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The Primary Structure of Hemoglobin D from the Aldabra Giant Tortoise, *Geochelone gigantea*

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ABSTRACT—The complete primary structures of α^D-2- and β-globin of hemoglobin D (Hb D) from the Aldabra giant tortoise, *Geochelone gigantea,* have been constructed by amino acid sequencing analysis in assistance with nucleotide sequencing analysis of PCR fragments amplified using degenerate oligonucleotide primers. Using computer-assisted sequence comparisons, the α^D-2-globin shared a 92.0% sequence identity *versus* α^D-globin of *Geochelone carbonaria*, a 75.2% *versus* α^D-globin of Aves (*Rhea americana*) and a 62.4% *versus* α^A-globin of Hb A expressed in adult red blood cells of *Geochelone gigantea*. Additionally, judging from their primary structures, an identical β-globin was common to the two hemoglobin components, Hb A and Hb D. The α^D-2- and β-globin genes contained the three-exon and two-intron configurations and showed the characteristic of all functional vertebrate hemoglobin genes except an abnormal GC dinucleotide instead of the invariant GT at the 5' end of the second intron sequence. The introns of α^D-2-globin gene were both small (224-bp/first intron, 227-bp/second intron) such that they were quite similar to those of adult α-type globins; the β-globin gene has one small intron (approximately 130-bp) and one large intron (approximately 1590-bp).

A phylogenetic tree constructed on primary structures of 7 α^D-globins from Reptilia (4 species of turtles, 2 species of squamates, and 1 species of sphenodontids) and two embryonic α-like globins from Aves (*Gullus gullus*) and Mammals (*Homo sapiens*) showed the following results: (1) α^D-globins except those of squamates were clustered, in which *Sphenodon punctatus* was a closer species to birds than turtles; (2) separation of the α^A- and α^D-globin genes occurred approximately 250 million years ago after the embryonic α-type globin-genes (π' and ζ) first split off from the ancestor of α-type globin gene family.

Key words: PCR, degenerate primer, nucleotide sequence, intron, exon

INTRODUCTION

Amniota (reptiles, birds and mammals), in general, have two or more hemoglobin components (Brown and Ingram, 1974; Moss and Hamilton, 1974; Lawn *et al*., 1978; Efstratiadis *et al*., 1980; Bunn and Forget, 1986; Fushitani *et al*., 1996; Gorr *et al*, 1998) that are expressed according to the demands of different physiological conditions. Among them, hemoglobin D (Hb D) was first found in birds as a minor component of the embryonic and adult definitive erythrocytes (Hagopian and Ingram, 1971; Brown and Ingram, 1974). Based on functional studies of Hb D, the presence of α^D-globin raises the oxygen affinity and might be one such adaptation of insufficient oxygen supply as observed in the embryonic stages (Dodgson *et al*., 1981; Chapman *et al*., 1982) or extreme hypoxic and even anoxic conditions (Rücknagel and Braunitzer, 1988). On the other hand, the primary structure of α^D-globin of Hb D shows closely resemblance with embryonic hemoglobins (Chapman *et al*., 1982) and thus, the Hb D is of interest for the study of the molecular evolution of Amniota globins because the distribution of the α^D-globin, to date, has been restricted in Aves and Reptilia (Rücknagel *et al*., 1984; Abbasi *et al*., 1988; Rücknagel *et al*., 1988; Matsuura *et al*., 1989; Fushitani *et al*., 1996; Gorr *et al*., 1998; Accession No. AF304335 in GenBank; Shishikura and Takami, 2001), except for Crocodilia (Leclercq *et al*., 1981; Leclercq *et al*., 1982). Most of the studies on globin gene structures have been carried out on birds and mammals (Bunn and Forget, 1986; Kleinschmidt and Sgouros, 1987), however, only one study has been conducted on reptilian α^D-globin cDNA structure from globin mRNA isolated from the red blood cells present in the adult *Geochelone carbonaria* (Accession No. AF304335 in GenBank). In addition to adult α^D-type globins, there are many genes related to α-globins such as embryonic α-like globins termed π'-globin (Chapman *et al*., 1980) for birds and ζ-globin (Aschauer *et al*., 1981) for mammals, all of

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which are important clues for understanding the molecular evolution of α- and α-related globins.

This study describes the primary structures of α^D-2- and β^D-globin of *G. gigantea* Hb D (hereafter the author uses β instead of β^D because the primary structure of β^D-globin prepared from Hb D was definitively shown to be identical when compared with that of β-globin prepared from the *G. gigantea* Hb A) in assistance with nucleotide sequences of the two globin genes of *G. gigantea*, and constructs a phylogenetic tree concerning the molecular evolution of α^D-type globins. The tree also shows the relationships of α- and embryonic α-related globins, π'- and ζ-globin, as well as a few representatives of α^A-type globins from vertebrates. This study first describes the genomic structures of globins amplified by PCR with degenerate primers, and then, the nucleotide sequences, to ascertain the amino acid sequences of α^D-2- and β-globin. During the course of this study, it was also demonstrated that an identical β-globin was shared in both Hb A and Hb D as predicted in the previous study (Shishikura and Takami, 2001).

MATERIALS AND METHODS

Materials

Hb D from the Aldabra giant tortoise, *G. gigantea*, was prepared as described in the previous study (Shishikura and Takami, 2001).

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). Separation columns, Alkyl Superose column HR5/5 and Resource column (prepackaged with 3 ml source 15 RPC gel matrix), were purchased and placed in a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Upsala, Sweden). Lysyl endopeptidase (*Achromobactor* protease I) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Taq DNA polymerase and GenElute Agarose Spin Columns were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). DNA molecular standard markers, pHY Marker (Takara Shuzo Co., Ltd., Shiga, Japan) and 100-bp DNA Ladder (New England Biolabs Inc., MA, USA) were used. Sequencing primers, M13 forward 17-mer (5'-GTA AAA CGA CGG CCA GT-3') and PUC/M13 reverse 17-mer (5'-CAG GAA ACA GCT ATG AC-3'), were obtained from Sigma-Aldorich Co. and Promega Co. (Madison, WI, USA), respectively. A BigDye Terminator Cycle Sequencing Ready Reaction Kit was purchased from Perkin-Elmer Japan Co. Ltd (Tokyo, Japan).

