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The Complete Primary Structure of the Marine Carnivora, Galapagoes Fur Seal (*Arctocephalus galapagoensis*, Otariidae) Hemoglobins

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The complete primary structure of the two hemoglobin components of the fur seal (*Arctocephalus galapagoensis*) is presented. The two components (HbI and HbII) occur in nearly equal amounts and have identical β-chains; whereas the two α-chains (αI/αII) differ by six exchanges Ile/Val, Met/Thr, Ser/Ala, Pro/His, Lys/Gly, and Thr/Ala at positions 10, 34, 35, 50, 78, and 131, respectively. The components were isolated by DEAE-Sephacel chromatography and were separated into the globin chains by RP-HPLC on a column of Nucleocil-C4. The sequences have been determined by Edman degradation in liquid- and gas-phase sequencer, using the native chains and tryptic peptides. The sequences compared with those of other Carnivora species and an adult human globin chains. An identical β-chain is found in fur seal and walrus, whereas larger differences were found between αI and αII compared to β-chains.

KEY WORDS: Fur seal; marine carnivora; hemoglobin; primary structure; homology.

1. INTRODUCTION

Otariid pinnipeds (eared seals) could spend most of their time in the water. For arctic and antarctic pinnipeds, reproduction is one of the main reasons for coming ashore because the young are unable to survive in the water for some time after birth; however, it is much less pronounced in galapagos species, in which even females come all year to inhabitual colonies without new born. Very little information is available on hemoglobin structure and function of pinniped seals. In the superfamily Otarioidea, amino acid sequences of the hemoglobin are so far known only for Pacific walrus (*Odobenus rosmarus divergens*) hemoglobin, which belongs to family Odobenidae (Lin *et al.*, 1989). In order to understand the structure–function relationship and evolution at the molecular level, this communication presents the complete primary structure of the αI, αII, and β-chains of the fur seal, *Arctocephalus galapagoensis*, which belongs to the family Otariidae.

2. MATERIALS AND METHODS

2.1. Isolation of Hemoglobin and Electrophoresis

Blood from fur seal (*Arctocephalus galapagoensis*) was collected at Galapagos Island in heparinized tubes. The erythrocytes were washed three times with physiological saline and lysed with cold distilled water. The hemolysate was examined for hemoglobin multiplicity by polyacrylamide gel disk electrophoresis in Tris/glycine buffer at pH 8.5 (Davis, 1964). Electrophoresis under dissociating conditions was carried out in the presence of Triton X-100 and 8 M urea (Alter *et al.*, 1980).

2.2. Separation of Hemoglobin Components

Whole hemolysate was subjected to ion exchange chromatography on a column (1.6 × 16 cm) of

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DEAE-Sephacel (Pharmacia), equilibrated with 50 mM Tris/HCl buffer containing 0.01% KCN *p*H 8.5. Elution of Hb was carried out with a gradient of 0–0.1 M NaCl. Flow rate was maintained at 20 ml/hr. The absorbance was monitored at 415 and 280 nm.

2.3. Separation of Globin Chains

2.3.1. *Ion-Exchange Method*

Hemoglobin was dehemed in cold acetone solution containing 2% HCl (Anson *et al.*, 1930). Globin was reduced under nitrogen for 3 h. The α- and β-chains were separated on a carboxymethylcellulose CM-52 (Whatman) column (1.6×15.7 cm), equilibrated with 25 mM sodium acetate, 0.2% mercaptoethanol, and 8 M urea, using a 0.02–0.08 M NaCl gradient (Clegg *et al.*, 1966).

2.3.2. *RP-HPLC Method*

Globin was prepared from CN-met-hemoglobin components by the acetone–HCl procedure (Anson *et al.*, 1930), and globin chains were separated by RP-HPLC using a 342-Gradient Liquid Chromatography, Controller 421 (Beckman) and a column of Nucleocil-C4 (Macherey & Nagel). The column was equilibrated with 0.1% aqueous TFA. Globin chains were eluted with a linear gradient of 35–60% acetonitrile over 60 min at a flow rate 1 ml/min. Absorbance was recorded at 230 nm.

