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The Primary Structure of Three Hemoglobin Chains from the Indigo Snake (*Drymarchon corais erebennus, Serpentes*): First Evidence for α^D Chains and Two β Chain Types in Snakes

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The hemoglobin of the indigo snake *(Drymarchon corais erebennus, Colubrinae*) consists of two components, HbA and HbD, in the ratio of 1:1. They differ in both their α and β chains. The amino acid sequences of both α chains (α^A and α^D) and one β chain (βI) were determined. The presence of an α^Dchain in a snake hemoglobin is described for the first time. A comparison of all snake β chain sequences revealed the existence of two paralogous β chain types in snakes as well, which are designated as βI and βII type. For the discussion of the physiological properties of *Drymarchon* hemoglobin, the sequences were compared with those of the human α and β chains and those of the closely related water snake *Liophis miliaris* where functional data are available. Among the heme contacts, the substitution α^D58(E7)His→Gln is unusual but most likely without any effect. The residues responsible for the main part of the Bohr effect are the same as in mammalian hemoglobins. In each of the three globin chains only two residues at positions involved in the α1/β2 interface contacts, most important for the stability and the properties of the hemoglobin molecule, are substituted with regard to human hemoglobin. On the contrary, nine, eleven, and six α1/β1 contact residues are replaced in the α^A, α^D, βI chains, respectively.

Key words: *Colubridae* / Heme contact / Hemoglobin sequence.

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Introduction

Among the large number of known hemoglobin sequences, those of reptiles are rare although they are at least as interesting as the majority of mammalian hemoglobins. The earliest undoubted reptile representatives appeared in the fossil record during the Carboniferous period (about 310–320 million years ago) and thus the origin of the different lineages of sauropsidan reptiles is ancient compared to intra-mammalian divergences. The inference of a reliable reptile (*Sauropsida*) system is additionally impeded by the radiation-like separation into the main branches. Sequence comparisons and the construction of phylogenetic trees will certainly improve our knowledge of their relationships. Moreover, the study of the primary structures of globin chains will lead to a better understanding of the physiology of oxygen uptake and delivery.

The snakes (*Serpentes*), including about 2700 extant species (Mattison, 1986), are so far represented only by 6 hemoglobin chains. These are an α chain from the European asp (*Vipera aspis, Viperidae*; Duguet *et al.*, 1974), a β chain from the Indian cobra (*Naja naja naja, Elapidae*; Naqui *et al.*, 1991) and an α and β chain from the sea snake (*Microcephalophis gracilis, Elapidae*; Islam *et al.*, 1990) as well as the water snake *Liophis miliaris, South American Xenodontinae*; Cadle, 1984; Matsuura *et al.*, 1989). Here we present the primary structures of two α chains and one β chain of the indigo snake (*Drymarchon corais erebennus, Colubrinae*) living in Texas. *Liophis* and *Drymachon* belong to the paraphyletic family of *Colubridae* (Cadle, 1987) which includes about one third of all living snake species. Together with *Viperidae* and *Elapidae*, they are combined in the superfamily of advanced snakes (*Colubroidea*).

The determination of α chains as α^D hemoglobin chains is described here for the first time in a snake hemoglobin. This type of α chain was first discovered in birds (Brown and Ingram, 1974) where it is widely distributed as a constituent of the adult HbD component (Kleinschmidt and Sgouros, 1987). But in some avian taxa such as cuckoo, pigeon, parakeet, penguin, white stork, blue and yellow macaw, and grey heron at least the α^D protein is missing (Chapman *et al.*, 1982; Oberthuer *et al.*, 1986). In birds, the synthesis of α^D chains starts already during embryonic development. The minor component HbM ($\alpha^D{}_2\varepsilon_2$) has been found in five day old chicken embryos. From day 7 of the chicken ontogenesis, HbD ($\alpha^D{}_2\beta_2$) is

found to be expressed (Brown and Ingram, 1974). Moreover, structural and functional resemblances of α^D chains and larval α type chains of tadpoles, birds (π) and mammals (ξ) have been described (Oberthuer *et al.*, 1986; Hiebl *et al.*, 1987). Indeed, α^D chains and these larval α type chains share several amino acid residues which are different from those in adult α chains (Gorr, 1993). They point to a common ancestry of α^D and larval α chains originating from early gene duplication roughly 400 million years ago, through which they became paralogous to the purely adult α subunits (Czelusniak *et al.*, 1982; Goodman *et al.*, 1982). Besides birds, α^D chains have so far only been described for two turtles (*Chrysemys picta belii* and *Phrynops hilarii*; Ruecknagel *et al.*, 1984), the tuatara (*Sphenodon punctatus*; Abbasi, 1988), and the Komodo dragon (*Varanus komodoensis*; Fushitani *et al.*, 1996). All α^D chains known so far are characterized by a number of specific residues. These are: position 8 – basic residue; 9 – hydrophobic residue; 11 – Gln; 24 – Phe or Ile; 53 – charged residue; 71 – basic residue; 89 – Tyr; 90 – Asn; 106 – Gln or His; 109 – Leu; 113 – Leu or Met; 116 – acidic residue; 117 – Tyr, 124 – Ala; 125 aromatic residue, 138 – Glu. Note the accumulation of α^D specific residues towards their C terminus, especially in their GH interhelical region (pos. 113 – 117). With very few exceptions, these synapomorphic residues are shared in α^D chains of reptiles including birds in opposition to their paralogous α^A counterparts.