All other chemicals and solvents used were the most purified grade commercially available.

Globin-chain separation

The Hb D was modified by reduction and *S*-pyridylethylation (Friedman *et al*., 1970) and then directly applied on a reversed-phase column (Resource column), which had been equilibrated with a 0.1% TFA solution. Removal of unincorporated reagents bound on the Resource column could be achieved by washing with an excess amount of 0.1% TFA solution until the base line was below 0.05 at 280 nm. The globin-peptides were, then, eluted from the column by a linear gradient with 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).

Enzymatic digestion and peptide separation

Lysyl endopeptidase digestion was performed essentially with modifications of Jekel *et al* (1983), the details of which were previously described (Shishikura and Takami, 2001). To obtain overlapping peptides, the globin (about 10 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.

All peptides derived from the parent molecules were separated using a reversed-phased column, Resource, in a linear gradient with 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). When necessary, rechromatography of selected peptides was performed as previously described (Shishikura *et al*., 1987).

Amino acid sequencing

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.

Isolation of genomic DNA

Prior to DNA extraction, fixed-tissue samples (80–120 mg) in absolute alcohol were dissolved in 600 μl DNA extraction buffer (10 mM Tris, 10 mM EDTA, 150 mM NaCl, pH 8.0) in a micro-centrifuge tube to obtain wet forms. Samples were treated with SDS (final concentration: 0.4%) and proteinase K (final concentration: 20 mg/ml), mixed well, and incubated for 60 min at 55°C, followed by overnight incubation at 37°C. The extraction of DNA was performed by the procedure described by Sambrook *et al*. (1989) with minor alterations: two rounds of precipitation with ethanol and spooling the precipitate purified DNA. DNA was then resuspended in 1 ml of TE buffer (10 mM Tris/HCl buffer containing 1 mM EDTA, pH 8.0) and stored at 4°C: about 0.1 mg/ml of high-molecular-weight genomic DNA was obtained, as evaluated by the absorption spectrum and by 0.8% agarose gel electrophoresis.

Primers design

Degenerate primers were designed based on the amino acid sequences of lysyl endopeptidase digested fragments of parent molecules. In order to sequence the PCR amplified fragments with a BigDye Terminator Cycle Sequencing Ready Reaction Kit, the degenerate oligo-nucleotide primers were tailed with the M13 forward or M 13 reverse sequencing primer tail (for the tail sequences shown above). A list of degenerate primers used in PCR amplifications is shown in Table 1.

PCR conditions

The PCR amplifications were performed in a 25-μl volume containing about 100 ng of genomic DNA template, 3 to 30 pmoles of each degenerate primer, deoxynucleotide triphosphates (400 μM) and 1.25 U of *Taq* DNA polymerase in the buffer conditions recommended by the manufacturer, 2.5 mM $MgCl_2$. The reactions started with denaturation at 95°C for 3 min, followed by 45 cycles and ended with 7 min of extension at 72°C on a DNA Thermal Cycler 9700 (Perkin-Elmer, Norwalk, CT, USA). The first five cycle profile began with denaturation for 1 min at 95°C, 5-stepwise different annealing temperatures (65°C, 62.5°C, 60°C, 57.5°C and 55°C) for 10 sec each, and ended with elongation for 1min every cycle at 72°C. The thermal profile including denaturation of the first 5 cycles modified the procedures described by Sachadyn *et al*. (1998), Skantar and Carta (2000), and Don *et al*. (1991). The remaining cycles were programmed according to the method recommended by the manufacturer.

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Agarose gel electrophoresis

A 1.5% agarose gel was used to examine the purity and the size range of the PCR products amplified from the *Geochelone* genomic DNA. In each lane, except lanes of DNA-markers, 10 μl of each of the amplified DNA samples were loaded. The two DNA molecular weight standard markers were used. The gel was run in TBE (Tris-Borate-EDTA) buffer at 110V for 50 min. The results were then recorded using a KODAK Electrophoresis Documentation and Analysis System 290 (EDAS 290), and analyzed by a 1D Image Analysis Software (v. 3.5.4; Eastman Kodak Co., Rochester, NY, USA).

Extraction of PCR products and nucleotide sequencing analysis

After trimming away excess agarose, the gel slices (<500 μg) containing the PCR products were placed into the GenElute Agarose Spin column and centrifuged for 10 min at 14,000 x g. The filtrate was concentrated by Microcon-100 and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with the following modifications to the manufacturer's recommended protocol: 3.6 picomoles of M13 sequencing primer (forward or reverse) were annealed with about 32 ng of PCR product by mixing primer and template with 8 μl of Terminator Ready Reaction Mix in a final volume of 20 μl. This mixture was placed in a GeneAmp PCR system 9700 and subjected to cycle sequencing depending on the manufacturer's recommended protocol: start with heating for 10 sec at 96°C, and then 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min and reactions allowed to end with rapid thermal ramp at 4°C. Purifying extension products and the removal of unincorporated dye terminators in sequencing reactions were subjected to Centri-Sep spin columns (Princeton Separations P/N CS-901). Sequences of the PCR fragments were determined for both strands with the BigDye Primer Cycle Sequencing Ready Reaction Kit and the samples were on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Japan Co. Ltd. Tokyo).

Computer analysis

A multiple alignment program, Clustal W (Thompson *et al*., 1994), was used in the alignment of reptilian and other vertebrate's globin primary structures. Pair-wise distances among the 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 globins. Pair-wise alignments of DNA sequences were carried out using softwares of DNASIS as well as DNA Strider (V. 1.0.1).