2.4. Enzymatic Cleavage and Fractionation of Peptides

The globin chains were oxidized with performic acid and digested with trypsin (Tos-PheCH2Cl-treated, Worthington) for 1 hr at *p*H 10.5, and for another 2 hr at *p*H 9.5 with an enzyme/substrate ratio of 5:100 (Hirs *et al.*, 1967). After 3 hr, the hydrolysate was titrated to *p*H 4 and centrifuged. The soluble fraction was subjected to gel filtration on a column of Sephadex G-25 fine (2.6×140 cm), equilibrated, and eluted with 0.1 M acetic acid at a flow rate of 20 ml/hr. Individual peaks were rechromatographed by RP-HPLC on Lichrosorb RP-2 column (4.6×25 mm), using an ammonium acetate/acetonitrile system (Kratzin *et al.*, 1980). Peptides were eluted with a gradient from 0–60% over a 60 min, at a flow rate 1 ml/min.

2.5. Sequence Analysis[6]

Sequence studies were carried out by automatic Edman degradation (Edman and Begg, 1967) in liquid-phase sequenator (models 890B and 890C Beckman Instruments). For intact polypeptides and lysine peptides, 0.25 M Quadrol was employed (Braunitzer *et al.*, 1970) after coupling with reagent IV (Pfletschinger and Braunitzer, 1980); whereas 3-diethylamino propyne was used in the case of arginine peptides (Braunitzer *et al.*, 1978). Some peptides were sequenced by the gas-phase method using the non-commercial sequenator (Begg *et al.*, 1986). Conversion of the thiazolinone to phenylthiohydantoin derivatives of amino acids was carried out in the presence of 3 M TFA at 80°C, and identified by HPLC (Zimmermann and Pisano, 1977).

3. RESULTS AND DISCUSSION

3.1. Hemoglobin Composition and Primary Structure

Alkaline polyacrylamide gel electrophoresis revealed two hemoglobin components occuring in nearly equal amounts (Fig. 1a). Electrophoresis under dissociating conditions (Fig. 1b) moreover showed the presence of one α- and one β-chain. During chain

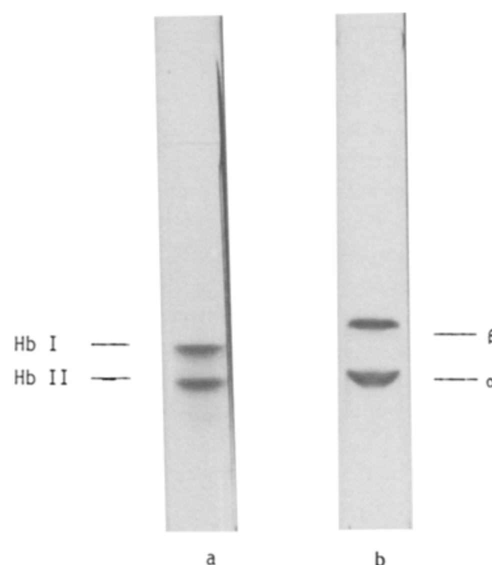

Fig. 1. Electrophoretic pattern of fur seal hemolysate on polyacrylamide gel. (a) Disk electrophoresis at *p*H 8.5; (b) under dissociating conditions in 8 M urea and Triton X-100.

[6] Quadrol = N,N,N,N-tetrakis(2-hydroxypropyl)ethylenediamine; Reagent IV = trisodium 7-(Isothiocyanato)-naphthalene-1,3,5-trisulphonate; Propyne = 3-(diethylamino)propyne.

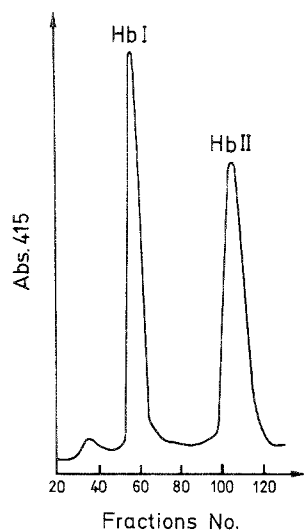

Fig. 2. Separation profile of the hemoglobin components on DEAE-Sephacel (1.6 × 16 cm), 50 mM Tris-HCl, *p*H 8.5, NaCl gradient 0–0.1 M.

separation by CM-cellulose chromatography two chains were eluted. Considering the results of disk electrophoresis and amino acids analysis of the tryptic peptides, which evidence two hemoglobin components, this suggests that the two components differ in the α-chains only. Subjecting the native α-chain to Edman degradation proves the presence of two α-chains with different in charges, explaining the presence of two bands in the alkaline polyacrylamide gel electrophoresis (Fig. 1a).