Results

The hemoglobin of the indigo snake (*Drymarchon corais erebennus*) consists of two components in approximately equal amounts as indicated by native PAGE (Figure 1). They have been designated as HbA and HbD. The electrophoresis under dissociating conditions revealed six bands (Figure 1, gels 2 and 9). More than the expected four peaks for two α and two β chains were also obtained during chain separation by reversed-phase HPLC (Figure 2). This might be due to aggregation caused by external cysteine residues which are more frequently found in reptilian than in mammalian hemoglobin. The individual peaks were characterized by Triton electrophoresis and N-terminal sequence determination up to position 10. The first three peaks (1 – 3) following the elution of the heme showed the same two bands in different ratios during Triton electrophoresis (gels 3 – 5) and the identical N-terminal sequence 'Val-His-Trp Ser-Ala-Glu-Glu-Lys-Gln-Leu' characterizing them as pure βII chain. An attempt to determine the primary structure of this chain with the combined material of peak 1 – 3 failed because

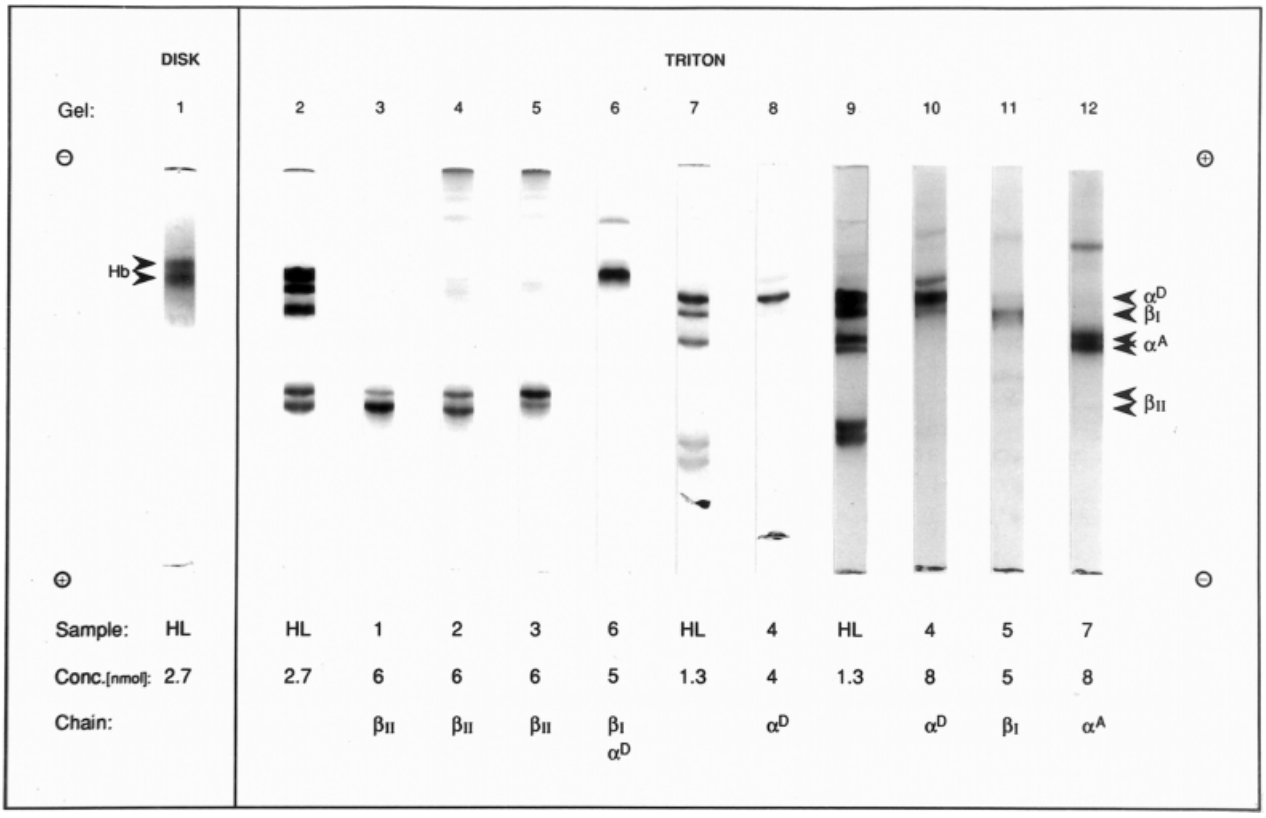

Fig. 1 Polyacrylamide Gel Electrophoresis of *Drymarchon* Hemolysate under Native Conditions (Disk) and Hemolysate and Globin Chains under Dissociating Conditions (Triton).
The sample numbers correspond to the peak numbers obtained by the HPLC chain separation shown in Figure 2. HPLC-purified chains were identified as indicated through N-terminal sequencing. HL = freshly prepared hemolysate.

