Structure, function and molecular adaptations of haemoglobins of the polar cartilaginous fish *Bathyrja eatonii* and *Raja hyperborea*

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Cartilaginous fish are very ancient organisms. In the Antarctic sea, the modern chondrichthyan genera are poorly represented, with only three species of sharks and eight species of skates; the paucity of chondrichthyans is probably an ecological consequence of unusual trophic or habitat conditions in the Southern Ocean. In the Arctic, there are 26 species belonging to the class Chondrichthyes. Fish in the two polar regions have been subjected to different regional histories that have influenced the development of diversity: Antarctic marine organisms are highly stenothermal, in response to stable water temperatures, whereas the Arctic communities are exposed to seasonal temperature variations. The structure and function of the oxygen-transport haem protein from the Antarctic skate *Bathyrja eatonii* and from the Arctic skate *Raja hyperborea* (both of the subclass Elasmobranchii, order

Rajiformes, family Rajidae) is reported in the present paper. These species have a single major haemoglobin (Hb 1; over 80 % of the total). The Bohr-proton and the organophosphate-binding sites are absent. Thus the haemoglobins of northern and southern polar skates appear functionally similar, whereas differences were observed with several temperate elasmobranchs. Such evidence suggests that, in temperate and polar habitats, physiological adaptations have evolved along distinct pathways, whereas, in this case, the effect of the differences characterizing the two polar environments is negligible.

Key words: Antarctic, Arctic, Bohr effect, haemoglobin, phosphate binding, skate.

INTRODUCTION

Oxygen carriers are ancient proteins, probably evolved from enzymes that protected the organism against oxygen toxicity [1]. When multicellular organisms increased in size and complexity, their surface/volume ratios diminished, and simple diffusion of oxygen across the body wall was inadequate to reach all cells. The evolution of simple oxygen-binding proteins into multisubunit circulating proteins, in combination with the advent of circulatory systems, made the transport of oxygen from the periphery of the organism to cells possible. The evolution of the tetramer, including duplication and differentiation of the globin gene into α and β genes, formation of subunit interfaces, and quaternary-structure allosteric changes took place within a relatively short period between the branching points of hagfish and lampreys from cartilaginous fish. The separation of sharks from bony fish occurred near the α/β gene duplication [2].

In Antarctica, the chondrichthyan genera are poorly represented in the modern fauna, with only three species of sharks and eight species of skates [3], representing approx. 4 % of the total fauna. The late-Cretaceous and late-Eocene fossils from Seymour and James Ross Islands are proof that many modern chondrichthyan genera inhabited the temperate waters of the Weddellian Province [4].

On the other hand, Notothenioidei are a large group of marine teleosts largely endemic to the Antarctic Ocean. Indirect indications suggest that notothenioids appeared in the early Tertiary,

filling the ecological void on the shelf left by most of the other fish fauna (which became locally extinct during maximal glaciation), and began to diversify in the middle Tertiary. Reduced competition and increasing isolation favoured speciation. Notothenioids fill a varied range of ecological niches normally occupied by taxonomically diverse fish communities in temperate waters.

In this environment, the low metabolic demand and the high oxygen concentration reduce the need for Hb. The vast majority of species of the dominant suborder Notothenioidei have a single Hb, sometimes accompanied by a minor component [5]. Of the 213 species living on the shelf or upper slope of the Antarctic continent, 96 are notothenioids [6].

Why are there so few chondrichthyans in Antarctica? There is no obvious physiological reason for their scarcity. The freezing point of the blood plasma of *Squalus acanthias* (spiny dogfish) is -1.95°C [7]. Since sea water freezes at -1.86°C , this would seem to provide adequate protection against freezing, provided there is no contact with ice. Perhaps the scarcity of chondrichthyans in the modern fauna is an ecological consequence of unusual trophic or habitat conditions in the Southern Ocean [8]. In addition, the reduced diversity of teleost fishes in the Antarctic midwaters may have restricted the entry of sharks into the ecosystem.

