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

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The complete amino acid sequences of the hemoglobins from the adult European mink (*Mustela lutreola*) are presented. The erythrocytes contain two hemoglobin components and three globin chains. The isolation of globin chains achieved by ion-exchange chromatography on a column of CM-cellulose in 8 M urea buffer. The primary structure of globin chains and of the tryptic peptides determined in liquid- and gas-phase sequenators. The alignment of the α- and β-chains with those of reported sequences from other carnivora species belonging to the family Mustelidae may give an insight into the evolution of this molecule.

Introduction

The Carnivora is a well defined order of mammalia, presenting various behavioural patterns and comprising many interesting species. They are therefore of great value for systematic and evolutionary studies. To achieve these goals primary structure of hemoglobins from various Carnivora species representing the family Mustelidae have been reported by our group [1–3]. The present study is another contribution to characterize the hemoglobins from European mink a member of the same family. On the basis of taxonomy mink is placed in the order Carnivora, sub-order Fissipedia, family Mustelidae and the genus *Mustela*. This report continues our study on molecular characterization of Carnivora at hemoglobin level.

Materials and Methods

Preparation of hemolysate

Blood from a European mink was collected in heparinized tubes at Zoological Garden, Helsinki. 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 acetone solution containing 2% HCl [7]. Globin obtained was reduced under nitrogen for 3 h. A column (1.6 × 15 cm) was packed with carboxymethylcellulose CM-52 in 0.025 M sodium acetate, 0.2% mercaptoethanol 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 ($TosPheCH_2Cl$-treated, Worthington) for 1 h at pH 10.5, and for another 2 h at pH 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 part was fractionated by gel filtration on Sephadex G-25 fine (2.6 × 150 cm) in 0.1 M acetic acid. The pre-fractioned peaks were re-chromatographed by

† Deceased on May 27th, 1989.

Enzyme: Trypsin (EC 3.4.21.4).
Abbreviations: $TosPheCH_2Cl$, (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.

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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 890 B and 890 C, 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 European mink consist of two components as verified by polyacrylamide gel disc electrophoresis (Fig. 1 a). Electrophoresis under dissociating conditions in the presence of Triton X-100 and 8 M urea revealed two bands corresponding to one α- and one β-chain (Fig. 1 b).

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

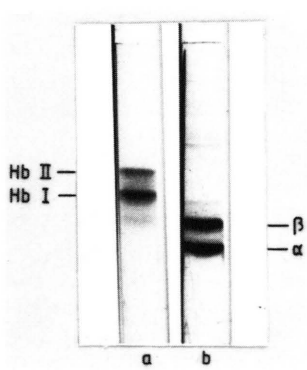

Fig. 1. Electrophoretic pattern of European mink hemolysate on polyacrylamide gel. (a) Disc at pH 8.3; (b) under dissociating conditions, in 8 M urea and Triton X-100.

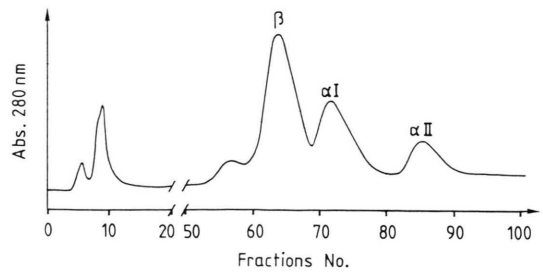

Fig. 2. Separation profile of the globin chains on a column of CM-cellulose (size, 1.6 × 15 cm). Buffer, 0.025 M sodium acetate, 0.2% mercaptoethanol and 8 M urea, pH 5.7.

The characterization of the α- and β-globin chains was achieved by sequencing the N-terminal regions of the native chains up to 42 residues, followed by sequencing of the tryptic peptides. The structural difference in the two hemoglobin components confined to the α-chain at α 15: α I/α II Asp/Gly. The complete primary structure of the α- and β-chains is presented in Fig. 3 and the amino acid compositions of all peptides are listed in Table I and II.