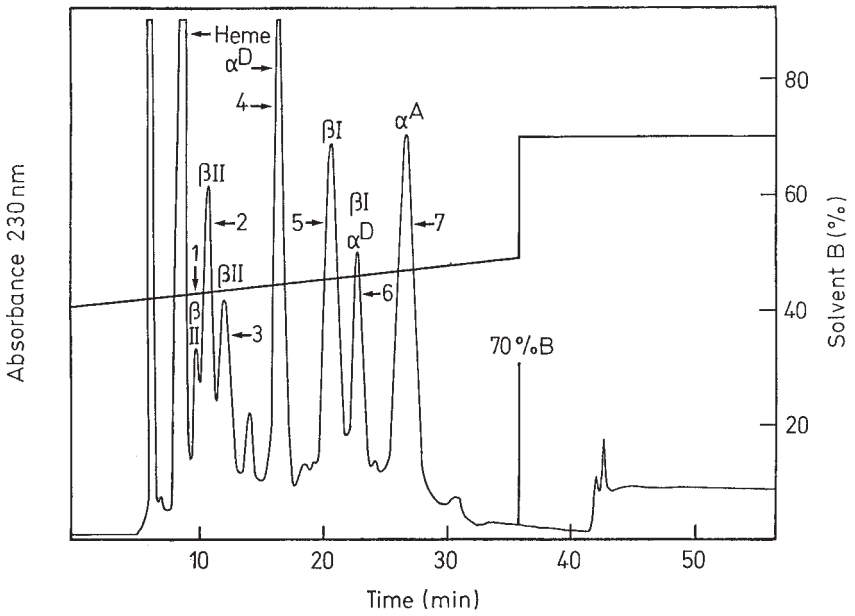

Fig. 2 HPLC Separation of the Globin Chains from *Drymarchon* Hemolysate.
The gradient profile is indicated. Numbers refer to the individual peaks.

of its low quantity. The peaks 4, 5 and 7 contained the pure α^D, βI and α^A chains, respectively, which could be completely sequenced (gel 8 or 10, 11 and 12, respectively). Peak 6 proved to be an aggregate of the α^D and the βI chain (gel 6). It is obvious that the number and purity of reptilian hemoglobin subunits cannot always be inferred from the electrophoretic banding pattern: aggregates may yield single bands (Figure 1, gel 6) and HPLC peaks (Figure 2, peak 6), pure chains instead more than one band (Figure 1, gels 3–5, 10 or 12) and peak (Figure 2, peak 1–3).

All N-terminal sequences were determined by automatic Edman degradation in the liquid phase. On the α^A and βI chain, respectively, 42 amino acid residues could be identified using the unmodified chains. The α^D chain was employed in the PE-form which allowed the unambiguos identification of the cysteine at position 19. The sequence determination was possible up to position 56 with the exeption of two serine residues at positions 49 and 52.

The main part of the primary structures was determined by examination of the tryptic peptides obtained from the native as well as the oxidized chains. The peptides could be isolated by reversed-phase HPLC. The advantage of using the intact chains was a higher yield of tryptophan containing peptides whereas the oxidized chains provided the peptides including cysteine.

The α^A chain proved to have two different amino acids at the same position in a 1:1 ratio (pos. 4 Glu/Asp, pos. 28 Thr/Ala, and pos. 35 Ala/Val) (Figure 3). The corresponding peptides Tp 1 (Tp 1/2a), Tp 4b and Tp 5 were isolated in nearly equal amounts. Due to incomplete cleavage, the ditryptic peptide Tp 4b/5 with 28 Ala and 35 Val could be isolated additionally. Its sequence allowed to assign these two residues to the same α^A chain. The alignment was done with the help of the N-terminal sequence, the ditryptic peptides Tp 6/7, Tp 10/11 and Tp 12a/12b as well as the homology to the α chain of *Vipera aspis* (Duguet *et al.*, 1974).

In the βI chain an unspecific tryptic cleavage at 139His provided the peptide Tp 14b (pos. 140–144). The isolation and sequencing of the ditryptic peptides Tp 4/5+6 and Tp 10b/11 as well as the N-terminal sequence and the homology with the β chain of the closely related *Liophis miliaris* (Matsuura *et al.*, 1989) allowed the alignment of the other peptides.

Although all tryptic peptides could be obtainend from the α^D chain as well, the yield of the cysteine containing fragments was too low – even when collecting those of the oxidized chain – for a definite determination of the cysteine residues at positions 104, 122 and 137. Therefore a chymotryptic digestion of the PE-chain was performed in addition. A cleavage time of 18 h yielded peptides of four to five residues which could be isolated analogously to the tryptic peptides. A diode-array detection allowed the identification of PE-cysteine containing fragments because of their specifically high 254/280 nm absorption ratio. In the peptides Cp 1 (pos. 102–104), Cp 2 (pos. 118–122) and Cp3 (pos.137–140) the cysteine residues at positions 104, 122 and 137 could be identified as PE-Cys phenylthiohydantoin derivatives during sequence analysis. An unspecific cleavage with trypsin was obtained after 89Tyr yielding the fragments Tp 9d+10I and Tp 9d+10II. The only ditryptic peptide which could be isolated was Tp 12c/13. Using this sequence as well as the N-terminal structure and the homology with the α chain of *Liophis miliaris* (Matsuura *et al.*, 1989) the alignment of all peptides could be achieved.