Besides the shark species, in the Antarctic region, there are two species of *Raja* and six species of *Bathyrja*, the dominant family south of 60°S [9]. *Bathyrja* lacks a fossil record. The genus includes 45 extant species distributed worldwide and is supposed to have originated at least 100 million years ago [10]. Stehmann

Abbreviations used: 2,3-BPG, 2,3-bisphosphoglycerate; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; PDB, Protein Data Bank.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY772716 (*Bathyrja eatonii* α -chain nucleotide sequence), AY772717 (*Bathyrja eatonii* β -chain nucleotide sequence), AY773131 (*Raja hyperborea* α -chain nucleotide sequence) and AY773132 (*Raja hyperborea* β -chain nucleotide sequence). The amino acid sequence data reported will appear in Swiss-Prot Protein Database under the accession numbers P84216 (*Bathyrja eatonii* α -chain amino acid sequence) and P84217 (*Bathyrja eatonii* β -chain amino acid sequence).

[10] proposed the following vicariance hypothesis: "During the Jurassic (213–144 million years ago), the *Bathyrāja* lineage had an extensive latitudinal distribution in the shelf waters of most continental areas. Waters at this time were warm and homogenous throughout the world. Tectonic processes split the *Bathyrāja* lineage into at least two stocks, one in the north-eastern Pacific and one off the South America/Antarctic component of Gondwana. At the same time waters were cooling toward the poles, and latitudinal climatic zones were developing".

In comparing the taxonomic composition of the Arctic and Antarctic faunas, the most obvious difference is that no single group in the Arctic dominates the fauna as do the Antarctic notothenioids, where the species endemism is 88 % for the benthic fauna and rises to 97 % when only notothenioids are considered [11]. Species endemism for marine fish in the Arctic is 20–25 % [12]. In the Arctic region, there are 26 species belonging to the class Chondrichthyes, approx. 6.3 % of the total fauna.

Hbs of cartilaginous fish have been studied less extensively than those of teleosts. Only temperate elasmobranch Hbs have been investigated so far. One difficulty that presumably hindered the elucidation of structure–function relationships is the high genetic polymorphism. When the X-ray structures of two cartilaginous fish Hbs were solved [13,14], the quaternary structure and its change upon ligand binding appeared to be preserved. The proximal effect linking the quaternary-structure change to the haems was also preserved, but the distal effect, especially the role of valine E11, was altered. The Bohr-proton and organophosphate-binding sites were absent, indicating that, in such cases, the stereochemical mechanisms other than the proximal effect have evolved independently in the different species.

The present paper reports the first molecular characterization of the oxygen-transport haemoproteins of two skates living in polar habitats, namely Antarctic *Bathyrāja eatonii* (Eaton's skate) and Arctic *Raja hyperborea*. The blood of the two species was found to contain two components. To assess the possible differential contribution of Hb within the evolutionary response to the two polar environments, the primary structure, the oxygen-equilibrium properties and the thermodynamic features of the major component Hb I of both species were investigated. The primary structures of *B. eatonii* and *R. hyperborea* Hb I reveal significant substitutions of many residues that are generally conserved in many Hbs and in human HbA. These are likely to be related to the functional properties of these Hbs, i.e. absence of pH and organophosphate regulation, indicating that teleost and cartilaginous fish Hbs have independently evolved stereochemical mechanisms to regulate ligand binding to the haems. Moreover, similar to all cartilaginous fish, Hb I of *B. eatonii* and *R. hyperborea* (i) lacks helix D in the β -subunits, and (ii) has relatively low co-operativity ($h \approx 1.6$ – 1.8) [15]. Thermodynamic analysis indicates that the oxygenation enthalpy change (ΔH) maintains a rather constant absolute value in the pH range 6.6–8.7 in both species.

Molecular modelling was used to characterize the haem environment and to explain the lack of Bohr and organophosphate effects in comparison with the solved X-ray structures of the two cartilaginous fish [13,14].

