Carnivora: The Amino Acid Sequence of the Adult European Polecat (*Mustela putorius*, Mustelidae) Hemoglobins

Aftab Ahmed, Meeno Jahan, Gerhard Braunitzer*

Max-Planck-Institut für Biochemie, Abt. Proteinchemie, D-8033 Martinsried bei München, West Germany

Helmut Pechlaner

Alpenzoo Weiherburg, A-6020 Innsbruck, Austria

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The complete amino acid sequences of the hemoglobins from the adult European polecat *(Mustela putorius)* are presented. The erythrocytes contain two hemoglobin components and three globin chains (αI, αII and β). The primary structure of globin chains and of the tryptic peptides determined in liquid- and gas-phase sequantors. Comparing the sequences of the globin chains of the polecat with that of human Hb-A, 17 (23.9%) substitutions were recognized in the αI, 16 (22.5%) in the αII and 14 (20.4%) in the β chain. A high degree of homology observed with other representatives of the family Mustelidae.

Introduction

The first primary structure of adult human hemoglobin reported by our group in 1961 [1]. After that various kind of hemoglobin have been characterized to date [2]. To study the evolutionary relationship among the different carnivora species, the available data is still limited. From the genus Mustela only amino acid sequence of the β chain from ferret *(Mustela putorius furo)* is reported [3]. In this communication we present complete primary structure of hemoglobins from European polecat, *(Mustela putorius)* a representative of the same genus. On the basis of taxonomy polecat is placed in the order Carnivora, sub-order Fissipedia, family Mustelidae and the genus Mustela. This work is part of our study on molecular characterization of carnivora at hemoglobin level.

Abbreviations: TosPheCH$_2$Cl = (N-tosyl-L-phenylanyl)-chloromethane; Quadrol = N,N,N′,N′-tetrakis-(2-hydroxypropyl)ethylenediamine; reagent IV = trisodium 7-(isothiocyanato)naphthalene-1,3,5-trisulfonate; RP-HPLC = reversed-phase high-performance liquid chromatography.

Enzyme: Trypsin (EC 3.4.21.4).

* Reprint requests to Prof. G. Braunitzer.

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Materials and Methods

Preparation of hemolysate

Blood from a European polecat was collected in heparinized tubes at Zoological Garden, Innsbruck. The erythrocytes were washed three times with physiological saline and lysed with distilled water in the cold [4]. The hemolysate was checked for heterogeneity by polyacrylamide gel disc electrophoresis in Tris/glycine buffer at pH 8.3 [5], as well as under dissociating conditions in the presence of Triton X-100 and 8 M urea [6].

Globin chain separation

Hemoglobin was dehemed in cold aceton solution containing 2% HCl [7]. Globin obtained was reduced under nitrogen for three hours. A column (1.6×15 Cm) was packed with carboxymethylcellulose CM-52 in 0.025 M sodium acetate, 0.2% mercaptoethnol and 8 M urea, pH 5.7. The globin chains were eluted by applying a linear gradient (0.02–0.08 M) NaCl [8].

Enzymatic cleavage and peptide separation

The globin chains were oxidized with performic acid and digested with trypsin (TosPheCH$_2$Cl-treated, Worthington) for 3 h at pH 10.5 and 9.5 with an enzyme/substrate ratio of 5:100 [9–10]. After 3 h, the hydrolysate was titrated to pH 4 and centrifuged. The soluble peptides were fractionated by gel filtration on Sephadex G-25 fine (2.6×150 cm) in 0.1 M acetic acid. The pre-fractioned peaks were rechromatographed by RP-HPLC on a column of LiChrosorb-RP2, (4.6×25 mm) equilibrated in

0.05 M ammonium acetate [11]. Peptides were eluted with a gradient of acetonitrile from 0–60% in 60 min.

Sequence determination

Amino acid sequences were determined by automated Edman degradation [12] in liquid phase sequenators (models 890B and 890C, Beckman Instruments). A modified Quadrol program [13] (0.25 M Quadrol) was applied for sequencing of the intact chains and large lysine peptides which had been reacted with reagent IV [14]. 3-Diethylamino propyne [15] was employed for sequencing of the arginine peptides. Some peptides were sequenced by gas phase method using a non-commercial sequenator [16, 17]. The thiazolinone derivatives were converted to phenylthiohydantoin derivatives in the presence of 3 M TFA at 80 °C, and identified by HPLC [18].

