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The amino acid sequence of the α- and β-chains of the two hemoglobins of the Antarctic fish *Notothenia coriiceps neglecta*

Rossana D'Avino, Carla Caruso, M. Eugenia Schininà*, Bruno Rutigliano, Mario Romano, Laura Camardella, Francesco Bossa*, Donatella Barra* and Guido di Prisco

Institute of Protein Biochemistry and Enzymology, CNR, Via Toiano 6, 80072 Arco Felice, Naples and **Department of Biochemical Sciences and CNR Center of Molecular Biology, University, 'La Sapienza', Rome, Italy*

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The blood of the Antarctic fish *Notothenia coriiceps neglecta* contains two hemoglobins, Hb 1 and Hb 2, which have a β-chain in common. We have elucidated the primary structure of the β-chain (146 residues) and of the α-chains (142 residues) of the two hemoglobins. The two α-chains differ from each other by 51 residues; in comparison with globin sequences of temperate fishes, the α-chain of Hb 1 is more similar to that of bluefin tuna than to the α-chain of Hb 2 of the same species.

Cold adaptation; Hemoglobin; Amino acid sequence; Antarctica fish; (*Notothenia coriiceps neglecta*)

1. INTRODUCTION

During the Paleozoic and Mesozoic, Antarctica, South America, Africa, India, Australia and New Zealand were part of the supercontinent of Gondwana, which began to break up near the Jurassic-Cretaceous boundary, approximately 135 million years ago. The drift then carried Antarctica close to its present position about 65 million years ago; glaciation and ice sheet formation may have begun 25 million years later [1–3]. Following the opening of the Drake Passage near the Oligocene-Miocene boundary 22–25 million years ago [4,5], which permitted the onset of the Circum-Antarctic Current and the resulting development of the Antarctic Convergence, the cooling of the environment south of the latter steadily proceeded to reach the present, extreme climatic conditions.

The four major families of Antarctic fishes (Nototheniidae, Bathydraconidae, Harpagiferidae and Channichthyidae) have remained isolated south of the Convergence for over 25 million years; their physiology has gradually become adjusted to the progressive cooling. Being exposed to unique ambient conditions (the water temperature is constantly near −1.87°C, the equilibrium temperature of the ice-salt water mixture: fishes from temperate waters would rapidly freeze), fishes are cold adapted [6] and, in fact, their exposure to water temperatures of a few degrees above zero has lethal effects [7]. Thus to marine organisms the Convergence acts as a natural barrier in both directions.

The blood of Antarctic fishes has acquired some features which clearly differentiate them from fishes of temperate and tropical climates. It contains fewer erythrocytes and less hemoglobin [8]; the resulting decrease in viscosity greatly facilitates the cardiac work. However, at the temperature of the environment, oxygen is more soluble in sea water and the oxygen affinity for hemoglobin is higher. On the other hand the few erythrocyte-like cells of the family Channichthyidae, which represent the extreme stage of such evolution, are completely hemoglobinless [9].

In view of these considerations, an investigation

Correspondence address: G. di Prisco, Institute of Protein Biochemistry and Enzymology, CNR, Via Toiano 6, 80072 Arco Felice, Naples, Italy

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was initiated on the relationship between the molecular structure and the oxygen-binding properties of Antarctic fish hemoglobin. This communication describes the complete primary structure of the two hemoglobins of *Notothenia coriiceps neglecta*, a cold-adapted Antarctic teleost.

2. EXPERIMENTAL

Specimens of *N. coriiceps neglecta* (genus, *Notothenia*; family, Nototheniidae) were collected as described [10]. Trypsin (treated with tosylphenylalanylchloromethane) and chymotrypsin were from Worthington Biochemical Co., *Staphylococcus aureus* V8 endoproteinase Glu-C from Boehringer Mannheim, carboxypeptidases A and B from Sigma Chemical Co., HPLC-grade acetonitrile from Carlo Erba, Sequencer reagents from Applied Biosystems.