EXPERIMENTAL

Materials

Toyopearl Super Q-650S was from TosoHaas, Mono P HR 5/20 was from Amersham Biosciences, trypsin (EC 3.4.21.4) treated with L-1-tosylamide-2-phenylethylchloromethylketone was from Cooper Biomedical, endoproteinase Asp-N and Glu-C (se-

quencing grade) were from Roche, 4-vinylpyridine was from Sigma, dithiothreitol was from Fluka, sequential-grade reagents were from Applied Biosystems, HPLC-grade acetonitrile was from Lab-Scan Analytical, oligonucleotides were from MWG, and Taq DNA polymerase was from EuroClone. All other reagents were of the highest purity commercially available.

Hb purification

Specimens of the two skates were collected by bottom trawling from the research vessel *L.M. Gould* near Low and Brabant Islands in the Palmer Archipelago (*B. eatonii*) and from the research vessel *Jan Mayen* near the coast of Greenland (*R. hyperborea*).

Blood samples were drawn by cardiac puncture by means of heparinized syringes. Haemolysates were prepared as described in [16]. Purification of Hb I was achieved by FPLC anion-exchange chromatography, with Toyopearl Super Q-650S (*B. eatonii*), and Amersham Biosciences Mono P HR 5/20 columns (*R. hyperborea*). The Hb-containing pooled fractions were dialysed against 10 mM Hepes, pH 7.7. All steps were carried out at 0–5 °C. Hb solutions were stored in small aliquots at –80 °C until use. For oxygen binding, aliquots of a solution of CO Hb I were stored at –80 °C before use within a maximum of 7 days. For each experiment, one aliquot was thawed, converted into the oxy form by exposure to light and oxygen, and was used immediately. For this purpose, the Hb solution was placed in an ice bath, and the gas phase was made 100 % in oxygen. With gentle agitation, the Hb was illuminated with a light source (Sylvania Model SG-50 with a DWY lamp). No oxidation was detectable spectrophotometrically in the Hb solution, indicating that final Met-Hb formation was negligible (< 2 %).

Amino acid sequencing

Alkylation of thiol groups with 4-vinylpyridine, and tryptic, Asp-N and Glu-C digestions were carried out as described in [17–19]. Globins and peptides were purified by reverse-phase HPLC on a micro-Bondapak-C₁₈ column (0.39 cm × 30 cm; Waters) as described in [17]. Cleavage of Asp-Pro bonds was performed on polybrene-coated glass-fibre filters in 70 % (v/v) methanoic (formic) acid, for 24 h at 42 °C [20]. Asp-Pro-cleaved α -globins were treated with *o*-phthalaldehyde before sequencing [21] in order to block the non-proline N-terminus and reduce the background. Sequencing was performed using an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids. Multiple alignment of globins was performed with CLUSTALW [22].

Cloning and sequence analysis of globin cDNAs

Total RNA was isolated from spleen using TRI[®] Reagent (Sigma-Aldrich), as described in [23]. First-strand cDNA synthesis was performed according to the manufacturer's instructions (Promega) using an oligo(dT)-adaptor primer in both species. The α - and β -globin cDNAs were amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Amplifications were performed with 2.5 units of Taq DNA polymerase, 5 pmol each of the above primers and 0.20 mM dNTPs buffered with 670 mM Tris/HCl, pH 8.8, 160 mM ammonium sulphate, 0.1 % Tween 20 and 1.5 mM MgCl₂. The PCR program consisted of 30 cycles of 1 min at 94 °C, 1 min at temperatures between 42 and 54 °C, and 1 min at 72 °C, and ending with a single cycle of 10 min at 72 °C. The cloning of the N-terminal regions was obtained by 5' RACE (rapid amplification of cDNA ends) using the Marathon[™]

cDNA Amplification Kit (BD Biosciences) and two internal primers. Amplified cDNA was purified and ligated in the pDrive vector (Qiagen). *Escherichia coli* cells (strain DH5 α) were transformed with the ligation mixtures. Standard molecular-biology techniques [24] were used in the isolation, restriction and sequence analysis of plasmid DNA. Both strands of the cloned cDNA fragments underwent automated sequencing.

MS

The molecular mass of S-pyridylethylated α - and β -chains and of peptides (less than 10 kDa) was measured by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation. Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration, 5 nmol $\cdot$ ml $^{-1}$) in 4 vol. of matrix, namely 10 mg $\cdot$ ml $^{-1}$ sinapinic acid in 30 % acetonitrile containing 0.3 % trifluoroacetic acid (globins), and 10 mg $\cdot$ ml $^{-1}$ α -cyano-4-hydroxycinnamic acid in 60 % acetonitrile containing 0.3 % trifluoroacetic acid (peptides).