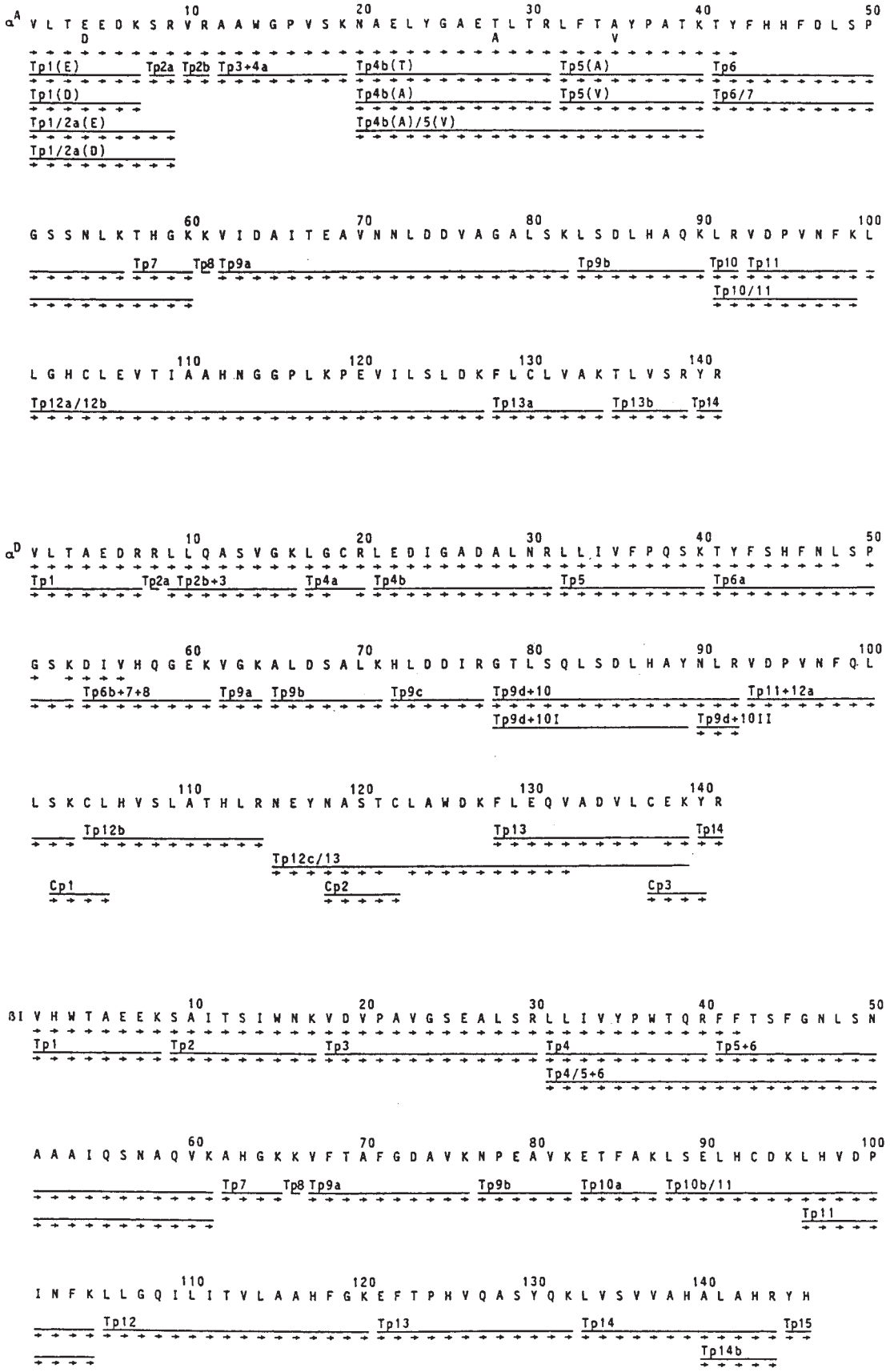

Fig. 3 Amino Acid Sequence of *Drymarchon* α^A, α^D and βI Globin Chains.
The N-terminally determined sequence of amino acid residues is given with the topmost line of arrows. The isolated peptides are marked and the amino acids identified after Edman degradation are indicated again by arrows. Tp = tryptic peptide, Cp = chymotryptic peptide.

The primary structures and sequencing schemes of the α^A, α^D and βI chain are presented in Figure 3.

Discussion

Composition of Snake Hemoglobins

The two components of the hemoglobin from the indigo snake (*Drymarchon corais erebennus*) comprise two α and two β chains. The α^A, α^D, and βI chain could be sequenced, whereas the lack of material impeded the determination of the primary structure of the βII chain. Unfortunately, it remains unclear whether the α^A or the α^D subunit forms a tetramer with the βI chain, because HbA and HbD could not be separated. The nearly equal amount of both components did not even allow an estimation from the chain quantities.