To obtain homogenous αI- and αII-chains, the individual components that were separated by DEAE-Sephacel chromatography (Fig. 2) were subjected to RP-HPLC, on Nucleosil column, as shown in Fig. 3. The amino acid compositions of the tryptic peptides obtained from the oxidized chains are listed in Tables I–III. The primary structure of the globin chains was

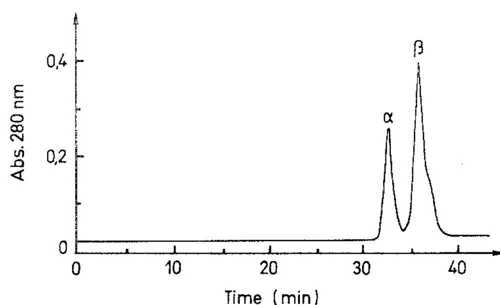

Fig. 3. Chain separation of globin from Hb II on a RP-HPLC. column: Nucleosil-C4 (4.6 × 250 nm); buffer: 0.1% TFA; gradient: 35–60% acetonitrile over 60 min; flow rate: 1 ml/min.

mainly determined by sequencing the N-terminal regions of the native chains (up to 63 residues of the α-chains and 42 of the β-chain), followed by sequencing of the tryptic peptides. The sequence analysis showed identical primary structures for the β-chains of both components. The complete primary structures of the αI-, αII-, and β-chains are given in Fig. 4. Interestingly, the structural differences in the two hemoglobin components were confined six positions in (four different tryptic peptides) to the α-chains, but were resolved neither by electrophoresis under dissociating conditions nor by any other chromatographic methods (CM-cellulose or RP-HPLC). The α-II differs from α-I at positions 10 (Ile/Val), 34 (Met/Thr), 35 (Ser/Ala), 50 (Pro/His), 78 (Lys/Gly), and 131 (Thr/Ala).

3.1. Structural and Evolutionary Aspects

A comparison of αI- and αII-chains from the fur seal and human hemoglobins indicates 20 (14.2%) and 19 (13.5%) amino acids exchanges, respectively. In the β-chains, the number of subsitutions is lower: 11 (7.5%) replacements distributed over the entire length of the β-chains. Some of the amino acid residues exchanged in the fur seal are involved in contacts with the heme or between the subunits in human hemoglobin. The heme contact affected is β70 (E14) Ala/Ser. β43 (CD2) Glu/Asp is an $\alpha 1\beta 2$ contact, whereas β125(H3) Pro/Gln, αI 34(B15) Leu/Met, αII 34(B15) Leu/Thr, αII35 (B16) Ser/Ala, α111 (G18) Ala/Cys are $\alpha 1\beta 1$ contacts. Most of these subsitutions are common in other mammals. None of the residues contributing to the 2,3-diphosphoglycerate contact sites was altered. The evolutionary relationships of marine mammals are not fully understood and there are some controversy about the origins of Pinnipedia, that are specialized for an aquatic existence, whether Otarioidea and Ursidae have common ancestors as well as Phocoidea and Mustalidea. More hemoglobin sequences in this suborder may help to clarify origins and evolutionary relationships at the molecular level.

In Tables IV and V, sequences for 12 carnivore α- and β-chains are compared to fur seal αI-, αII-, and β-chains. The Carnivora species are: harbor seal (Watanabe *et al.*, 1986); Weddell seal, pacific walrus (Lin *et al.*, 1989*a*, *b*); polecat (Ahmed *et al.*, 1989), European mink (Ahmed *et al.*, 1990); racoon (Brimhall *et al.*, 1978); badger (Hombrados *et al.*, 1976, 1978); lesser panda, giant panda (Tagle *et al.*, 1986); polar bear (Hofman *et al.*, 1987; dog (Brimhall *et al.*, 1977*a*); and coyote (Runkel *et al.*, 1974). The