Results and Discussion

The hemoglobin of polecat consist of two components as verified by polyacrylamide gel disc electrophoresis (Fig. 1a). Electrophoresis under dissociating conditions in the presence of Tritone-X100 and 8 M urea revealed two bands corresponding to one α and one β chain (Fig. 1b).

The globin subjected to the column of CM-cellulose resulted in the separation of three polypeptide chains namely αI, αII and β (Fig. 2). This result supports the presence of two hemoglobin components detected by disc electrophoresis.

Pre-fractionation of the tryptic peptides on sephadex-G25 gave some of the peptides in pure form. The peptides mixture were rechromatographed on RP-HPLC led to pure peptides (Fig. 3a and 3b).

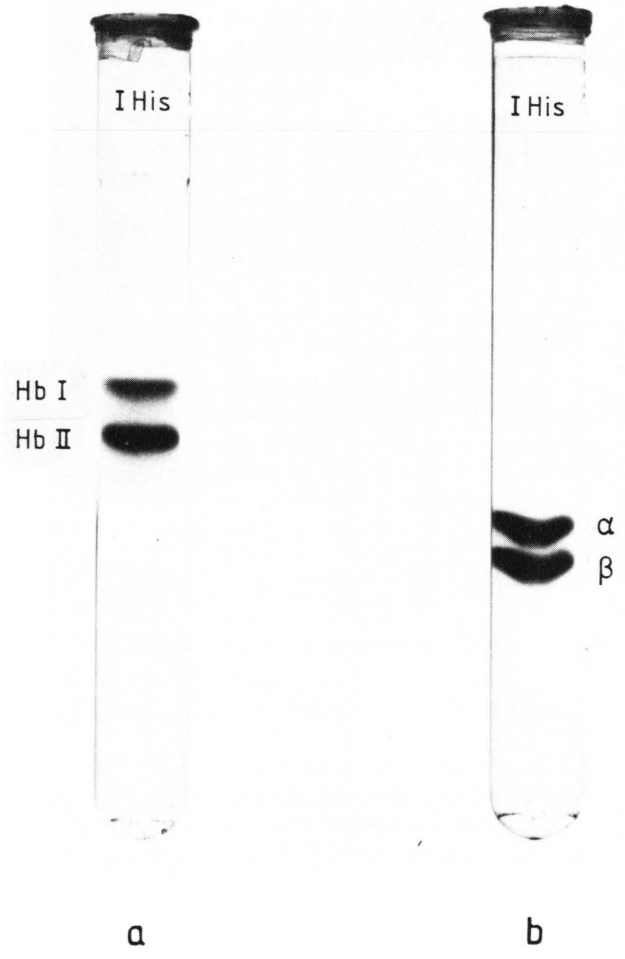

Fig. 1. Electrophoretic pattern of European polecat hemolysate on polyacrylamide gel. (a) Disc at pH 8.3. (b) Under dissociating conditions, in 8 M urea and Triton X-100.

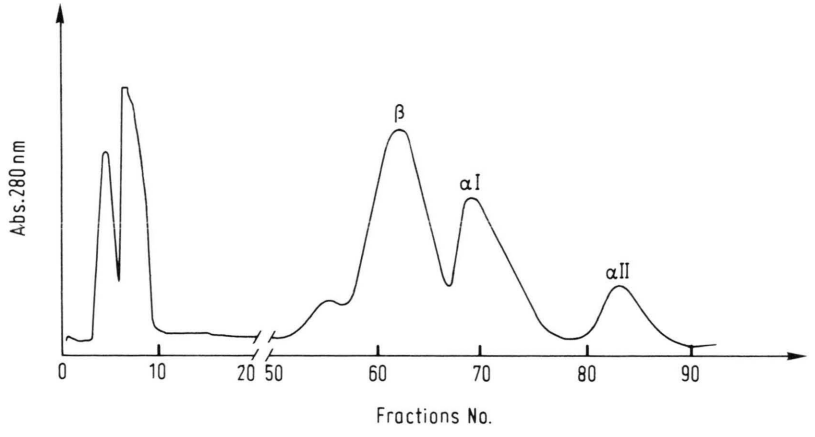

Fig. 2. Separation profile of the globin chains on a column of CM-cellulose (size; 1.6×15 cm). Buffer: 0.025 M sodium acetate, 0.2% mercaptoethanol and 8 M urea, pH 5.7.