Oxygen binding

Haemolysate stripping was carried out as described in [25]. Oxygen equilibria were measured in 100 mM Hepes in the pH range 6.2–8.7, at 2°C and 10°C (keeping the pH variation as a function of temperature in due account) at a final Hb concentration of 0.5–1.0 mM on a haem basis. An average S.D. of ± 3 % for values of p_{50} (partial pressure of oxygen required to saturate 50 % of the haems) was calculated; experiments were performed in duplicate. In order to obtain stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air [26]. pH was measured with a Radiometer BMS Mk2 thermostatically controlled electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP, GTP and inositol hexakisphosphate were measured at a final ligand concentration of 3 mM, namely a large excess over tetrameric Hb concentration. Oxygen affinity (p_{50}) and co-operativity (h) were calculated from the linearized Hill plot of $\log S/(1 - S)$ against $\log pO_2$ at half saturation; S denotes fractional oxygen saturation.

The overall ΔH (kcal $\cdot$ mol $^{-1}$; 1 kcal = 4.184 kJ), corrected for the heat of oxygen solubilization (-3 kcal $\cdot$ mol $^{-1}$), was calculated by the integrated van't Hoff equation:

$$\Delta H = -4.574[(T_1 \cdot T_2)/(T_1 - T_2)]\Delta \log p_{50}/1000$$

Molecular modelling

A specific BLAST sequence search of the Protein Data Bank (PDB) using default parameters was used to identify candidate parent structures of *B. eatonii* and *R. hyperborea* Hb 1 for homology modelling. Next, sequence alignments were performed using CLUSTALW for sequences of the temperate skate *Dasyatis akajeii* (red stingray) Hb (PDB code 1CG5) [13], the shark *Mustelus griseus* (spotless smooth-hound) Hb (PDB code 1GCV) [14], human HbA (PDB code 2HHB) [27] and the Antarctic teleost *Trematomus bernacchii* (emerald rockcod) Hb (PDB code 1HBH) [28].

Models of α - and β -chains of *B. eatonii* and *R. hyperborea* Hb 1 were built using both the multiple alignments obtained by CLUSTALW and the single structure of *D. akajeii* Hb, which, among the chosen sequences, shows the highest identity with the target (62 % and 59 % for α -chain, 56 % and 58 % for β -chain of *B. eatonii* and *R. hyperborea* Hb 1 respectively). Three different models for both alignments were made using the comparative modelling program MODELER [29] as implemented in InsightII

(Accelrys). The zone of β -chain around $\beta 50$, which corresponds to the region of removal of helix D in cartilaginous fish, was optimized further (loop modelling in MODELER). The structural validity of the models of α - and β -chains of *B. eatonii* and *R. hyperborea* Hb 1 was assessed by several criteria. A representative model from each set was selected by reference to the MODELER objective function (F, molecular probability density function violation), which describes the degree of fit of the model to the input structural data used in its construction, as well as by use of the structure verification program PROCHECK [30]. The co-ordinates of the generated models were then subjected to the Verify3D algorithm [31] using the Verify3D Structure Evaluation Server (available at http://www.doe-mbi.ucla.edu/Services/Verify_3D.html) to identify regions of improper folding. Sequence-structure compatibility was also assessed with the ProsaII program [32].

After addition of hydrogen atoms, the quaternary structures of *B. eatonii* and *R. hyperborea* Hb 1 were created using InsightII (Accelrys) and CHARMM force field for energy minimization. Energetically favourable positions for the binding of organophosphates in *B. eatonii* Hb 1, *R. hyperborea* Hb 1 and *D. akajeii* Hb were evaluated using the docking program GROUP implemented in GRID version 21 [33]. The program GRID determines the interaction energy of a 'probe', representing a chemical fragment, at each point of an orthogonal grid which encloses the structure of a target macromolecule. A grid which surrounds each protein structure exceeding it by 5 Å (1 Å = 0.1 nm) in each dimension and a grid spacing of 1.0 Å were selected. The standard GRID energy function and parameters were used to calculate the interaction energy. The program GROUP, by fitting the maps generated by GRID for the probes which all together represent the structure of ATP, positions the atoms of the ligand at favourable places on the protein.