The most interesting result of our study is the identification of an α^D chain in a snake hemoglobin. A sequence comparison (Table 1) of the α^A and α^D chain from *Drymarchon* with other snake α chains makes very clear that α globins of *Vipera, Microcephalophis* and *Python* (Stoeckelhuber, 1992) are of the α^A type. On the contrary, the *Liophis* α chain clearly represents the α^D type. This fact was not realized by the authors of the *Liophis* α chain sequence (Matsuura *et al.*, 1989) although they found unusually large differences with regard to the α chains of other sauropsidans (α^A type) and of adult amphibians but lower ones to tadpoles (see above). Therefore, they discussed a different genetic origin of both α chain types and the 'intriguing possibility that an adult α chain might have been recruited from an early stage of development'. The presence of α^D chains could also be verified in two more snake hemoglobins. The squamate-specific α^D residues 4Ala, 7Arg, and 8Arg (Gorr, 1993) could be identified among the N-terminal sequences of the globin chains from the gray rat snake (*Elaphe obsoleta spiloides, Colubrinae*; Schroeder *et al.*, 1985) and from the Indian python (*Python molurus bivittatus, Boidae*; Stoeckelhuber, 1992). The α^D chain of *Drymarchon* contains four cysteine residues at the positions 19, 104, 122, and 137 but shares only the first two of them with *Liophis*. The external cysteine at position 19 might be responsible for the aggregation tendency of this hemoglobin, which might also be valid for the partly buried α^A130Cys. The additional α^D122Cys and α^D137Cys are internal which holds also true for 104Cys in both α chains and for βI93Cys. A comparison of the β chains from *Drymarchon* and *Liophis* showed 13 substitutions, whereas 49 and 46 replacements were found when *Drymarchon* was compared with *Microcephalophis* and *Naja*, respectively. Such large differences can hardly be explained by more distant relationships. The reason for these discrepancies became obvious when the sequences of both β chains of the Indian python (Stoeckelhuber, 1992) were considered as well (Table 2). One of the *Python* β chains is more closely related to the already sequenced β chains of *Drymarchon* and *Liophis*, whereas the other shows more similarities to *Microcephalophis* and *Naja*. This stands for two paralogous types of β chains in snake hemoglobins which we designated as βI (*Python* HbI, *Drymarchon* and *Liophis*) and βII (*Python* HbII, *Microcephalophis* and *Naja*). Even when the most distant *Boidae* and *Colubridae* are compared, the number of amino acid substitutions does not exceed 29 for orthologous chains, but it increases to values between

Table 1 Pairwise Comparison of the Sequences from Snake α^A and α^D Chains.

Species and chain type	Dc α^A	Mg α^A	Nn α^A	Va α^A	Pm α^A	Dc α^D	Lm α^D
Drymarchon (Dc) α^A	–	17.0	17.7	39.7	27.0	52.5	51.8
Microcephalophis (Mg) α^A	24	–	19.1	36.9	27.7	53.2	53.9
Naja (Nn) α^A	25	27	–	34.8	29.8	53.9	53.9
Vipera (Va) α^A	56	52	49	–	41.8	53.9	53.9
Python (Pm) α^A	38	39	42	59	–	55.3	55.3
Drymarchon (Dc) α^D	74	75	76	76	78	–	5.7
Liophis (Lm) α^D	73	76	76	76	78	8	–

Number (below left) and percentage (top right) of substitutions are given.

Table 2 Pairwise Comparison of the Sequences from Snake βI and βII Chains.

Species and chain type	Dc βI	Lm βI	Pm βI	Pm βII	Mg βII	Nn βII
Drymarchon (Dc) βI	–	8.9	17.1	30.8	33.6	31.5
Liophis (Lm) βI	13	–	18.5	28.8	32.9	36.3
Python (Pm) βI	25	27	–	27.4	31.5	31.5
Python (Pm) βII	45	42	40	–	19.9	16.4
Microcephalophis (Mg) βII	49	48	46	29	–	19.2
Naja (Nn) βII	46	53	46	24	28	–

Number (below left) and percentage (top right) of substitutions are given.

40 and 53 if comparisons between the paralogous βI and βII are made. These data are further supported by the published number of 45 exchanges between both β chains of *Liophis* (Matsuura *et al.*, 1992) as well as by the N-terminal sequence of a second *Naja* β chain representing the βI type (Naqui *et al.*, 1987). All complete sequences of snake α and β chains published up to now are aligned with the human globin chains in Figure 4.