In comparison with human hemoglobin it shows 19 (26.7%) substitution in α I-chain, 18 (25.3%) in α II- and 13 (18.9%) 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. All of the four 2,3-diphosphoglycerate contact sites in the β-chain are found to be invariant.

The $\alpha_1\beta_1$ contacts are very important for the dimeric structure of hemoglobin. Analysis of the relevant residues show a total of three differences occur two in the α-chain at α 34 Leu/Ala and α 111 Ala/Cys whereas in the β-chain only one residue is altered at β 125 Pro/Gln.

The $\alpha_1\beta_2$ interface that is important for the stability of tetramer and also for the cooperative binding of the oxygen. In mink hemoglobin one of these contacts is exchanged in the β-chain at β 43 Glu/Asp, however in the α-chain all contact points are identical with human hemoglobin.

One residue, involved in the heme contact, is substituted in the β-chain at β 70 Ala/Ser. This substitution is found frequently in the mammalian

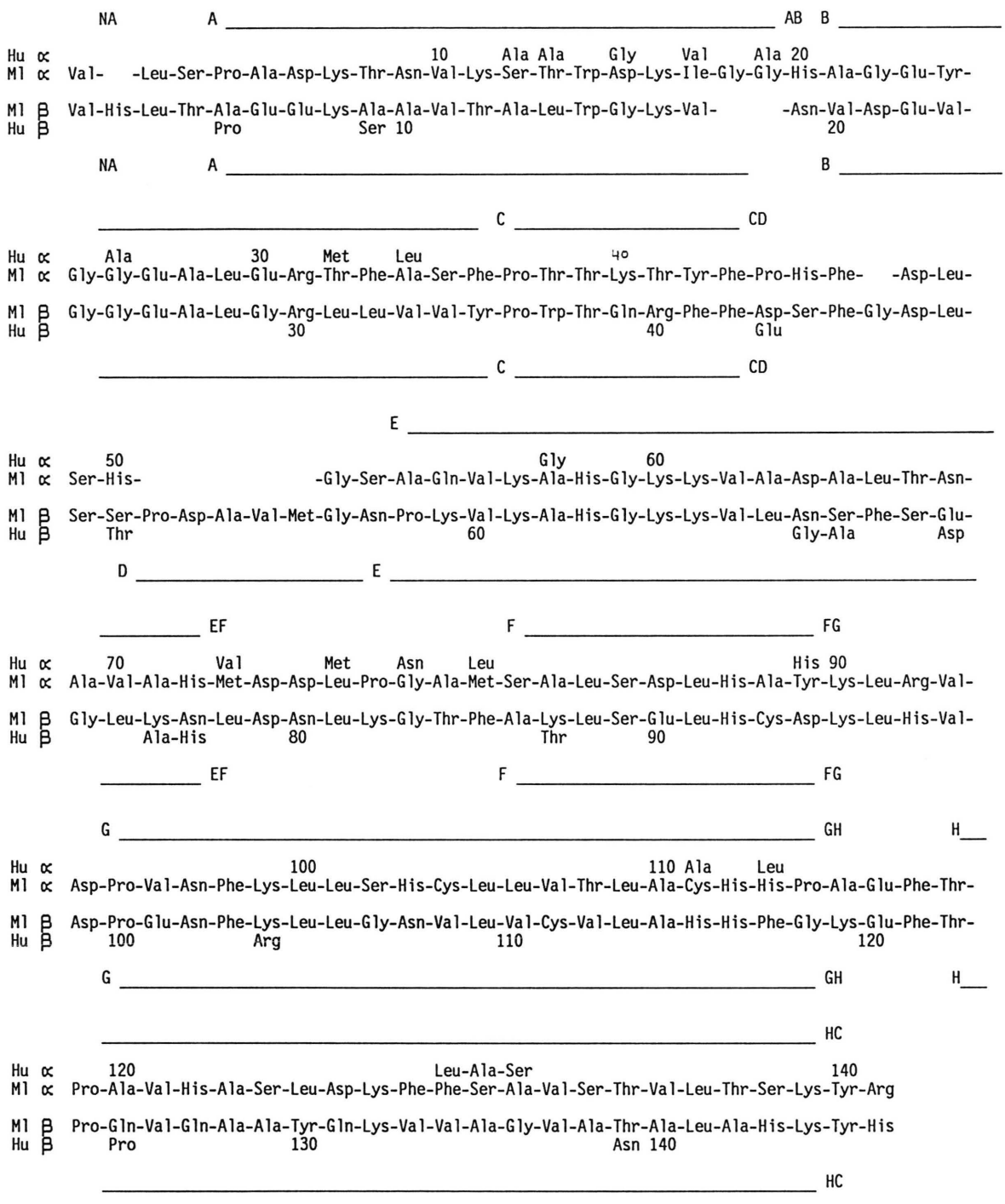

Fig. 3. Amino acid sequence of the α- and β-chains from European mink (M l) hemoglobins. The sequences are aligned with the chains of adult human (Hu) hemoglobin. In case of human hemoglobin only the substituted residues are given. The helices are indicated. The Hb-II differ from the Hb-I at α I/α II: α 15 Asp/Gly.

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

Peptides:	Tp 1	Tp 2	Tp 3	Tp 4	Tp 5	Tp 6	Tp 7
Position:	1–7	8–11	12–16	17–31	32–40	41–56	57–60
Asp	1.13	1.07	1.00	–	–	1.08	–
Thr	–	0.78	0.99	–	2.69	0.95	–
Ser	0.76	–	0.84	–	0.98	1.66	–