RESULTS AND DISCUSSION

Purification of Hbs and separation of globins

Ion-exchange chromatography of the haemolysate (results not shown) showed two components in both skates. Purification of Hb 1 was achieved by gradient elution with Tris/HCl, pH 7.6 (*B. eatonii*) and Tris/HCl, pH 7.6, containing NaCl (*R. hyperborea*). The first peak corresponded to Hb 1, the second to Hb 2 (contaminated by Hb 1). The approximate amount ratio was 80:20. The literature suggests that the various components present in cartilaginous-fish haemolysates are often structurally and functionally similar. It appears that such haemolysates contain products of genetic polymorphism, which can be regarded as a single type of Hb which merely exhibits microheterogeneity in the amino acid sequence.

The separation of the globins of *B. eatonii* and *R. hyperborea* Hb 1 was obtained by reverse-phase HPLC (results not shown). HPLC, SDS/PAGE (15 % polyacrylamide) and MS indicated the presence of four different subunits in the haemolysate of both species. However, five cDNAs for α -chains and three cDNAs for β -chains were identified in *B. eatonii* (E. Cocca and G. di Prisco, unpublished work); in keeping with the hypothesis of genetic polymorphism, these sequences differ from one another at very few positions.

Primary structure

The amino acid sequences of the α - and β -chains of Hb 1 of *B. eatonii* and *R. hyperborea* are shown in Figure 1. The primary structure of Hb 1 of *B. eatonii* was established by alignment of

Figure 1 Amino acid sequences of the α - and β -chains of *B. eatonii* and *R. hyperborea* Hb 1

Similar residues are indicated in grey boxes. Forward and reverse primers were designed using the amino acid sequences of the α - and β -chains: 5'-EIHHVAE-3' and 5'-AEHLDDL-3' (forward and reverse *B. eatonii* α -chain respectively), 5'-DKAAY-3' and 5'-AGDSGVQGH-3' (forward and reverse *B. eatonii* β -chain respectively), 5'-EADKHAI-3' (forward *R. hyperborea* α -chain), and 5'-HITADEA-3' (forward *R. hyperborea* β -chain).

tryptic, Asp-N and Glu-C peptides (results not shown), and on the basis of nucleotide sequences (E. Cocca and G. di Prisco, unpublished work) to confirm the amino acid sequence and to complete the C-terminal region of the β -chains. The primary structure of Hb 1 of *R. hyperborea* was established by nucleotide sequencing, using primers designed on sequence stretches. The N-terminus of the α -chains in both species was available for Edman degradation. The sequence-deduced molecular masses of globins were 15680 Da for α - and 15668 Da for β -chains (*B. eatonii*), and 15564 Da for α - and 15399 Da for β -chains (*R. hyperborea*). These values are in agreement with MALDI-TOF MS.

B. eatonii Hb 1 has 141 amino acid residues in the α - and β -chains; *R. hyperborea* has 139 amino acid residues in the α -chain and 141 in the β -chain. By comparison with the α -chain of human HbA, one deletion (α GH3) and one insertion (between α CD8 and α CD9) were found in *B. eatonii* Hb 1, and three deletions (α B1, α B2 and α GH3) and one insertion (between α CD8 and α CD9) were found in *R. hyperborea* Hb 1. The α -chains of both fish, similar to human HbA and of the temperate skate *D. akajei* Hb, have free valine at the N-terminus. In both β -chains, four residues are missing between helices C and E, corresponding to helix D (Figure 1).

Remarkably, in *B. eatonii* Hb 1, there is a serine residue at position H21 (143 β), compared with histidine in mammals and

lysine or arginine in other fish (except trout Hb 1, which also has serine and lacks organophosphate regulation); *R. hyperborea* Hb 1 has alanine at H21 (143 β) (Figure 1).