Table I. Amino Acid Composition of Tryptic Peptides of αI-Chain of Fur Seal Hemoglobin

Tp	1	2	3	4	5	6	7	8	9a	9b	10	11	12	13	14
Pos.	1–7	8–11	12–16	17–31	32–40	41–56	57–60	61	62–78	79–90	91–92	93–99	100–127	128–139	140–141
Asp	1.07	1.02	0.86	—	—	1.06	—	—	2.79(3)	1.14	—	1.99	1.11	—	—
Thr	—	1.04	2.12	—	3.10	1.01	—	—	2.13	—	—	—	1.94	2.92	—
Ser	0.82	—	—	—	1.28	1.78(2)	—	—	—	1.78(2)	—	—	1.78(2)	2.78(3)	—
Glx	—	—	—	3.07	—	1.04	—	—	—	—	—	—	1.07	—	—
Pro	1.13	—	—	—	0.67	1.98	—	—	0.94	—	—	1.19	2.22	—	—
Gly	—	—	—	4.88	—	1.08	0.95	—	—	—	—	—	—	—	—
Ala	0.99	—	—	2.08	—	1.03	1.01	—	4.15	3.04	—	—	4.08	—	—
Cys[a]	—	—	—	—	—	—	—	—	—	—	—	—	1.84	—	—
Val	0.98	—	—	—	—	1.04	—	—	2.14	—	—	1.85	2.10	2.07	—
Met[a]	—	—	—	—	0.96	—	—	—	0.95	—	—	—	—	—	—
Ile	—	1.07	—	—	—	—	—	—	—	—	—	—	—	—	—
Leu	1.03	—	—	1.98	—	1.00	—	—	2.00	3.00	1.00	—	5.92	1.10	—
Tyr	—	—	—	0.90	—	0.66(1)	—	—	—	0.90	—	—	—	—	0.91
Phe	—	—	—	—	2.12	2.02	—	—	—	—	—	0.94	0.99	2.03	—
His	—	—	—	1.07	—	1.19	0.97	—	1.00	1.10	—	—	3.97	—	—
Trp[a]	—	—	1.00	—	—	—	—	—	—	—	—	—	—	—	—
Lys	0.97	1.10	1.01	—	1.18	1.08	1.06	1.00	1.02	1.02	—	1.06	1.02	1.10	—
Arg	—	—	—	1.00	—	—	—	—	—	—	0.99	—	—	—	1.01
Sum	7	4	5	15	9	16	4	1	17	12	2	7	28	12	2

[a] Determined after performic acid oxidation. Values within parentheses are taken from sequence analysis.

Table II. Tryptic Peptides from αII-Chain of Fur Seal Hemoglobin

Tp	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pos.	1–7	8–11	12–16	17–31	32–40	41–56	57–60	61	62–90	91–92	93–99	100–127	128–139	140–141
Asx	1.08	1.02	0.89	—	—	1.02	—	—	3.83	—	1.98	1.00	—	—
Thr	—	1.04	2.11	—	3.97	0.91	—	—	1.94	—	—	1.92	2.03	—
Ser	0.80	—	—	—	—	1.68(2)	—	—	1.79(2)	—	—	1.84	2.79	—
Glx	—	—	—	3.02	—	1.12	—	—	—	—	—	1.03	—	—
Pro	1.13	—	—	—	1.12	1.18	—	—	1.29	—	1.09	—	—	—
Gly	—	—	—	4.89	—	1.16	0.98	—	1.08	—	—	2.11	—	—
Ala	0.99	—	—	2.09	0.99	0.98	1.00	—	6.82	—	—	4.06	1.05	—
Cys[a]	—	—	—	—	—	—	—	—	—	—	—	2.01	—	—
Val	0.98	0.98	—	—	—	0.91	—	—	1.97	—	1.85	2.11	2.06	—
Met[a]	—	—	—	—	—	—	—	—	0.92	—	—	—	—	—
Leu	1.01	—	—	1.99	—	0.99	—	—	5.23	1.11	—	5.92	1.03	—
Tyr	—	—	—	0.89	—	0.85	—	—	1.00	—	—	—	—	1.00
Phe	—	—	—	—	1.89	1.89	—	—	—	—	0.98	0.99	2.09	—
His	—	—	—	1.06	—	2.19	0.89	—	2.02	—	—	3.91	—	—
Trp[a]	—	—	1.00	—	—	—	—	—	—	—	—	—	—	—
Lys	0.98	1.05	1.11	—	1.03	1.08	1.02	1.00	1.09	—	1.02	1.01	1.11	—
Arg	—	—	—	1.00	—	—	—	—	—	1.01	—	—	—	1.01
Sum	7	4	5	15	9	16	4	1	29	2	7	28	12	2