Glu	–	–	–	3.13	–	0.99	–
Pro	1.03	–	–	–	1.08	1.18	–
Gly	–	–	–	5.04	–	1.01	1.09
Ala	0.89	–	–	2.02	1.20	1.22	1.00
Cys*	–	–	–	–	–	–	–
Val	0.92	0.99	–	–	–	1.05	–
Met*	–	–	–	–	–	–	–
Ile	–	–	–	0.99	–	–	–
Leu	1.06	–	–	1.02	–	1.17	–
Tyr	–	–	–	0.84	–	0.84	–
Phe	–	–	–	–	1.90	1.80	–
Trp	–	–	1.00	–	–	–	–
His	–	–	–	1.03	–	2.02	0.89
Lys	1.09	1.14	1.15	–	1.13	0.99	1.02
Arg	–	–	–	0.96	–	–	–
Sum	7	4	5	15	9	16	4

Peptides:	Tp 8	Tp 9	Tp 10	Tp 11	Tp 12	Tp 13	Tp 14
Position:	61	62–90	91–92	93–99	100–127	128–139	140–141
Asp	–	4.94	–	2.00	1.23	–	–
Thr	–	1.04	–	–	1.90	1.89	–
Ser	–	1.88	–	–	1.88	2.60	–
Glu	–	–	–	–	1.17	–	–
Pro	–	1.15	–	1.06	2.24	–	–
Gly	–	1.15	–	–	–	–	–
Ala	–	7.02	–	–	3.92	1.25	–
Cys*	–	–	–	–	1.92	–	–
Val	–	2.01	–	1.94	2.03	2.07	–
Met*	–	1.98	–	–	–	–	–
Ile	–	–	–	–	–	–	–
Leu	–	3.93	0.91	–	5.75	1.31	–
Tyr	–	0.92	–	–	–	–	0.76
Phe	–	–	–	0.91	1.08	1.92	–
Trp	–	–	–	–	–	–	–
His	–	2.03	–	–	3.91	–	–
Lys	1.00	1.10	–	1.06	1.17	1.04	–
Arg	–	–	1.08	–	–	–	0.99
Sum	1	29	2	7	28	12	2

* Determined after performic acid oxidation.

hemoglobins. The heme binding sites in the α-chain correspond to human hemoglobin.

Comparison of the primary structure of α- and β-chains of mink hemoglobin with those of other Carnivora entails the following three genera of the family Mustelidae, mink, polecat, ferret *(Mustela)* [1, 19], otter *(Lutra)* [2], ratel *(Mellivora)* [3], and bager *(Meles)* [20–21].

The sequence alignment revealed high degree of homology. The total differences α/β obtained are as follow; polecat (1/4), bager (8/4), ratel (9/1), otter (2/2) and in ferret β-chain (4).