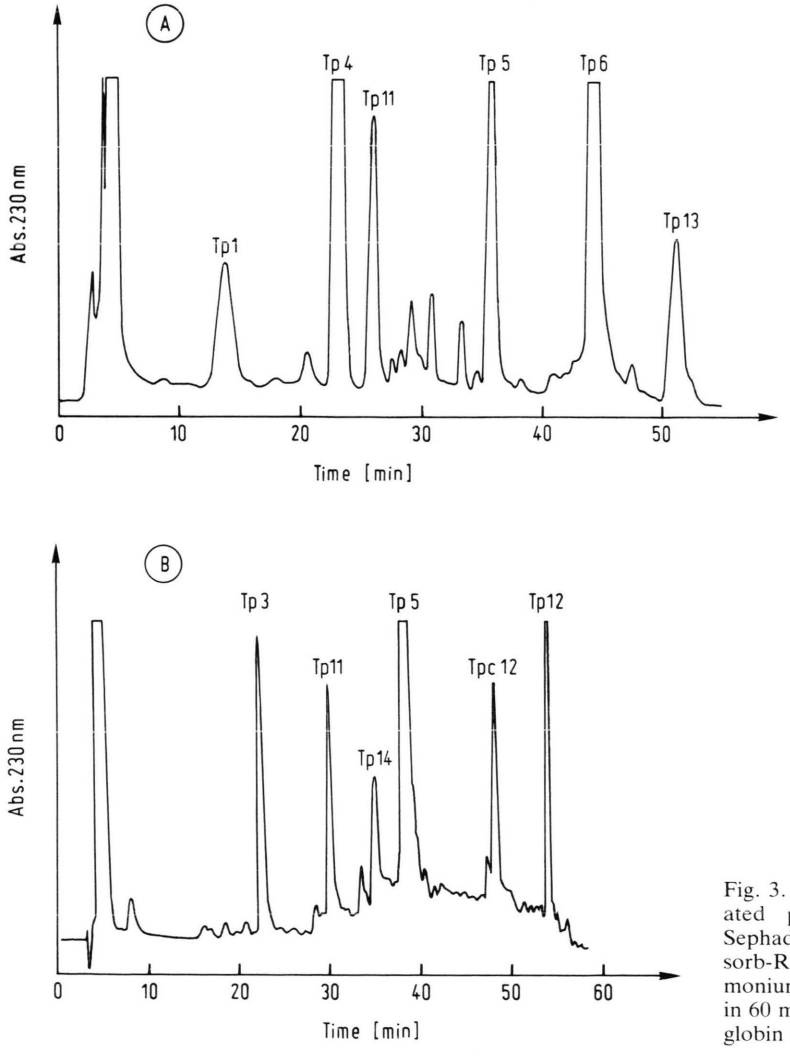

Fig. 3. Rechromatography of the pre-fractionated peaks of the tryptic peptides from Sephadex-G25 on RP-HPLC. Column: LiChrosorb-RP2 (4.6×250 mm). Buffer: 50 mM ammonium acetate; gradient: 0–60% acetonitrile in 60 min; flow rate 1 ml/min. A) Peptides from globin α-chain. B) Peptides from globin β-chain.

The primary structure determination of the globin chains was achieved by sequencing the N-terminal regions of the native chains up to 42 amino acid residues, followed by sequencing of the tryptic peptides. The complete primary structure of the α and β chains is presented in Fig. 4 and the amino acid compositions of all peptides are listed in Table I and II.