[a] Determined after performic acid oxidation. Values within parentheses are taken from sequence analysis.

Table III. Amino Acid Composition of Tryptic Peptides of β-Chain of Fur Seal Hemoglobin

Tp	1	2	3	4	5	6	7	8	9a	9b	10a	10b	11	12	13	14	15
Pos.	1–8	9–17	18–30	31–40	41–59	60–61	62–65	66	67–76	77–82	83–87	88–95	96–104	105–120	121–132	133–144	145–146
Asp	1.04	—	2.17	—	4.88[a]	—	—	—	2.16	2.88	—	1.02	2.12	1.25	—	1.11	—
Thr	0.81	1.03	—	1.06	—	—	—	—	—	—	1.00	—	—	—	0.93	—	—
Ser	—	—	—	—	2.48	—	—	—	1.68(2)	—	—	0.79(1)	—	—	—	—	—
Glu	1.10	—	2.23	1.07	—	—	—	—	—	—	—	1.02	1.08	—	3.94	—	—
Pro	—	—	—	1.14	2.32	—	—	—	—	—	—	—	0.90	—	1.27	—	—
Gly	—	1.26	2.88	—	2.03	—	1.09	—	1.08	—	0.97	—	—	1.94	—	1.12	—
Ala	0.93	2.88	0.94	—	1.16	—	1.00	—	—	—	0.98	—	—	1.20	1.93	3.90	—
Cys[b]	—	—	—	—	—	—	—	—	—	—	—	0.99	—	0.84	—	—	—
Val	0.91	1.03	2.67	1.90	1.02	0.99	—	—	1.08	—	—	—	0.96	3.16	1.11	2.78	—
Met[b]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Leu	1.06	1.03	1.07	2.09	1.10	—	—	—	1.97	1.98	—	2.10	1.02	3.64(4)	—	1.01	—
Tyr	—	—	—	0.83	—	—	—	—	—	—	—	—	—	—	1.00	—	0.99
Phe	—	—	—	—	2.84	—	—	—	0.98	—	0.87	—	0.96	1.00	0.97	—	—
His	1.00	—	—	—	—	—	1.10	—	—	—	—	1.04	0.96	1.69(2)	—	0.98	0.89
Trp[b]	—	0.82	—	0.81	—	—	—	—	—	—	—	—	—	—	—	—	—
Lys	1.12	0.81	—	—	1.10	1.00	1.11	1.00	1.02	1.12	1.14	1.09	0.98	0.97	1.11	1.02	—
Arg	—	—	1.05	0.92	—	—	—	—	—	—	—	—	—	—	—	—	—
Sum	8	9	13	10	19	2	4	1	10	6	5	8	9	16	12	12	2

[a] Aspartic acid + $MetSO_2 = 5$.
[b] Determined after performic acid oxidation. Values within parentheses are taken from sequence analysis.

Table IV. Amino Acid Differences Between αI/αII Chains of Fur Seal with Those of Other Carnivora Sequences

A. Fur seal αI chain

Pos:	8	10	12	17	19	23	30	34	35	50	57	68	70	71	72	73	78	80	85	89	100	111	115	116	129	130	131	Diff.
FSI	T	I	T	L	G	E	E	M	S	P	A	T	V	A	H	M	K	L	A	Y	L	C	A	Q	F	S	T	0
FSII		V						T	A	H							G										A	6
HS		V	A	I				T	A	H							G			H				D			A	10
WS		V		I					A					S		I	G							D			A	8
WR		V														I	G											3
PC		V	S	I				A		H		N					G		D								A	9
EM		V	S	I				A		H		N					G	M	D								A	10
RA	A		A	I				A				L			G	L	G		D					E	L	T	S	13
EB	A		A					A		H	G	N				L	G		D					E	L		S	12
LP		V	S					A				L		G		L	G		D	H				E			A	11
GP			A	I				A				L		G		L	G		D	H		S		E			A	12
PB	S		A	I	S			A					A	G		L	G		D	H	F	S		E			A	15
DD			S			D	D	Q								L	G		D				A	E		A	A	11
CO			S			D	D	Q								L	G		D				T	E		T	A	11