RESULTS AND DISCUSSION

Globin isolation

In a preceding paper (Shishikura and Takami, 2001) we have described the isolation of the two hemoglobin components of the Aldabra giant tortoise *G. gitantea*, in which the two were designated as Hb A and Hb D. The nomenclature of Hb A and Hb D was adopted in Ingram's laboratory (Hagopian and Ingram, 1971; Brown and Ingram, 1974) where the various domestic fowl hemoglobins were defined. Among them, the adult definitive erythrocytes contained the major adult hemoglobin (Hb A) and the minor definitive hemoglobin (Hb D). After establishing the complete amino acid sequences of globins as described below, the presence of Hb D in the Aldabra giant tortoise, *G. gigantea*, was completely confirmed when compared with the known primary structures of α^D-globins (Kleinshmidt and Sgouros, 1987) specific to the Hb D. The advantage of modifying the protein by reduction and *S*-pyridylethylation also applied for separation of globin-constituents from the Hb D. As the results, three major fractions, α-1, α-2 and β in the order of elution, were separated as shown in Fig. 1: the two peaks, α-1 and α-2, were identical to each other having characteristics of α^D-type globins so far sequenced until the first 20 N-terminal amino acid residues, but in contrast their chromatograms on reversed-phase column were shown as distinctly different. There might be sequence microheterogeneity of their primary structures as found in those of Hb A (Shishikura and

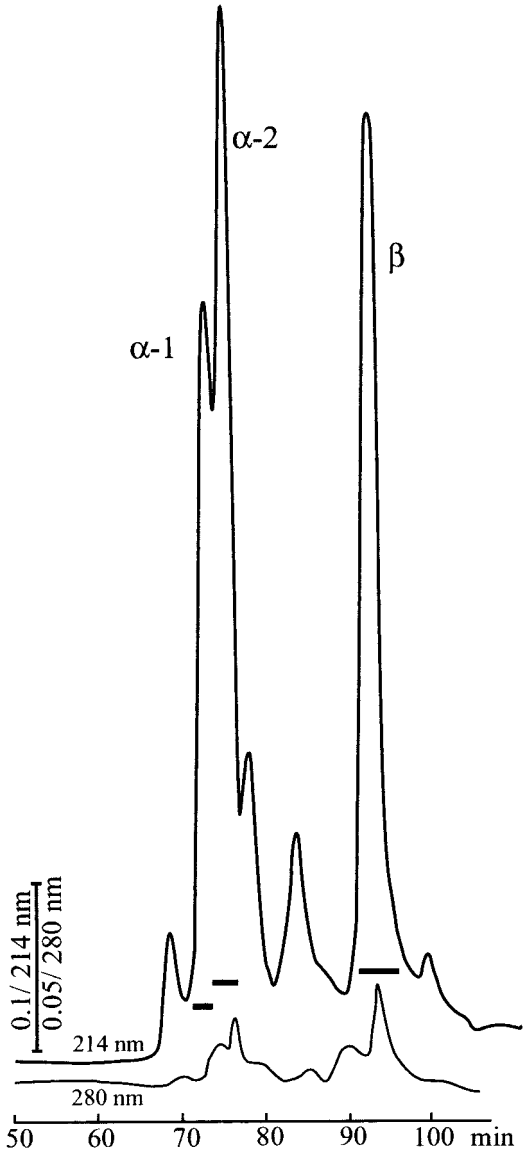


Fig. 1. Separation of globin-constituents from reduced and *S*-pyridylethylated *G. gigantea* Hb D (about 2 mg) on Resource column. A linear gradient was used between 0.1% TFA in water and 60% acetonitrile in 0.08% at a flow rate of 0.5 ml/min. Major peaks are designated as α-1, α-2 and β, respectively. Bars indicate fractions used for sequencing.

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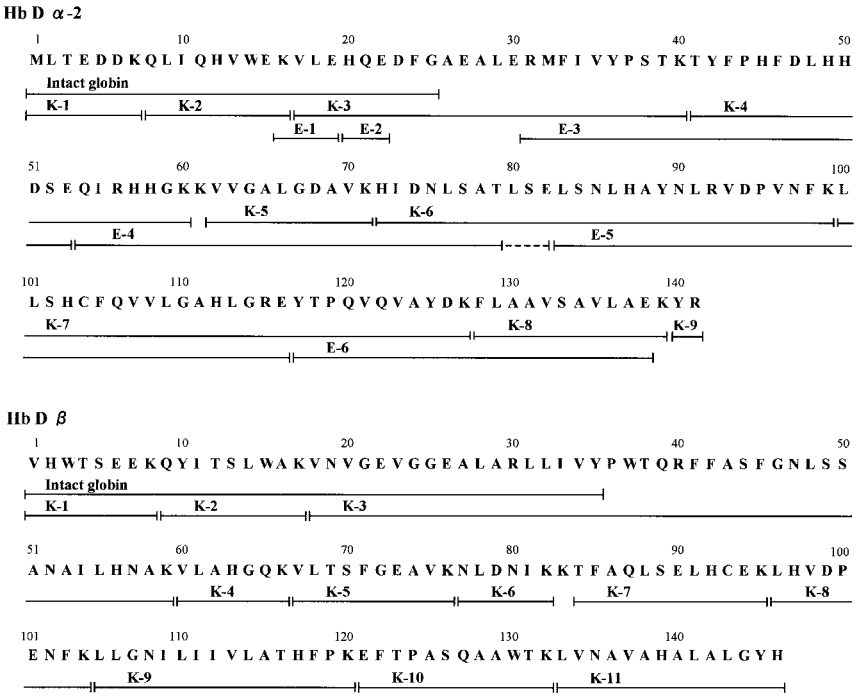


Fig. 2. Strategies and complete amino acid sequences of α-2 (*top*) and β (*bottom*) globins of Hb D from the Aldabra giant tortoise, *G. gigantea*. The complete amino acid sequences of the α-2 (*top*), and β (*bottom*) globins of *G. gigantea* Hb D have been established. Fragments generated by cleavage with lysyl endopeptidase and overlapping peptides obtained by *V8* protease are used for the amino acid sequence determination. 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. Peptide nomenclatures are as follows: lysyl endopeptidase; K, *V8* protease; E.