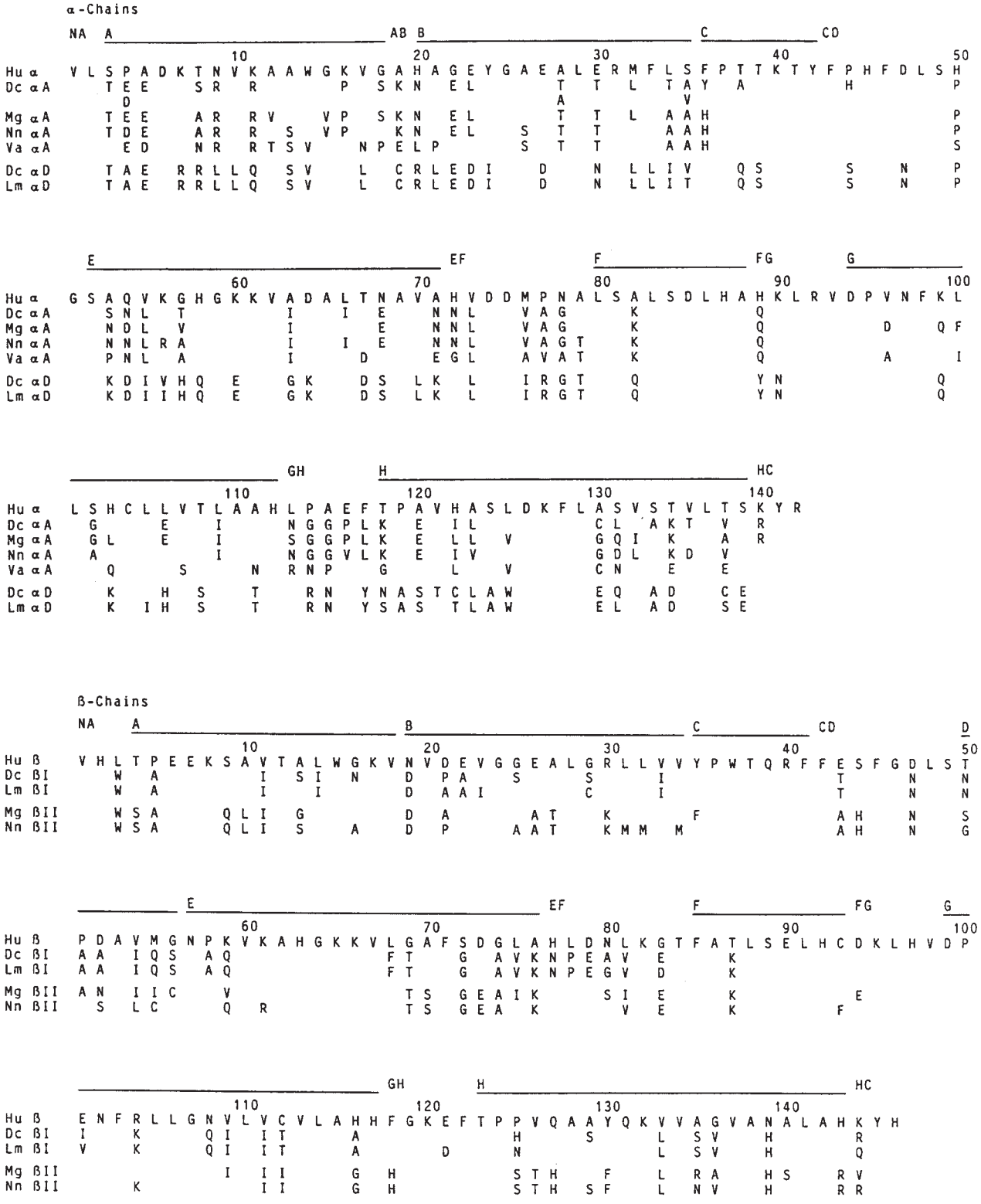

Fig. 4 Comparison of the Amino Acid Sequences of All Known Snake α^A, α^D, βI, and βII Globin Chains with the α and β Chains of Human Hemoglobin.

For the snake globin chains, only substitued residues are given. Helices are indicated. Species: Hu, human; Dc, *Drymarchon corais erebennus*; Mg, *Microcephalophis gracilis*; Va, *Vipera aspis*; Lm, *Liophis miliaris*; Nn, *Naja naja naja*.

In general, our knowledge of the composition of snake hemoglobins is limited and the evidence obtained by electrophoretic or chromatographic methods is partly contradictory. Electrophoretic surveys including quite a number of species from different families were done mainly on starch gel (Schwantes, 1972; De Smet, 1978). They showed multiple bands for most hemoglobins, but only a single one for *Liophis* (Schwantes, 1972) and *Elaphe* (De Smet, 1978) where the presence of two components (four chains) was demonstrated using HPLC-techniques (Schroeder *et al.*, 1985; Matsuura *et al.*, 1989). Similarly, the results of earlier chromatographic chain separations might be misleading if aggregation had not been prevented. The presence of two hemoglobin components could so far be proved for *Colubridae* (*Drymarchon, Liophis*, and *Elaphe*) and *Boidae* (*Python*) and is likely for the viperide species *Vipera* (*Viperinae*) and *Bothrops* (*Crotalinae*) as well as for *Naja* (*Elapidae*) because of the presence of more than two chains. For *Vipera*, three peaks have been found during chain separation by counter-current distribution (Duguet *et al.*, 1971). *Bothrops alternatus* hemoglobin showed two components on a DE-52 cellulose chromatography profile (Oyama *et al.*, 1993). *Naja* revealed one α^A and two β chains during chromatography on CM-cellulose (Naqui *et al.*, 1987). In this case, the α^D chain might have resisted elution as it was the case when python globin chains were separated by this method (Bittner, 1984). So far *Microcephalophis* (*Elapidae*) is the only snake where just two chains (α^A and βII) could be detected using CM-cellulose chromatography and PAGE under dissociating conditions in the presence of Triton X-100 and urea (Islam *et al.*, 1990). Like in the majority of snakes investigated, two different types of α and β chains, respectively, constituting HbA and HbD, were also found in the lizards *Varanus komodoensis* (Fushitani *et al.*, 1996), *Varanus exanthematicus, Varanidae* (Abbasi and Braunitzer, 1991) and *Uromastix hardwickii, Agamidae* (Naqui *et al.*, 1983). It is likely that the four different chains (α^A, α^D,βI, and βII) compose HbA and HbD in most snake hemoglobins, but it is not yet known which β chain is present in HbA or HbD, respectively.