B. Fur seal αII chain

Pos:	8	10	12	15	17	19	23	30	34	35	50	57	68	70	71	72	73	80	85	89	100	111	115	116	129	130	131	Diff.
FSII	T	V	T	D	L	G	E	E	T	A	H	A	T	V	A	H	M	L	A	Y	L	C	A	Q	F	S	A	0
HS			A		I															H				D				4
WS				G	I				M		P	T			S		I							D				8
WR									M	S	P						I										T	5
PC			S		I				A	S			N						D									6
EM			S		I				A	S			N					M	D									7
RA	A	I	A		I				A	S	P		L			G	L		D					E	L	T	S	15
EB	A	I	A						A	S		G	N				L		D					E	L		S	12
LP			S						A	S	P		L		G		L		D	H				E				10
GP		I	A		I				M	S	P		L		G		L		D	H		S		E				13
PB	S	I	A		I	S			M	S	P			A	G		L		D	H	F	S		E				16
DD		I	S				D	D	Q	S	P						L		D				T	E		A		12
CO		I	S				D	D	Q	S	P						L		D				T	E		T		12

[a] Only differences are marked by the one-code designation of the respective amino acids. Abbreviations used for species are: FS, fur seal; HS, harbor seal; WS, Weddell seal; WR, walrus; PC, polecat; EM, mink; RA, racoon; EB, badger; LP, lesser panda; GP, giant panda; PB, polar bear; DD, dog; and CO, coyote.

Table V. Comparison of Fur Seal and Other Carnivora β-Chains[a]

β-chains Pos:	5	6	9	10	12	13	16	21	27	33	41	43	50	51	52	54	56	58	73	95	104	125	135	139	Diff.
FS	A	D	A	A	T	A	G	D	E	V	F	D	S	P	D	V	G	P	D	K	K	Q	A	N	0
HS	G	E	S											A		I									5
WS		E	S												N	I	S			Q					6
WR																									0
PC	G	E							T										E				T		5
EM		E																	E					T	3
RA			T			T		E				E		A		I			E		R	P			9
EB		E	S			S					Y		T						E						6
LP	G	E				G	S												E						5
GP	G	E				G	S						T				N		E						7
PB	G	E	S			G	G						T	A		I	N								9
DD		E	S	L	S	G				I			T				S	A							9
CO		E	S	L	S	G				I			T				S	A							9

[a] For abbreviations, see Table IV.