Takami, 2001). Hence, the author first sequenced the α-2-globin from the two α-types of globins.

Sequence strategies

For establishing complete primary structures of α-2- and β-globin, two sequencing methodologies, protein and DNA sequencing, were carried out. First, the parent molecules and their peptide fragments were sequenced and aligned tentatively with the assistance of sequence similarities toward the known primary structures of reptilian α- and β-globins, in particular, those obtained from the *G. gigantea* Hb A (Shishikura and Takami, 2001). Fig. 2 shows the results of amino acid sequence analyses of α^D-2- and β-globin. Appendix provides the data supporting the amino acid sequences in Fig. 2. The α^D-2-globin chain was composed of 141 amino acid residues and the β-globin chain was composed of 146 residues. Two lysine-lysine residues appeared in positions 60-61 of α^D-2-globin chain and 82–83 in β-globin chain were difficult to determine by analyzing the peptide fragments derived from digestions with lysyl endopeptidase. To complete the primary structure, peptide fragments containing the lysine-lysine residues generated by another enzymatic digestion such as *V8* protease are required to be sequenced. This was done in the construction of α^D-2-globin structure (Fig. 2, *top*) but required time-consuming work. To cope with time-consuming problems in

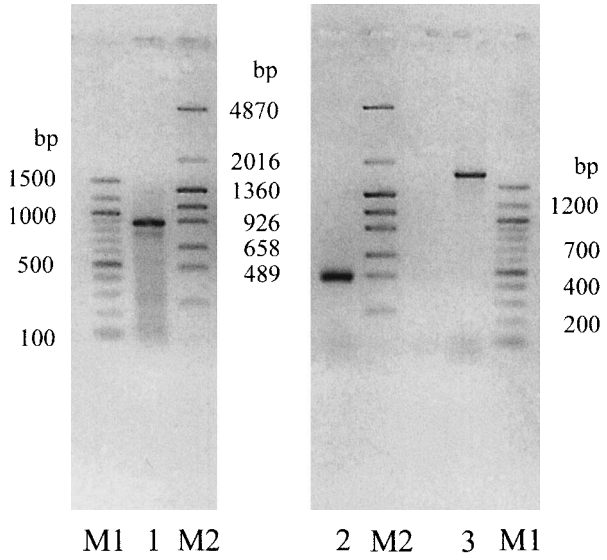


Fig. 3. Agarose gel electrophoreses of PCR products amplified from *G. gigantea* genomic DNA using degenerate primers. *Lane 1*; an 870-bp fragment amplified with degenerate PCR primers M13a-1 and M13a-2, *Lane 2*; a 480-bp fragment amplified with degenerate PCR primers M13b-1 and M13b-2, *Lane 3*; 1.75-kbp fragment amplified with degenerate PCR primers M13b-3 and M13b-4, *Lanes M1* and *M2*; DNA molecular standard markers, 100-bp DNA Ladder (M1) and pHY Marker (M2)

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determining primary structures, the following methods were used: (1) based on sequencing information of both intact globins and digested fragments, degenerate oligo-nucleotide primers were synthesized with a M13 forward or M13 reverse sequencing primer tail; (2) using these primers (forward and reverse), a target gene was amplified by PCR from genomic DNA as a template; (3) the PCR fragment was purified and sequenced by cycle sequencing with the M13 forward or reverse sequencing primer. Fig. 3 shows amplified fragments on agarose gel electrophoresis: An 870-bp fragment was generated from the PCR-amplification of genomic DNA using primers M13a-1 and M13a-2, assuming amplified complete coding regions (three exons) and interviewing regions (two introns), and the remaining, 480-bp fragment and 1.75-kbp fragment, were amplified using primer-sets of M13b-1/M13b-2 and M13b-3/M13b-4, respectively; (4) nucleotide sequences of the three PCR fragments were determined with manufactured M13 sequencing primers, the BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 310 Genetic Analyzer; (5) finally, both protein and DNA sequencing data were complementary combined to establish complete structures of the α-2- and β-globin chains of *Geochelone* Hb D. As shown in Fig. 2 and Table 2 (2A and 2B), the two primary structures reinforced each other by the two different methods.

In comparison with the structural data of β-globin of Hb A (Shishikura and Takami, 2001), the primary structure of β-globin derived from the Hb D was completely identical, indicating that the β-globin was common in the construction of the two adult hemoglobin components, Hb A and Hb D. This finding supports the studies of Rücknagel and Braunitzer (1988) who described that the red blood cells shared the same β-globin chains in Hb A and Hb D. The sharing of identical β-globin chains has also been demonstrated in crocodiles (Leclercq *et al*., 1981; Leclercq *et al*., 1982), while lizards and snakes express two adult β-types of globins (Rücknagel *et al*., 1988; Matsuura *et al*., 1989; Abbasi and Braunitzer, 1991; Naqvi *et al*., 1994; Fushitani *et al*., 1996; Gorr *et al*., 1998). In this context, adult mammals (Braunitzer *et al*., 1961; Leclercq *et al*., 1981) and birds (Rücknagel *et al*., 1984; Oberthür *et al*, 1983; Oberthür *et al*., 1986) have been reported to have one kind of β-globin, but adult frogs (Knöchel *et al*., 1983; Patient *et al*., 1983) contained two subtypes of β-globin chains. Due to an inconsistency in the number of subtypes of adult β-type globin-chains among amphibians, reptiles, birds and mammals, reinvestigations are needed, especially, in regards to the evolution of Tetrapoda (Benton, 1990; Hardison, 1998).