The presence of histidine at NA2 (2 β) in *R. hyperborea* is not exceptional for elasmobranchs. It also occurs in Hbs of the sharks *S. acanthias* [34] and *Heterodontus portusjacksoni* (Port Jackson shark) [35,36]. The episodic occurrence of histidine in NA2 (2 β) in elasmobranchs suggests that it may be a phylogenetically primitive character.

The alignment of the polar α - and β -chains with those of HbA and of Hbs of *D. akajei*, the shark *M. griseus* and the Antarctic bony fish *T. bernacchii* is shown in Figures 2 and 3. The sequences of the two chains of Hbs from polar cartilaginous Hbs have comparatively higher identity with other temperate cartilaginous Hbs than with polar teleosts (Table 1). The sequence identity between Hbs of the Arctic and Antarctic skates is even higher (70–73 %).

The amino acid sequences of the α - and β -chains of *B. eatonii* Hb 2 were also established (results not shown). The identity with the homologous chains of Hb 1 was over 95 %, supporting the microheterogeneity hypothesis in cartilaginous fish Hbs.

Oxygen-binding properties

B. eatonii and *R. hyperborea* Hb 1 are devoid of Bohr effect at 10 and 2°C, both in the absence and the presence of effectors (Figures 4A, 4C, 5A and 5C respectively). The chloride-independent part of the Bohr effect of human HbA largely arises from the β -subunit C-terminal residue His¹⁴⁶ (β HC3), which forms salt bridges with Asp⁹⁴ (β FG1) through the imidazole group and with Lys⁴⁰ (α C5) across the $\alpha_1\beta_2$ contact through the carbonyl group [37]. In *B. eatonii* and *R. hyperborea* Hb 1, positions β (HC3) and α (C5) are occupied by the same residues, but Asp⁹⁴ (β FG1) is replaced by leucine in *B. eatonii* Hb 1 and by threonine in *R. hyperborea* Hb 1, uncommon in other fish Hbs (Figure 1). Moreover, both species lack organophosphate regulation (see below).

The changes in oxygen-transport protein synthesis, whether up- or down-regulation or expression of a particular combination of gene products, are often considered to be a long-term response to developmental or environmental change. On the other hand, the regulation by allosteric effectors (protons and organophosphates) is thought to be responsible for more immediate short-term perturbations.

At first glance, the p_{50} values of polar skate Hb 1 appeared to be low when compared with some other cartilaginous Hbs, in both the absence and the presence of allosteric effectors (Table 2). However, this difference should be considered in the light of the fact that Hbs of some cartilaginous fish display the Bohr effect. Therefore, at alkaline pH, their affinity is much higher, and, at low pH, it becomes much lower [13,14]. Moreover, if the affinities are compared, taking the respective environmental temperatures and physiological pH into account, this difference becomes insignificant. As shown in Figures 4(B), 4(D), 5(B) and 5(D), co-operativity was relatively low in both species, reaching a maximum of approx. 1.8, as in all cartilaginous fish [15], indicating that a highly co-operative oxygen carrier is not needed.

Enthalpy change

Oxygen-binding equilibria were investigated in the range 2–10°C in both Hbs (Figure 6). In Hb 1 of both fish, where the Bohr effect and phosphate regulation is absent, the ΔH , which includes the heat of oxygen solubilization and the heats of processes linked to oxygen binding, such as proton and anion dissociation, kept a rather constant absolute value as a function of pH, in line with

Figure 2 Sequence features of α - (A) and β - (B) chains of *B. eatonii* Hb 1

Alignments of the closest homologues found by a BLAST search. The α - (A) and β - (B) chains of the Antarctic skate *B. eatonii* Hb 1, the temperate skate *D. akajei* Hb, the shark *M. griseus* Hb, human HbA and the Antarctic fish *T. bernacchii* were aligned using CLUSTALW and modified for print representation using ESPrnt. The residues in black boxes are identical in all homologues, whereas the residues in white boxes are similar in all homologues.