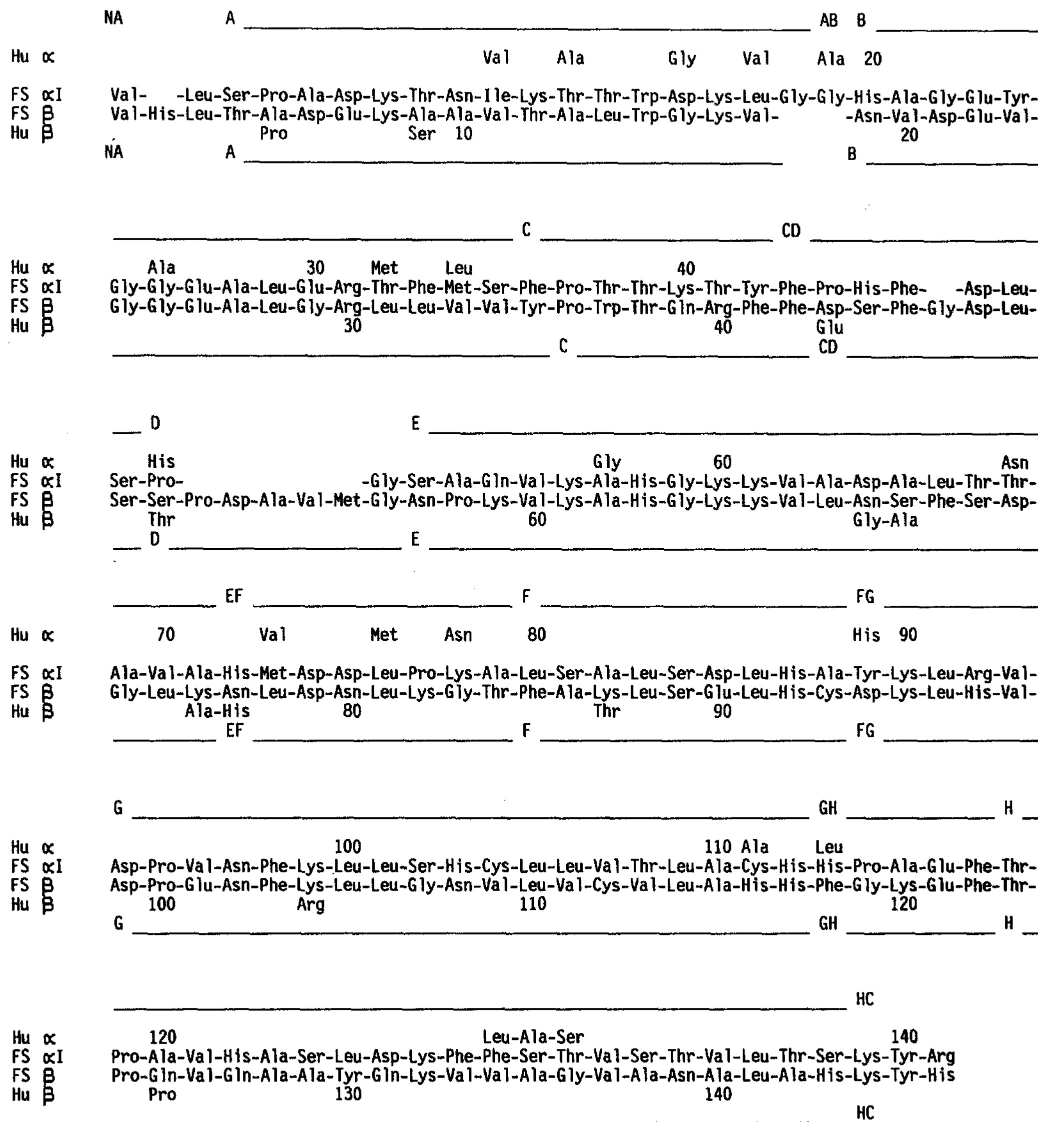

Fig. 4. Amino acids sequence of the α and β chains from fur seal (FS) hemoglobins. The sequences are aligned with the chains of adult human (Hu) hemoglobin. For the latter, only the substituted residues are given. The helices are indicated. The Hb-II differ from the Hb-I at αI/αII: α10 Ile/Val, α34 Met/Thr, α35 Ser/Ala, α50 Pro/His, α78 Lys/Gly, and α131 Thr/Ala.

comparisons in Tables IV and V disclose very interesting results, viz., that within marine Carnivora, an identical β-chain is found in fur seal and walrus; whereas, 3 and 5 differences are seen comparing the αI- and αII-chains, respectively. This is the only identical β-chain is so far detected in any marine Carnivora. The data shows a close relationship in marine carnivora and favors the classification in a common superfamily. It is very surprising, that 6 exchanges are found within two fur seal α-chains (αI and αII), whereas only 4 exchanges are seen when αII from fur seal is compared with α-chain from harbor seal. Lower numbers of exchanges are moreover found when Pinnipedia hemoglobins are compared to mink and polecat (Mustalidae) globin chains sequences. On the contrary, the difference between the polar bear (Ursidae) and pinnipeds is clearly larger. These differences suggest a relationship between Pinnipedia and Mustalidae but not especially between Pinnipedia and Ursidae. Concentrating on the Pinnipedia, there is no common residue that is found only in these four representatives (Tables IV and V). In marine Carnivora, position α34, which involves $\alpha1\beta1$ contacts, is unique, in having Met and Thr that are not in any other representative of terrestrial Carnivora studied so far.

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