Comparison of the primary structure of α-type globins within *G. gigantea*, α^D-2-globin of *G. gigantea* differs from α^A-globin in 53 amino acid residues (62.4% identity), but when compared with homologous globin chains found in adult *Geochelone carbonaria* (a different species of tortoises) and adult *Rhea Americana* (a species of birds), only 7 (95.0% idnetity) and 35 (75.2% identity) amino acid residues were substituted, respectively.

PCR amplification of globin gene by degenerate primers

Two degenerate oligo-nucleotides (M13a-1 and M13a-2 in Table 1) which were designed from the regions of N-terminal (8 amino acid residues in length) and C-terminal (8 amino acid residues in length) of α^D-2-globin successively amplified a PCR-product with 870-bp estimated by migration distance on agarose gel electrophoresis (Fig. 3, *lane 1*). On the contrary, in the case of amplification of β-globin using M13b-1 and M13b-4 primers no product was observed on agarose gel electrophoresis, indicating that the whole coding region of β-globin gene was impossible to amplify at once using two degenerate primers designed by its N-terminal and C-terminal amino acid sequences. It seems to be difficult to amplify extremely long nucleotides such the case over 1.75-kbp PCR-fragment. Hence, several sets of sense and anti-sense degenerate primers were synthesized and used for amplification of β-globin gene in total with the genomic DNA: the two sets of sense and anti-sense primers (M13b-1/M13b-2 and M13b-3/M13b-4 shown in Table 1) produced a single fragment in each PCR, in which nucleotide-sized fragments were determined to be a 480-bp fragment and a 1.75-kbp fragment, respectively (Fig. 3, *lane 2* and *3*). Both products and the 870-bp fragment of α^D-2-globin gene were sequenced from both sides and aligned by computer-assisted programs. Table 2A and 2B show alignments of nucleotide sequences in encompassing whole

Table 1. Oligo-nucelotide primers used in this study

Gene	Primer Name	Nucleotide sequences																								Reference	Degeneracy
		1									10										20						(fold)
Hb D α-2	M13a-1(Forward)	M13-	A	T	G	Y	T	N	A	C	N	G	A	R	G	A	Y	G	A	Y	A	A	R	C	A	N-terminal	512
	M13a-2(Reverse)	M13-	A	A	Y	T	T	R	T	C	R	T	A	N	G	C	N	A	C	Y	T	G	N	A	C	C-terminal	1024
		1									10						20				20						
Hb D β	M13b-1(Forward-1)	M13-	G	T	G	C	A	C	T	G	G	A	C	Y	W	S	N	G	A	R	G	A	G	A	A	G	N-terminal	64
	M13b-2(Reverse-1)	M13-	C	T	T	G	A	A	G	T	T	C	T	C	R	G	G	R	T	C	C	A	C	R	T	G	104-97	8
	M13b-3(Forward-2)	M13-	C	A	Y	G	T	G	G	A	Y	C	C	Y	G	A	G	A	A	C	T	T	C	A	A	G	97-104	8
	M13b-4(Reverse-2)	M13-	G	T	G	G	T	A	V	C	C	S	A	G	R	G	C	C	A	G	R	G	C	R	T	G	C-terminal	48

M13 forward sequence: 5'-GTA AAA CGA CGG CCA GT-3'
M13 reverse sequence: 5'-CAG GAA ACA GCT ATG AC-3'
The International Union of Pure and Applied Chemistry Symbols used to denote multiple nucleotides are as follows: N=A+G+C+T; R=A+G; S=C+G; V=A+G+C; W=A+T; Y=C+T.

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Table 2A. Nucleotide sequences of three exons and exon-intron boundaries of α^D-2-globin gene

Exon-1	1	ATG	CTA	ACA	GAG	GAC	GAC	AAG	CAG	CTG	ATC	CAA	CAT	GTG	TGG	GAG
	46	AAG	GTG	CTG	GAG	CAC	CAG	GAG	GAC	TTT	GGG	GCC	GAG	GCC	CTG	GAG
	91	AG														
Intron-1	1	gta	ggg	ccc	ggg	gca	ggc	ggc	ccg	ggc	gca	ggg	tgc	agg	gag	gaa
		(46-168 not shown)														
	169	tgg	ggg	act	ttg	gga	ttc	act	gtc	tct	gac	ctc	cct	ccc	ccg	cag
Exon-2	1	G	ATG	TTC	ATC	GTC	TAC	CCC	TCC	ACC	AAG	ACC	TAC	TTC	CCC	CAC
	44	TTC	GAC	CTG	CAT	CAT	GAC	TCG	GAA	CAG	ATC	CGC	CAC	CAC	GGC	AAG
	89	AAG	GTG	GTG	GGC	GCC	CTG	GGG	GAC	GCC	GTG	AAG	CAC	ATC	GAC	AAC
	134	CTC	AGC	GCG	ACG	CTC	TCC	GAG	CTC	AGC	AAC	CTG	CAC	GCC	TAC	AAC
	179	TTG	CGC	GTG	GAC	CCG	GTC	AAC	TTC	AAG						
Intron-2	1	gca	agt	gca	ggc	tac	ggc	cag	gaa	gag	ttc	ccg	ggg	ggt	gcg	gga
		(46-181 not shown)														
	182	ggc	cga	ggg	ctg	gct	gcc	gct	gac	cca	gtg	cac	ttt	gct	ttg	cag
Exon-3	1	CTG	CTG	TCC	CAC	TGC	TTC	CAG	GTG	GTG	CTG	GGC	GCG	CAC	TTG	GGC
	46	CGC	GAG	TAC	ACC	CCG	CAG	GTG	CAA	GTC	GCC	TAT	GAC	AAG	TTC	CTG
	91	GCC	GCC	GTC	TCG	GCG	GTG	CTG	GCT	GAG	AAG	TAC	CGG			