The sequence is aligned with that of human hemoglobin, revealing 17 (23.9%) substitution in αI chain, 16 (22.5%) in αII and 14 (20.4%) in the β chain. The exchanges are resulting in the alteration of five functional important positions, $\alpha 1\beta 1$, $\alpha 1\beta 2$ and heme contact points.

For the dimeric structure of hemoglobin $\alpha 1\beta 1$ contacts are very important. At these positions three substitutions were found at: $\alpha 34$ Leu/Ala, $\alpha 111$ Ala/Cys, $\beta 125$ Pro/Gln.

Table I. Amino acid composition of tryptic peptides from European polecat α-chain.

Peptides:	Tp1	Tp2	Tp3	Tp4	Tp5	Tp6	Tp7	Tp8
position:	1–7	8–11	12–16	17–31	32–40	41–56	57–60	61
Asp	0.83	1.2	0.82	–	–	1.15	–	–
Thr	–	0.85	1.02	–	2.83	1.05	–	–
Ser	0.93	–	0.92	–	0.92	1.89	–	–
Glu	–	–	–	3.03	–	1.09	–	–
Pro	0.93	–	–	–	1.27	1.09	–	–
Gly	–	–	–	4.90	–	1.11	1.05	–
Ala	1.06	–	–	2.06	0.97	1.14	1.09	–
Cys*	–	–	–	–	–	–	–	–
Val	1.01	0.88	–	–	–	1.06	–	–
Met*	–	–	–	–	–	–	–	–
Ile	–	–	–	0.99	–	–	–	–
Tyr	–	–	–	0.93	–	1.01	–	–
Phe	–	–	–	–	1.79	2.11	–	–
Trp	–	–	1.00	–	–	–	–	–
His	–	–	–	1.05	–	2.11	0.86	–
Lys	1.13	1.02	1.28	–	1.19	1.13	0.99	1.00
Arg	–	–	–	0.99	–	–	–	–
Sum	7	4	5	15	9	16	4	1

Peptides:	Tp9	Tp10	Tp11	Tp12	Tp13	Tp14
position:	62–90	91–92	93–99	100–127	128–139	140–141
Asp	5.11	–	2.05	1.18	–	–
Thr	1.03	–	–	1.93	2.04	–
Ser	1.84	–	–	1.77	2.74	–
Glu	–	–	–	1.03	–	–
Pro	1.00	–	0.92	2.10	–	–
Gly	1.05	–	–	–	–	–
Ala	7.16	–	–	4.22	1.04	–
Cys*	–	–	–	1.77	–	–
Val	1.96	–	1.98	1.97	2.03	–
Met*	0.97	–	–	–	–	–
Ile	–	–	–	–	–	–
Leu	5.34(5)	1.00	–	6.14	1.02	–
Tyr	0.94	–	–	–	–	0.83
Phe	–	–	0.98	0.96	2.03	–
Trp	–	–	–	–	–	–
His	2.38(2)	–	–	3.87	–	–
Lys	1.19	–	1.06	1.04	1.09	–
Arg	–	0.99	–	–	–	1.14
Sum	29	2	7	28	12	2

* Determined after performic acid oxidation. Values in brackets are taken from sequence data.

Table II. Amino acid composition of tryptic peptides from European polecat β-chain.

Peptides: position:	Tp1 1–8	Tp2 9–17	Tp3 18–30	Tp4 31–40	Tp5 41–59	Tp6 60–61	Tp7 62–65	Tp8 66	Tp9a 67–76
Asp	–	–	2.02	–	3.89	–	–	–	0.98
Thr	1.10	1.04	0.94	0.98	–	–	–	–	–
Ser	–	–	–	–	3.11	–	–	–	1.61
Glu	2.03	–	2.04	1.08	–	–	–	–	0.99
Pro	–	–	–	1.15	2.19	–	–	–	–
Gly	1.03	1.00	2.95	–	1.54(2)	–	0.98	–	1.09
Ala	–	2.83	–	–	1.09	–	0.94	–	–
Cys*	–	–	–	–	–	–	–	–	–
Val	0.59	1.01	2.99	2.05	1.11	0.98	–	–	1.10
Met*	–	–	–	–	0.58(1)	–	–	–	–
Ile	–	–	–	–	–	–	–	–	–
Leu	1.05	1.12	1.04	2.25	1.08	–	–	–	2.08
Tyr	–	–	–	0.68	–	–	–	–	–
Phe	–	–	–	–	2.79	–	–	–	0.96
His	1.15	–	–	–	–	–	0.94	–	–
Trp	–	1.00	–	0.81	–	–	–	–	–
Lys	1.11	0.99	–	–	1.11	1.02	1.01	1.00	1.16
Arg	–	–	0.99	1.06	–	–	–	–	–
Sum	8	9	13	10	19	2	4	1	10