Table II. Amino acid composition of tryptic peptides from European mink 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	–	4.21	–	–	–	1.00
Thr	0.89	0.89	–	0.88	–	–	–	–	–
Ser	–	–	–	–	2.35 (3)	–	–	–	1.71
Glu	2.06	–	2.03	1.08	–	–	–	–	1.05
Pro	–	–	–	1.01	2.12	–	–	–	–
Gly	–	1.04	2.99	–	2.04	–	0.78	–	1.09
Ala	1.05	2.64	1.03	–	1.28	–	0.84	–	–
Cys*	–	–	–	–	–	–	–	–	–
Val	0.94	1.18	2.92	1.95	1.19	1.02	–	–	1.02
Met*	–	–	–	–	0.58 (1)	–	–	–	–
Ile	–	–	–	–	–	–	–	–	–
Leu	1.02	1.16	1.00	2.21	1.13	–	–	–	2.01
Tyr	–	–	–	0.78	–	–	–	–	–
Phe	–	–	–	–	2.78	–	–	–	0.95
Trp	–	1.00	–	–	–	–	–	–	–
His	0.94	–	–	–	–	–	0.82	–	–
Lys	1.06	1.10	–	–	1.11	1.08	1.21	1.00	1.11
Arg	–	–	1.00	1.04	–	–	–	–	–
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	2.93	–	1.08	1.92	1.01	–	–	–
Thr	–	0.96	–	–	–	1.03	1.04	–
Ser	–	–	0.81	–	–	–	–	–
Glu	–	–	1.09	1.05	–	3.98	–	–
Pro	–	–	–	1.11	–	–	–	–
Gly	–	0.84	–	–	2.05	–	1.04	–
Ala	–	0.93	–	–	1.04	2.19	4.05	–
Cys*	–	–	0.93	–	0.93	–	–	–
Val	–	–	–	1.00	2.99	1.08	2.81	–
Met*	–	–	–	–	–	–	–	–
Ile	–	–	–	–	–	–	–	–
Leu	1.99	–	1.91	0.98	3.96	–	0.99	–
Tyr	–	–	–	–	–	0.58	–	0.81
Phe	–	0.98	–	0.87	0.90	0.91	–	–
Trp	–	–	–	–	–	–	–	–
His	–	–	0.97	0.99	2.00	0.64	0.99	1.02
Lys	1.09	1.06	1.17	1.04	1.07	1.12	1.05	–
Arg	–	–	–	–	–	–	–	–
Sum	6	5	8	9	16	12	12	2

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

As shown in the Table III, positions α80 Leu and β139 Asn are invariant in all other members of the family Mustelidae. In mink hemoglobin both of these residues are altered, α80 Leu/Met and β139 Asn/Thr. The total substitutions between mink and otter are four whereas the maximum is thirteen which is found between mink and badger hemoglobin. It is interesting to note that mink and polecat which belong to the same genus *(Mustela)*, have five amino acid differences whereas mink compared with otter, representing the genus *(Lutra)*, has only four substitutions.

Table III. Amino acid differences between globin chains of European mink and other carnivora. Sequences are compared to the reference sequence (mink) 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	80	82	129	131	Difference
Mink*	Ala	Thr	Val	Ser	Ala	His	Ala	Met	Gly	Met	Ala	Phe	Ala	0
Polecat*										Leu				1
Badger		Ala	Ile	Ala			Gly	Leu		Leu		Leu	Ser	8
Ratel*	Ser	Ala				Pro	Gly	Gly	Met	Leu	Thr		Thr	9
Otter					Val					Leu				2

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

β-chains

Positions:	5	9	13	27	41	50	56	135	139	Difference
Mink	Ala	Ala	Ala	Ala	Phe	Ser	Gly	Ala	Thr	0
Polecat	Gly			Thr				Thr	Asn	4
Ferret	Gly			Thr			Ser		Asn	4
Badger		Ser	Ser		Tyr	Thr			Asn	5
Ratel									Asn	1
Otter			Ser						Asn	2

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