Table 2B. Nucleotide sequences of three exons and exon-intron boundaries of β-globin gene

Exon-1	1	GTG	CAC	TGG	ACC	AGC	GAG	GAG	AAG	CAG	TWC	ATT	ACC	AGT	CTG	TGG
	46	GMC	AAG	GTC	AAC	GTG	GRG	GAA	GTG	GGT	GGC	GAA	GCC	CTG	GCC	AG
Intron-1	1	gta	ggc	tcg	agc	ctc	aca	tgg	ata	tct	gcc	tcg	cat	tgc	tcc	tct
		(approximately 40 nucleotides not shown)														
		gca	gta	acc	ctg	tgt	ctg	tct	ctg	ctc	ctg	tct	ccc	tct	ctc	tag
Exon-2	1	G	CTG	CTG	ATC	GTC	TAC	CCC	TGG	ACC	CAG	AGG	TTT	TTC	KCT	TCC
	44	TTT	GGG	AAC	CTS	TCC	AGC	SCC	AAC	GCC	ATC	MTG	SRC	AAC	GCC	AAG
	89	GTG	STT	GCC	CAT	GGC	MAG	AAA	GTG	CTG	ACC	TCS	TTT	GGG	GAA	GCT
	134	GTG	AAG	AAC	CTG	GAC	AAC	ATC	AAG	RMM	ACG	TWC	GCC	CAG	CTG	AGC
	179	GAG	CTG	CAC	TGC	SAR	AAG	CTG	CAT	GTG	GAT	CCT	GAG	AAC	TTC	AAG
Intron-2	1	gtg	agt	ccg	gct	ctg	ggt	tga	ccc	tct	tcc	cag	ccc	cct	ttc	cat
		(approximately 1590 nucleotides not shown)														
		cag	agc	ggt	gct	gac	cca	gcg	ggt	atc	ttc	ttc	ctt	ctc	ctc	cag
Exon-3	1	CTC	CTG	GGC	AAT	ATC	CTC	ATC	ATC	GTC	CTG	GCC	ACC	CAC	TTC	CCA
	46	AAG	GAG	TTC	ACT	CCT	GCC	AGT	CAG	GCC	GCC	TGG	ACA	AAG	CTC	GTC
	91	AAT	GCA	GTG	GCC	CAT	GCT	CTG	GCT	CTC	GGT	TAC	CAC			

The International Union of Pure and Applied Chemistry Symbols used to denote multiple nucleotides are as follwos: K=G or T; M=A or C; R=A or G; S=G or C; W=A or T.

exon regions of the α^D-2- and the β-globin gene and exon-intron boundaries of the two genes. Breathnach and Chambon (1981) stated that there was no exception to the GT-AG rule according to which all intron sequences start with GT and end with AG. However, Table 2A shows that a unique structural feature of the α^D-2-globin gene is a GC instead of a GT dinucleotide at the 5' end of the second intron sequence. This finding is the first exception found in reptilian hemoglobin gene and supported the previous studies on gene structures of bird's hemoglobin (Erbil and Niessing, 1983; Dodgson and Engel, 1983). Erbil and Niessing (1983) found the T to C transition at the second intron position 2 of α^D-globin gene from a duck, *Cairina moschata*. This evidence together with the unique structure of α^D-2-globin gene found in the tortoise strongly indicates that the two animals, tortoises and birds, are the closest living relatives to each other.

When compared with the intron lengths among the four α-types of globin genes (Table 3), it was clearly determined that the *Geochelone* α^D-2-globin gene structure corresponded to that of the adult chicken α^D-globin gene and not to the embryonic chicken π'-globin gene (Engel *et al*., 1983) nor the embryonic human ζ-globin gene (Proudfoot *et al*., 1982). On the contrary, the *G. gigantea* β-globin gene was hard to classify since the second intron length (about 1.59-kbp) was large compared with those of the adult β-globin gene (Lawn *et al*., 1980; Dolan *et al*. 1983), including embryonic β-like globins (Efstratiadis *et al*., 1980; Chapman *et al*., 1981). In addition, the evolutionary relatedness of the intron sizes of the *Geochelone* globin genes to the other amniotes

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Table 3. Comparison of exson and intron sizes (in bp) of α^D -2- and β-globin genes

Class	Globin-gene Name	Exon-1	1st Intron	Exon-2	2nd Intron	Exon-3	Total	Reference
Reptilia	***G. gigantea* α^D-2**	**92**	**214**	**205**	**227**	**126**	**864**	**This study**
	G. carbonaria α^D	92	n.d.	205	n.d.	126	n.d.	AF304335 in GenBank
Aves	*Gullus gullus* α^D	92	148	205	261	126	832	Dodgson and Engel, 1983
	Gullus gullus π'	92	577	205	294	126	1294	Engel *et al.*, 1983
Mammalia	*Homo sapiens* ζ	92	886	205	239	126	1548	Proudfoot *et al.*,1982
	Homo sapiens α	92	117	205	141	126	681	Liebhaber *et al.*,1980
Reptilia	***G. gigantea* β**	**89**	**130[1)]**	**223**	**1590[1)]**	**126**	**2158[1)]**	**This study**
	G. carbonaria β	89	n.d.	223	n.d.	126	n.d.	Bordin *et al.*,1997
Aves	*Gullus gullus* β	82	92	223	810	126	1333	Dolan *et al.*, 1983
Mammalia	*Homo sapiens* β	89	130	223	850	126	1418	Lawn *et al.*, 1980

n.d.; not determined. Intron sizes could not be determined as this sequence is only represented by an RT-PCR product.
1) Intron sizes were estimated by migration distances on agarose gel electorphoresis (Fig. 4).

globin genes was defined for the first time.