Peptides: position:	Tp9b 77–82	Tp10a 83–87	Tp10b 88–95	Tp11 96–104	Tp12 105–120	Tp13 121–132	Tp14 133–144	Tp15 145–146
Asp	3.22	–	1.14	2.16	1.03	–	1.12	–
Thr	–	0.96	–	–	–	0.94	0.93	–
Ser	–	–	0.81	–	–	–	–	–
Glu	–	–	1.00	0.96	–	3.85	–	–
Pro	–	–	–	0.95	–	1.12	–	–
Gly	–	0.99	–	–	1.93	–	1.11	–
Ala	–	0.98	–	–	1.13	2.11	3.01	–
Cys*	–	–	0.86	–	0.69	–	–	–
Val	–	–	–	0.93	3.15	1.11	2.80	–
Met*	–	–	–	–	–	–	–	–
Ile	–	–	–	–	–	–	–	–
Leu	1.84	–	1.98	0.96	4.16	–	1.10	–
Tyr	–	–	–	–	–	0.82	–	0.84
Phe	–	0.98	–	0.92	0.98	1.00	–	–
His	–	–	1.16	0.93	1.94	–	0.92	1.15
Trp	–	–	–	–	–	–	–	–
Lys	0.93	1.06	1.03	0.98	0.93	1.03	1.02	–
Arg	–	–	–	–	–	–	–	–
Sum	6	5	8	9	16	12	12	2

* Determined after performic acid oxidation. Values in brackets are taken from sequence data.

The $\alpha_1\beta_1$ binding sites are responsible for the construction of tetrameric structure. One alteration is observed at β43 Glu/Asp. The binding sites in the α chain is identical with human hemoglobin.

Among the heme contact points, only one substitution was found at β70 Ala/Ser. This substitution is found frequently in the mammalian hemoglobins. The binding sites in the α chain correspond to human hemoglobin.

In the 2,3-diphosphoglycerate binding sites, which play an important role in the oxygen affinity of hemoglobin, no substitution was detected.