Molecular Basis of Physiological Properties

The comparison of the orthologous α^D and βI chains of the colubrid snakes *Drymarchon* and *Liophis* revealed only 8 and 13 replacements, respectively, thus indicating a close relationship of both species. It remains to be seen whether the *Drymarchon* hemoglobin – and possibly those of other snakes – also display the unnusual dissociation of the tetrameric molecule to dimers during oxygenation as it was reported for *Liophis miliaris* (Matsuura *et al.*, 1987, 1989; Focesi *et al.*, 1990), *Bothrops alternatus* and *Boa constrictor*, respectively (Focesi *et al.*, 1992). The overall similarity and the excellent correspondence of residues at positions of structural and functional importance in the hemoglobin subunits of *Drymarchon* and *Liophis*, however, gives reason to expect that such physiological properties should be conserved as well. This certainly holds also true for the heme contacts (Fermi *et al.*, 1984) including the rare but ineffective exchange of the distal His(E7) by Gln in the α^D chains, and most of the residues at the rigid $\alpha1/\beta1$ subunit interface, where 8 out

Table 3 Contact Positions Affected by Replacements in *Drymarchon* (Dc) and *Liophis* (Lm) Hemoglobin Compared to Human (Hn) Deoxyhemoglobin.

	$\alpha1/\beta1$-contacts									$\alpha1/\beta2$-contacts							
	Hu	Dc	Dc	Lm		Hu	Dc	Lm		Hu	Dc	Dc	Lm		Hu	Dc	Lm
Position	α	α^A	α^D	α^D	Position	β	βI	βI	Position	α	α^A	α^D	α^D	Position	β	βI	βI
31 B12	R				30 B12	R			37 C2	P				34 B16	V		
34 B15	L	T*	I	I	33 B15	V	I	I	38 C3	T	A*	Q	Q	35 C1	Y		
35 B16	S	V*A*	V*	T	34 B16	V			40 C5	K				36 C2	P		
36 C1	F	Y			35 C1	Y			41 C6	T				37 C3	W		
103 G10	H		K	K	55 D6	M	Q	Q	42 C7	Y				40 C6	R		
106 G13	L	E	H	H	108 G10	N	Q	Q	44 CD2	P	H	S	S	41 C7	F		
107 G14	V				112 G14	C	T*	T*	88 F9	A				43 CD2	E	T*	T*
110 G17	A				115 G17	A			91 FG3	L				97 FG4	H		
111 G18	A		T	T	116 G18	H	A*	A*	92 FG4	R				98 FG5	V		
114 GH2	P	G	R	R	119 GH2	G			94 G1	D				99 G1	D		
117 GH5	F	L	Y	Y	122 GH5	F			95 G2	P				100 G2	P		
118 H1	T	K	N	S	123 H1	T			96 G3	V				101 G3	E	I	V
119 H2	P		A	A	124 H2	P			97 G4	N				102 G4	N		
122 H5	H	I	C	T	125 H3	P	H	N	140 HC2	Y				105 G7	L		
123 H6	A	L	L	L	127 H5	Q			141 HC3	R				145 HC2	Y		
126 H9	D				128 H6	A								146 HC3	H		
					131 H9	Q											

Amino acid replacements which are not suitable for contact formation are marked by an asterisk.

of 11 replacements compared to human hemoglobin are identical in the α^D chains and 5 out of 6 in the βI chains (Table 3). The more conservative α1/β2 contacts which are involved in the shift of the subunits during the T→R transition (Fermi and Perutz, 1981) show only two replacements in each subunit: Thr(C3)Gln and Pro(CD2)Ser in both α^D chains and Glu(CD2)Thr and Glu(G3)Ile/Val in the β1 chains of *Drymarchon* and *Liophis* (Ile in *Drymarchon* and Val in *Liophis* are isopolar residues with closely related properties). The α^A chain of *Drymarchon* reveals a comparable ratio of mutabilities at its interfaces (α1/β1: 9 substitutions, α1/β2: 2 substitutions, *i.e.* Thr(C3)Ala, Pro(CD2)His; see Table 3).

Both β1 chains share the amino acid residues responsible for the alkaline Bohr effect in human hemoglobin. Like in other snakes, the allosteric effectors regulating hemoglobin oxygen affinity in *Liophis miliaris* are guanosine triphosphate (GTP) and adenosine triphosphate (ATP) in the ratio 1:0.7 (Ogo *et al.*, 1984). These phosphates are well known from fish hemoglobins where their binding sites in the central cavity between the β chains are Val(NA1), Glu(NA2), Lys(EF6) and Arg(H21). Only Val(NA1) and Lys(EF6) are present in the *Liophis* and *Drymarchon* βI chains, whereas NA2 and H21 have His in common both snakes. Investigations of the interaction of ATP with snake hemoglobins, especially that of *Liophis*, showed a single ATP binding site per tetramer up to pH 7.5 (Bonilla *et al.*, 1994). Although the measurement was carried out with the total hemoglobin containing equal amounts of βI and βII chains (Matsuura *et al.*, 1989), the molecular discussion is based only on the known sequence of the βI chain. The authors explained the high ATP binding affinity compared to that of human hemoglobin by two amino acid substitutions in the βI chains: Leu(NA3)Trp and Glu(G3)Val. The introduction of a more hydrophobic residue at position NA3 is expected to increase the modulator effect by a more tightly binding of its adenine moiety. The replacement of the negatively charged Glu(G3) by Val provides the neighbouring positively charged Lys(G6) unneutralized, ready for a pH-dependent binding of the ATP phosphate groups. The effector binding can be explained in the same way for *Drymarchon* βI chains with Ile(G3) instead of Val, but it should be different for the βII chains where Glu(G3) is conserved in all known cases (*Microcephalophis*, *Naja* and *Python*).

