EHGQKVLASFGEAV	KHLDNIKGF	FAHLSKLH	CEKHFVDPEN	FKLLG	DIILIVLA	GMHHPKDF
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