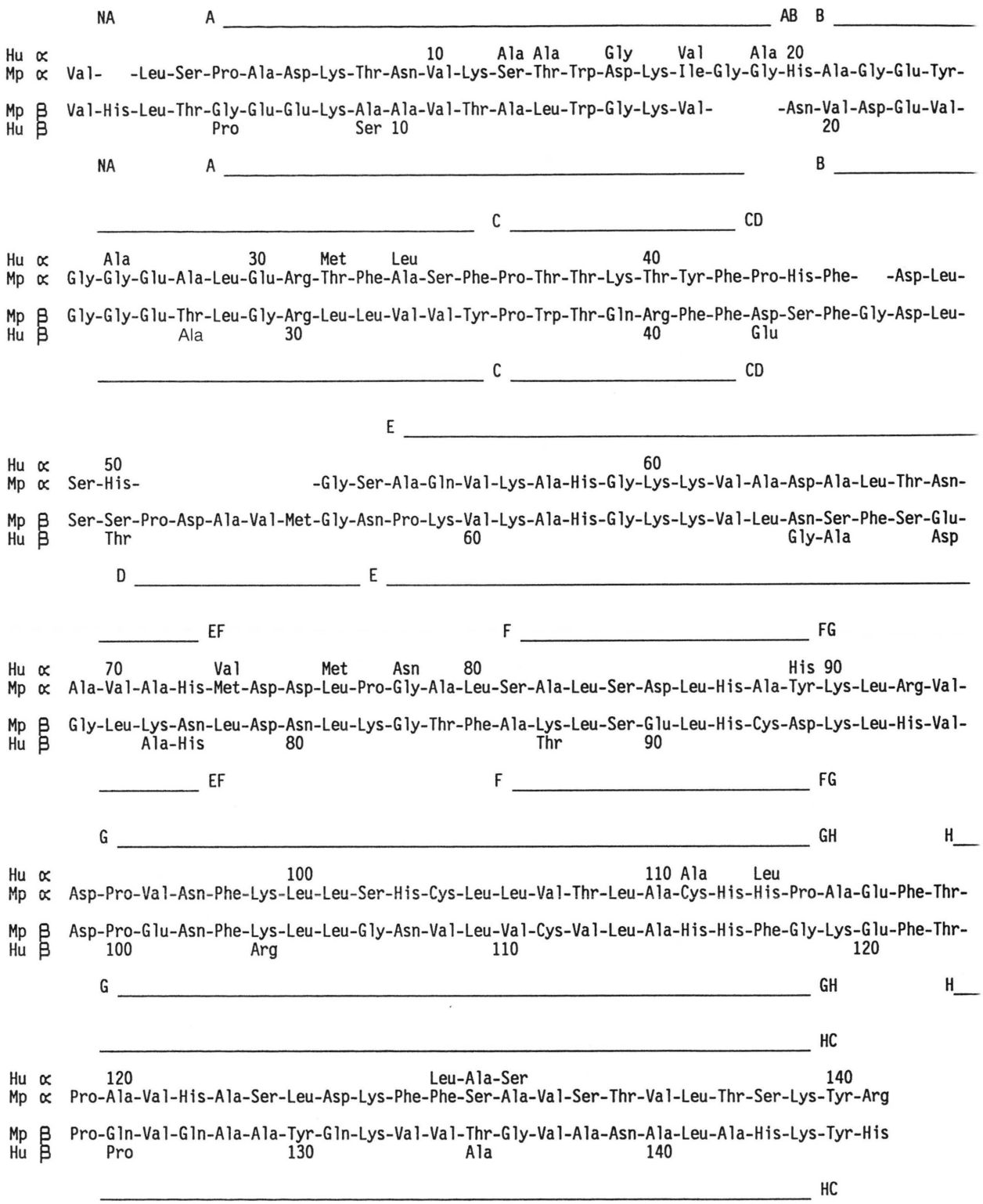

Fig. 4. Amino acid sequence of the α and β chains from European polecat (Mp) hemoglobins. The sequences are aligned with the chains of adult human (Hu) hemoglobin. For the latter only the substituted residues are given. The helices are indicated. The Hb-II differ from the Hb-I at αI/αII: α15 Asp/Gly.

Table III. Amino acid differences between globin chains of polecat and other carnivora. Sequences are compaired to the reference sequence (polecat) in top line. Only differences are marked. Ferret α-chain has not been reported.

α-chains

Positions:	5	8	10	12	34	50	57	73	78	82	129	131	Difference
Polecat*	Ala	Thr	Val	Ser	Ala	His	Ala	Met	Gly	Ala	Phe	Ala	0
Badger		Ala	Ile	Ala			Gly	Leu			Leu	Ser	7
Ratel*	Ser	Ala				Pro	Gly	Gly	Met	Thr		Thr	8
Otter					Val								1

* Polecat at position 15 has αI/αII: Asp/Gly. Ratel at position 34 has αI/αII: Ala/Val.

β-chains

Positions:	5	9	13	27	41	50	56	135	Difference
Polecat	Gly	Ala	Ala	Thr	Phe	Ser	Gly	Thr	0
Ferret							Ser	Ala	2
Badger	Ala	Ser	Ser	Ala	Thr	Thr		Ala	7
Ratel	Ala			Ala				Ala	3
Otter			Ser	Ala				Ala	3

A comparison of primary structures of α and β chains of polecat with those of badger [19–20], ratel [21], common otter [22], and the β chain of ferret [23] is shown in Table III. The total exchanges (α/β) found are as follow; badger (7/7), ratel (8/3), common otter (1/3) and in the β chain of ferret (2). The values obtained shows that more substitutions are located in the α chain except in case of otter where β chain has more substitutions.