Reptilian phylogeny and diversity based on α^D-Globin structures

Shishikura and Takami (2001) have constructed a phylogenetic tree based on α- and β-globins of 28 reptilian Hb As, by which the molecular phylogeny of Reptilia is highly correlated at the level of orders with the traditional phylogeny established mainly upon their morphological characteristics (Carroll, 1969; Benton, 1990). To date, there have been four different types of α-globins in amniotes reported: α^A, α^D, π' and ζ. The former two are adult α-type globins and the remaining are embryonic α-like globins. Fig. 4. shows a molecular tree of reptilian evolution constructed mainly by α^D-globins of 7 reptiles as well as relatedness among representatives of adult and embryonic α-type globins. The tree also strongly supports the previous molecular studies (Goodman *et al*., 1975; Fushitani *et al*., 1996; Gorr *et al*., 1998; Shishikura and Takami, 2001), however, it is reasonable to note the following two points: (1) the two kinds of embryonic globins, π' and ζ, first split off from the ancestor of the α-type of globins and formed a cluster; (2) the ancestor of squamates (snakes; *L. miliaris*, lizards; *Varanus komodoensis*) occupied unusual positions since α^D-globins of squamates began to diverge approximately 335 million years ago, much earlier than the separation of the three other clusters of α-type globin families.

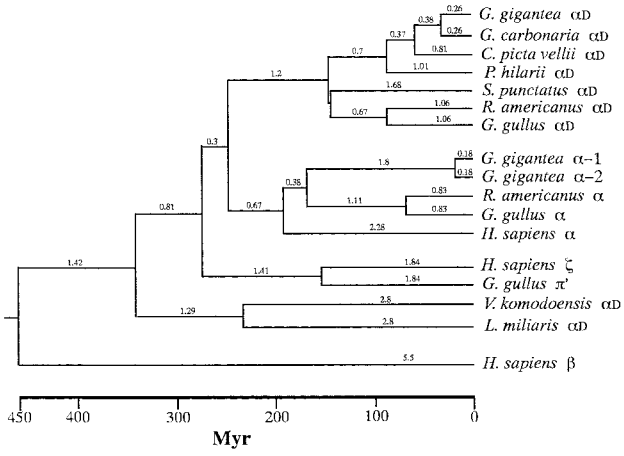


Fig. 4. A phylogenetic Tree based on primary structures of α^D-types of globins including some representative Amniota embryonic α-like globins and *Homo sapiens* β-globin as an outer group. Branch lengths are proportional to protein distances(×1/10) and shown on the individual branches of the tree. The 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 primary structures of globins used in the present analysis are as follows: α^D-globins: *G. gigantea* (this study), *G. carbonaria* (Accession No. AF304335 in GenBank), *C. picta vellii* (Rüchnagel *et al*., 1984), *S. punctatus* (Abassi *et al*., 1998), *R. americanus* (Oberthür *et al*., 1986), *G. gullus* (Takei *et al*., 1975), *V. komodoensis* (Fushitani *et al*., 1996), *L. miliaris* (Matsuura *et al*., 1989). α^A-globins: *G. giantea* α-1 and α-2 (Shishikura and Takami, 2001), *R. americanus* (Oberthür *et al*., 1983), *G. gullus* (Knöchel *et al*., 1982), *H. sapiens* (Braunitzer *et al*., 1961). Embryonic α-like globins: *H. sapiens* ζ (Aschauer *et al*., 1981), *G. gullus* π' (Chapman *et al*., 1980; 1982). *H. sapiens* β-globin (Braunitzer *et al*., 1961).

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Appendix. Sequence analysis of peptides obtained by cleavage with lysyl endopeptidase and *V8* protease