The hemoglobins and their globin chains were purified by ion-exchange chromatography and by reverse-phase HPLC, respectively [10]. *S*-Carboxymethylated globins were digested with trypsin and chymotrypsin [11]; Hb 2 α-chains were cleaved by CNBr [12] and some of the CNBr fragments further digested with endoproteinase Glu-C [13]. Deacylation of the amino-terminus of α-chains was achieved as described in [11]. Cleavage of the Asp-Pro bond was performed according to [14].

Peptide mixtures from proteolytic and chemical cleavages were separated by reverse-phase HPLC [11]. Amino acid analysis and amino acid sequence analysis by manual Edman degradation were performed as in [11]. Automated Edman degradation was carried out using protein-peptide sequencing systems, models 470 and 477A from Applied Biosystems, equipped with a 120A PTH analyser.

The oxygen saturation of hemoglobin was measured calculating the average of the absorbance difference at three wavelengths (540, 560 and 575 nm) between the spectra observed at a given pH before and after addition of a few crystals of sodium dithionite, which caused complete deoxygenation of the hemoglobin. Oxygen equilibrium curves were obtained tonometrically as described [15]. Buffers were 100 mM Tris-HCl, pH 8.5–7.5, and Bistris-HCl, pH 7.5–6.0.

3. RESULTS AND DISCUSSION

The erythrocytes of *N. coriiceps neglecta* contain two major hemoglobins [10], Hb 1 (85–90% of total) and Hb 2 (5–10%). In Antarctic fishes, hemoglobin diversity is very limited: one or two components have been detected in many species [16,17]. In contrast, most non-Antarctic fishes have multiple components [18], that in fast swimmers often show functional differences in oxygen binding. Such a variety is probably not required in an environment with relatively constant physicochemical features, therefore Antarctic fishes do not need more than one major hemoglobin.

Hb 1 and Hb 2 have the β-chain in common and differ by the α-chain [10]. The amino acid sequence of the α-chain of Hb 1 had been established previously [11].

The sequence of the α-chain of Hb 2 was elucidated by automated Edman degradation of HPLC-purified peptides, formed after cleavage of the protein with CNBr, endoproteinase Glu-C, trypsin, and after cleavage of the Asp-Pro bond [14] at position 96, followed by reaction with *o*-phthalaldehyde [19] before sequencing. In addition to analogy with the sequences of α-chains of hemoglobins from other fishes [11,20–23], these procedures provided all the necessary overlaps to reconstruct the sequences. Similar to Hb 1 α-chain [11], the N-terminus was acetylated, as demonstrated by fast-atom-bombardment mass spectrometry.

The sequence of the β-chain, common to Hb 1 and Hb 2, was established by automated Edman degradation of the intact protein (47 residues, starting from the N-terminus). Tryptic peptides were fractionated by HPLC and sequenced to position 104 by manual and automated degradation; their alignment was unequivocally established by analogy with the corresponding sequences of β-chains of hemoglobin from other fishes [20, 24–26]. The cleavage of the peptide bond between Asp-99 and Pro-100 [14], followed by reaction with *o*-phthalaldehyde [19], allowed the determination of the sequence between position 100 and 134 by automated Edman degradation. A manually sequenced, overlapping tryptic peptide extended the sequence to position 143. The C-terminal sequence of the globin chain, determined by carboxypeptidase digestion and by manual sequence of a tryptic peptide, is Gln-Tyr-His.

Fig.1 illustrates the complete sequence of the β-chain and of the two α-chains.