It is interesting to note among these five carnivora, representing the same family Mustelidae only polecat and ratel have two α chains (αI and αII). In polecat the alteration observed between two α chains is at αI/αII: 15 Asp/Gly, while in ratel αI/αII: 34 Ala/Val.

The high degree of homologies obtained in the α and β chains of human and of the carnivora chains compared. This support the early observation [23–24] slowed down in the evolutional rate of primate hemoglobin.

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[1] G. Braunitzer, R. Gehring-Muller, N. Hilschmann, K. Hilse, G. Hobom, V. Rudoloff, and B. Wittmann-Liebold, Hoppe-Seyler's Z. Physiol. Chem. **325,** 283 (1961).
[2] T. Kleinschmidt and J. G. Sgouros, Biol. Chem. Hoppe-Seyler **368,** 579 (1987).
[3] Y. Pauplin, Thesis, L'Université de Bordeaux II (1979).
[4] D. L. Drabkin, J. Biol. Chem. **146,** 703 (1946).
[5] B. J. Davis, Ann. N.Y. Acad. Sci. **121,** 404 (1964).
[6] B. P. Alter, S. C. Goff, G. D. Efremov, M. E. Gravely, and T. H. J. Huisman, Br. J. Haematol. **44,** 527 (1980).
[7] M. L. Anson and A. F. Mirsky, J. Gen. Physiol. **13,** 469 (1930).
[8] J. B. Clegg, M. A. Naughton, and D. J. Weatherall, J. Mol. Biol. **19,** 91 (1966).
[9] C. H. W. Hirs, W. H. Stein, and S. Moore, J. Biol. Chem. **221,** 151 (1956).
[10] C. H. W. Hirs, Methods Enzymol. **11,** 218 (1967).
[11] H. Kratzin, C. Yang, J. U. Krusche, and N. Hilschmann, Hoppe-Seyler's Z. Physiol. Chem. **361,** 1591 (1980).
[12] P. Edman and G. Begg, Eur. J. Biochem. **1,** 80 (1967).
[13] G. Braunitzer, B. Schrank, and A. Ruhfus, Hoppe-Seyler's Z. Physiol. Chem. **351,** 1589 (1970).

[14] J. Pfletschinger and G. Braunitzer, Hoppe-Seyler's Z. Physiol. Chem. **361,** 925 (1980).
[15] G. Braunitzer, B. Schrank, A. Stangl, and U. Scheithauer, Hoppe-Seyler's Z. Physiol. Chem. **359,** 137 (1978).
[16] R. M. Hewick, M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer, J. Biol. Chem. **256,** 7990 (1981).
[17] G. Begg, P. Rücknagel, J. Godovac-Zimmermann, and G. Braunitzer, Biol. Chem. Hoppe-Seyler **367,** 81 (1986).
[18] C. L. Zimmermann and J. J. Pisano, Methods Enzymol. **47,** 45 (1977).
[19] I. Hombrados, S. Ducastaing, A. Iron, E. Neuzil, B. Debuire, and K. Hank, Biochim. Biophys. Acta **427,** 107 (1976).
[20] I. Hombrados, E. Neuzil, B. Debuire, and K. Han, Biochim. Biophys. Acta **535,** 1 (1978).
[21] K. Rodewald, G. Braunitzer, and R. Göltenboth, Biol. Chem. Hoppe-Seyler **369,** 1137 (1988).
[22] H. Lin, T. Kleinschmidt, G. Braunitzer, and H. G. Scheil, Biol. Chem. Hoppe-Seyler **369,** 349 (1988).
[23] M. Goodman, J. Barnabas, G. Matsuda, and W. Moore, Nature **233,** 604 (1971).
[24] G. Matsuda, Prog. Mol. Anthropol. (Goodman and R. E. Tashian (eds)), pp. 223–237, Plenum Press, New York (1976).