Step	Hb D α-2 Amino Acid (*p* moles)				Hb D β Amino Acid (*p* moles)		
	Intact	**K-1**			**Intact**	**K-1**	
1	M (254)	M (6160)			V (687)	V (3918)	
2	L (297)	L (5178)			H (272)	H (884)	
3	T (117)	T (2209)			W (383)	W (104)	
4	E (291)	E (4423)			T (265)	T (1340)	
5	D (178)	D (2857)			S (91)	S (322)	
6	D (229)	D (3461)			E (436)	E (1164)	
7	K (232)	K (1208)	**K-2**		E (498)	E (1472)	
8	Q (127)		Q (2241)		K (458)	K (354)	**K-2**
9	L (151)		L (2614)		Q (315)		Q (2687)
10	I (117)		I (2241)		Y (247)		Y (2053)
11	Q (111)		Q (1782)		I (294)		I (2608)
12	H (59)		H (459)		T (150)		T (1210)
13	V (122)		V (2006)		S (49)		S (347)
14	W (56)		W (1206)		L (247)		L (1855)
15	E (104)		E (1360)	**E-1**	W (85)		W (645)
16	K (101)	**K-3**	K (989)	K (542)	A (256)		A (1722)
17	V (103)	V (1448)		V (1039)	K (178)	**K-3**	K (787)
18	L (94)	L (1357)		L (800)	V (205)	V (2667)	
19	E (94)	E (1219)	**E-2**	E (295)	N (186)	N (2406)	
20	H (42)	H (263)	H (368)		V (204)	V (2489)	
21	Q (60)	Q (870)	Q (871)		G (145)	G (1667)	
22	E (83)	E (1075)	E (831)		E (136)	E (2434)	
23	D (68)	D (532)			V (338)	V (1899)	
24	F (68)	F (814)			G (140)	G (1323)	
25	G (47)	G (535)			G (163)	G (1354)	
26		A (730)			E (386)	E (1726)	
27		E (727)			A (235)	A (1896)	
28		A (695)			L (211)	L (1716)	
29		L (581)			A (252)	A (1665)	
30		E (592)		**E-3**	R (123)	R (840)	
31		R (400)		N.D.	L (350)	L (1430)	
32		M (435)		M (785)	L (316)	L (1600)	
33		F (389)		F (761)	I (200)	I (1171)	
34		I (337)		I (1979)	V (185)	V (1140)	
35		V (314)		V (762)	Y (122)	Y (934)	
36		Y (265)		Y (487)	P (104)	P (776)	
37		P (270)		P (587)		W (332)	
38		S (44)		S (107)		T (439)	
39		T (134)		T (295)		Q (640)	
40	**K-4**	K (58)		K (803)		R (412)	
41	T (4005)			T (268)		F (650)	
42	Y (6603)			Y (279)		F (757)	
43	F (7549)			F (492)		A (541)	
44	P (4645)			P (256)		S (93)	
45	H (2838)			H (109)		F (413)	
46	F (5030)			F (308)		G (232)	
47	D (4682)			D (256)		N (334)	
48	L (4563)			L (216)		L (291)	
49	H (2133)			H (107)		S (58)	
50	H (2659)			H (142)		S (63)	
51	D (3410)			D (167)		A (254)	
52	S (570)			S (15)		N (175)	
53	E (2847)		**E-4**	E (68)		A (225)	
54	Q (2077)		Q (336)			I (136)	
55	I (2060)		I (480)			L (156)	
56	R (1581)		N.D.			H (65)	
57	H (947)		N.D.			N (119)	
58	H (1391)		H (95)			A (137)	
59	G (1365)		G (108)			K (42)	**K-4**
60	K (1344)		K (228)				V (3698)
61		**K-5**	K (218)				L (3684)
62		V (7177)	V (296)				A (3476)
63		V (6907)	V (170)				H (1136)
64		G (4722)	G (97)				G (1916)
65		A (6187)	A (138)				Q (2164)
66		L (5590)	L (200)			**K-5**	K (1299)
67		G (3871)	G (135)			V (1036)	
68		D (3735)	D (130)			L (1090)	
69		A (4395)	A (187)			T (949)	
70		V (3403)	V (169)			S (182)	
71	**K-6**	K (2660)	K (122)			F (719)	
72	H (917)		H (48)			G (421)	
73	I (2647)		I (74)			E (1007)	

Step	Hb D α-2 Amino Acid (*p* moles) Continued				Hb D β Amino Acid (*p* moles) Continued	
74	D (1667)		D (167)		A (790)	
75	N (1990)		N (59)		V (397)	
76	L (2351)		L (85)		K (319)	**K-6**
77	S (360)		S (13)			N (3797)
78	A (2010)		A (78)			L (3545)
79	T (798)		T (97)			D (2082)
80	L (1669)					N (2410)
81	S (235)					I (1914)
82	E (1070)		**E-5**			K (741)
83	L (1085)		L (896)		**K-7**	
84	S (164)		S (145)		T (1846)	
85	N (702)		N (535)		F (3200)	
86	L (862)		L (664)		A (3206)	
87	H (259)		H (139)		Q (2612)	
88	A (742)		A (604)		L (2743)	
89	Y (477)		Y (412)		S (421)	
90	N (582)		N (504)		E (1800)	
91	L (615)		L (364)		L (1788)	
92	R (625)		R (182)		H (860)	
93	V (483)		V (465)		C/pe-cys	
94	D (N.D)		D (293)		E (1547)	
95	P (259)		P (314)		K (831)	**K-8**
96	V (254)		V (359)			L (559)
97	N (218)		N (291)			H (174)
98	F (177)		F (267)			V (400)
99	K (117)	**K-7**	K (372)			D (315)
100		L (4545)	L (284)			P (280)
101		L (4673)	L (423)			E (519)
102		S (793)	S (43)			N (258)
103		H (1551)	H (114)			F (279)
104		C/pe-cys	C/pe-cys		**K-9**	K (259)
105		F (2856)	F (196)		L (2391)	
106		Q (2256)	Q (174)		L (1757)	
107		V (2454)	V (181)		G (674)	
108		V (2869)	V (217)		N (790)	
109		L (2279)	L (155)		I (425)	
110		G (1458)	G (117)		L (247)	
111		A (2129)	A (153)		I (252)	
112		H (656)	H (69)		I (416)	
113		L (1709)	L (142)		V (141)	
114		G (1272)	G (114)		L (174)	
115		R (1150)	R (97)		A (185)	
116		E (1564)	E (52)	**E-6**	T (128)	
117		Y (1098)		Y (3742)	H (53)	
118		T (601)		T (259)	F (127)	
119		P (974)		P (286)	P (50)	
120		Q (956)		Q (314)	K (34)	**K-10**
121		V (989)		V (306)		E (3554)
122		Q (878)		Q (249)		F (3406)
123		V (919)		V (244)		T (849)
124		A (889)		A (272)		P (2086)
125		Y (626)		Y (200)		A (2568)
126		D (535)		D (216)		S (521)
127	**K-8**	K (387)		K (316)		Q (1504)
128	F (3065)			F (212)		A 1090)
129	L (2272)			L (238)		A (1885)
130	A (2211)			A (213)		W (683)
131	A (2732)			A (219)		T (559)
132	V (1917)			V (202)	**K-11**	K (731)
133	S (273)			S (33)	L (1518)	
134	A (1525)			A (174)	V (1388)	
135	V (1329)			V (159)	N (1116)	
136	L (1186)			L (189)	A (1411)	
137	A (1268)			A (151)	V (1157)	
138	E (930)			E (100)	A (1267)	
139	K (740)	**K-9**			H (253)	
140		Y (2427)			A (1722)	
141		R (620)			L (787)	
142					A (1019)	
143					L (804)	
144	N.D.; not determined				G (523)	
145					Y (545)	
146					H (41)	

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