Table 1 shows the similarity among the α- and β-chains of Hb 1 and Hb 2 and most of the few available sequences of teleosts living under totally different conditions: carp (*Cyprinus carpio*), trout (*Salmo irideus*), catostomid fish (*Catostomus clarkii*) and bluefin tuna (*Thunnus thynnus*). The degree of sequence identities is a further indication that in this protein the primary structure is highly conserved, as shown also by the conservation of

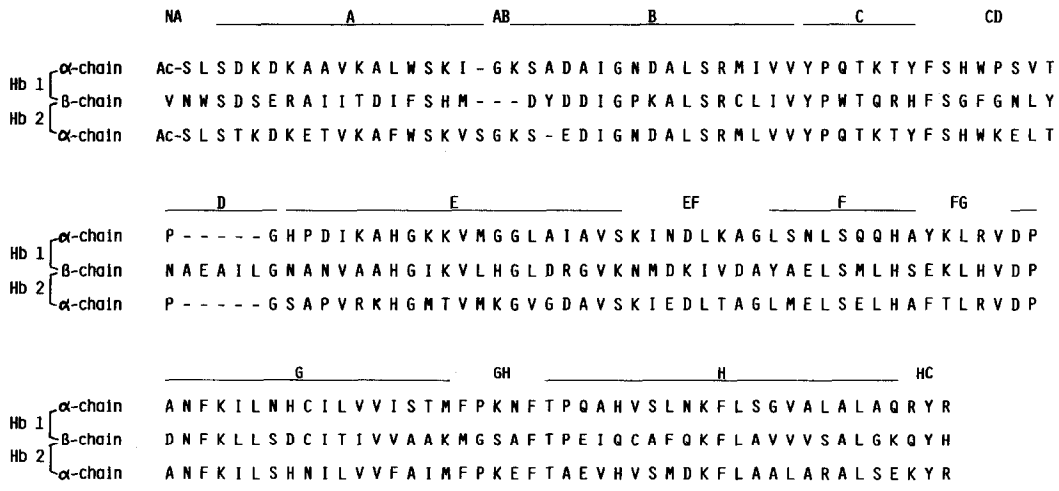

Fig.1. Alignment of the amino acid sequence of α- (142 residues) and β-chain (146 residues) of *N. coriiceps neglecta* Hb 1 and Hb 2. The helical (A, B, C, D, E, F, G, H) and non-helical (AB, CD, EF, FG, GH and HC) regions, as established for mammalian hemoglobins, are indicated above; in α-chains, helix D is lacking.

the domains of structural importance, including the residues known to be invariant in the vertebrate globin chains [27]. It is worth noting that the α-chain of Hb 1 of *N. coriiceps neglecta* shows a higher sequence identity with the α-chain of bluefin tuna hemoglobin (phylogenetically less remote from the Nototheniid than the others) than with the α-chain of Hb 2. This peculiarity exists, to a larger extent, in trout β-chain sequences [26]: trout Hb IV is more similar to carp hemoglobin than to trout Hb I. However, Hb I and Hb IV are functionally different.

Both Hb 1 and Hb 2 of *N. coriiceps neglecta* displayed a large, negative, alkaline Bohr effect, indicating a sharp pH dependence (enhanced by the presence of the effectors ATP and inositol hexaphosphate) of the oxygen affinities and cooperativity; the Root effect [28] was also observed in both components [10,29], as indicated by a decrease in oxygen saturation at lower pH, even

Table 1

Sequence identity (%) in α- and β-chains of fish hemoglobins

Species	*S. irideus* I	*C. carpio*	*C. clarkii*	*T. thynnus*	*N. coriiceps negl.* Hb 2
α-chains					
N. coriiceps neglecta Hb 1	55	59	58	73	64
N. coriiceps neglecta Hb 2	62	63	58	68	
T. thynnus	65	66	66		
C. clarkii	65	91			
C. carpio	66				

	S. irideus IV	*S. irideus* I	*C. carpio*	*T. thynnus*
β-chains				
N. coriiceps neglecta	63	53	57	66
T. thynnus	61	55	60	
C. carpio	73	64		
S. irideus I	59			

in the presence of the ligand at atmospheric pressure (over-stabilisation of the low affinity T state). The β-chain, common to Hb 1 and Hb 2, contains His-146 and Ser-93, postulated to be responsible for the Root effect [30]. The physiological meaning of Hb 2, a relatively minor component, functionally similar to Hb 1, is not clear; however, Hb 2 might conceivably be an evolutionary remnant.

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