Materials and Methods

Hemoglobin Composition and Chain Separation

Blood from an 1.8 m long indigo snake (*Drymachon corais erebennus*) was kindly provided by Mr. H. Pace from the exhibition 'Snakes and Reptiles' in Munich, Germany. The erythrocytes were separated by centrifugation, washed three times with isotonic saline and stored at –30 °C. For hemolysis, they were thawed and extracted with about five volumes of 20 mM Tris-HCl, 10 mM 1,4-dithiothreitol, pH 8.5. The composition of the hemoglobin was checked by polyacrylamide gel electrophoresis under native and dissociating conditions (Rovera *et al.*, 1978). For native conditions we used 10% polyarcrylamide in 375 mM Tris-HCl, pH 8.3; buffer: 57 mM glycine, 7.4 mM Tris-HCl, pH 8.3; current intensity: 4 mA/tube; staining: amido black 10B in 7% acetic acid. The dissociating conditions were performed in the presence of Triton X-100 and 8 M urea.

The globin chains were isolated from the entire hemoglobin (0.4 – 1.0 mg) by reversed-phase HPLC using a column (8 × 250 mm) of Nucleosil C-4, 300 – 7 (Machery-Nagel, Düren, Germany) in 0.08% TFA containing 40% acetonitrile. The following gradients of acetonirile/0.08% TFA were applied for elution: 0 – 2 min 40%, 2 – 36 min 40 – 48.5%, and 36 – 60 min 70%. The flow rate was 1.5 ml/min and the peaks were detected at 230 nm. A 340 Gradient Liquid Organizer and a Controller 421 (Beckman Instruments) were used.

Enzymatic Digestion and Peptide Separation

About 1 mg of native or performic acid oxidized globin chains was dissolved in 200 µl of 10 mM NaOH (pH ~ 10) and digested with 5% of (N-tosyl-L-phenylalanyl)chloromethane treated trypsin for 4 h at room temperature. If the protein did not dissolve completely during the digestion, the insoluble part was isolated by centrifugation, resuspended in 100 µl 10 mM NaOH, containing 0.05% SDS and treated again with 5% trypsin for 15 h.

The α^D chain (2.5 mg) was additionally modified by S-pyridylethylation (PE) of its cysteine residues (Friedmann *et al.*, 1970; Hawke and Yuan, 1987) and submitted to a chymotryptic hydrolysis. 1.5 mg of that alkylated sample were suspended in 15 µl of 0.1 M ammonium hydrogen carbonate, pH 9.0, and treated with ultrasound for 5 min to obtain a more homogenous suspension. A cleavage with 5% of α-chymotrypsin at 37 °C for 30 min under constant stirring yielded in a very limited degree of hydrolysis (HPLC determination of cleaved and noncleaved α^D-fractions) and thus the mixture was stirred at room temperature for another 18 h. The rest of the precipitate was removed by centrifugation.

All peptides were isolated from the hydrolysates by reversed-phase HPLC on Nucleosil C-4, 300 – 5 (column: 4.6 × 250 mm) in 0.1% TFA. Three linear acetonitrile/0.08% TFA gradients were used in succession after a 2 min period without acetonitrile, allowing for the binding of the peptides to the matrix: 0 to 30% in 90 min, 30 to 60% in 30 min and 60 to 70% in 28 min. One third of a 1 mg digest could be applied at once. The flow rate was 0.6 ml/min and the peaks were detected at 225 nm.

Globin chains and peptides were hydrolysed in 5.7 M HCl at 110 °C *in vacuo* for 20 h and analyzed in an Amino Acid Analyzer LC 5000 (Biotronic, Maintal, Germany). Cysteine was determined after performic acid oxidation, whereas for tryptophan the hydrolysis was performed in the presence of 1% thioglycolic acid. Pyridylethylcysteine was coeluted with histidine.

Sequence Determiniation

The N-terminal sequences of the chains were determined by automated degradation in a liquid phase sequencer (Model 890 C, Beckman Instruments) using a modified Quadrol program (Edman and Begg, 1967). For some large tryptic peptides, a 3-(diethylamino)propyne program (Braunitzer *et al.*, 1978) was employed. Most peptides were sequenced by the gas phase method (Hewick *et al.*, 1981) using a non-commercial sequencer (Begg *et al.*, 1986). Conversion was performed for 15 min at 80 °C in 1.9 M TFA. The phenylthiohydantoin derivatives were identified by HPLC with isocratic elution (Lottspeich, 1980) on a system consisting of a model 114 M Solvent Delivery Module (Beckman Instruments) and an Intelligent Sample Processor (Millipore/Waters). The extinction was monitored at 265 nm us-

ing an UV-detector (Biotronik) and a Shimadzu Model C-R3A Chromatopac recorder. The phenylthiohydantoin derivative of PE-cysteine was eluted between those of threonine and methionine.

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