Figure 3 Sequence features of α - (A) and β - (B) chains of *R. hyperborea* Hb 1

Alignments of the closest homologues found by a BLAST search. The α - (A) and β - (B) chains from Arctic *R. hyperborea* Hb 1, temperate *D. akajei* Hb, shark *M. griseus* Hb, human HbA and Antarctic *T. bernacchii* Hb were aligned using CLUSTALW and modified for print representation using ESPrnt. The residues in black boxes are identical in all homologues, whereas the residues in white boxes are similar in all homologues.

the absence of the endothermic contributions due to the Bohr effect and ATP regulation. The evolution of polar fish appears to have often favoured a decrease in the temperature sensitivity of Hb oxygen affinity, surprisingly similar to that observed in some species whose lifestyle is highly different, and which must

adapt to large temperature fluctuations, e.g. tuna [25]. Thus these Hbs do not require significant amounts of energy during the oxygenation–deoxygenation cycle. This energy-saving mechanism may facilitate Hb function in the constantly low temperature of sea water.

Table 1 Sequence identities of the two chains of Hb of various fish species compared with *B. eatonii* and *R. hyperborea*(a) α -Globin

	Sequence identity (%)	
	<i>B. eatonii</i>	<i>R. hyperborea</i>
Antarctic teleosts*	34–36	30–37
Arctic teleosts*	34–39	30–37
<i>Homo sapiens</i>	45	41
<i>Latimeria chalumnae</i> (coelacanth)	37	35
<i>M. griseus</i>	51	46
<i>D. akajei</i>	62	59
<i>H. portusjacksoni</i>	48	44
<i>S. acanthias</i>	50	49
<i>B. eatonii</i>	–	73
<i>R. hyperborea</i>	73	–

(b) β -Globin

	Sequence identity (%)	
	<i>B. eatonii</i>	<i>R. hyperborea</i>
Antarctic teleosts*	34–37	28–36
Arctic teleosts*	31–36	26–33
<i>H. sapiens</i>	34	36
<i>L. chalumnae</i>	36	37
<i>M. griseus</i>	39	41
<i>D. akajei</i>	56	58
<i>H. portusjacksoni</i>	41	48
<i>S. acanthias</i>	43	44
<i>B. eatonii</i>	–	70
<i>R. hyperborea</i>	70	–

* Sequences are from [44,45].

Figure 4 Oxygen-equilibrium isotherms (Bohr effect) (A, C) and subunit co-operativity (B, D) as a function of pH of *B. eatonii* Hb 1 at 10 °C (A, B) and at 2 °C (C, D)

(A, B, C, D) Hb was incubated in Hepes (100 mM), in the absence of effectors (●), in the presence of 100 mM NaCl (○), 100 mM NaCl and 3 mM ATP (△, ▽), 100 mM NaCl and 3 mM GTP (■), and 100 mM NaCl and 3 mM inositol hexakisphosphate (▲, ▼).

Temperature-dependence, which is governed by the associated overall enthalpy change, is an important feature of the reaction of Hbs with oxygen. Heat absorption and release can be considered to be physiologically relevant modulating factors, similar to hetero- and homo-tropic ligands. The affinity and co-operativity of all Hb

Figure 5 Oxygen-equilibrium isotherms (Bohr effect) (A, C) and subunit co-operativity (B, D) as a function of pH of *R. hyperborea* Hb 1 at 10 °C (A, B) and at 2 °C (C, D)

(A, B, C, D) Hb was incubated in Hepes (100 mM), in the absence of effectors (●), in the presence of 100 mM NaCl (○), 100 mM NaCl and 3 mM ATP (△, ▽), 100 mM NaCl and 3 mM GTP (■), and 100 mM NaCl and 3 mM inositol hexakisphosphate (▲, ▼).

systems are strictly correlated with the extent of heat absorption accompanying oxygenation. In human HbA, the apparent overall ΔH is more exothermic at alkaline pH values, where the Bohr effect is not operative and the endothermic contribution of the Bohr protons is abolished. For fish, relying upon Hbs with constant ΔH values may thus be a frequent evolutionary strategy of molecular adaptation to extreme life conditions.

Molecular modelling

In order to gain additional insight into the functional properties of *B. eatonii* and *R. hyperborea* Hb 1, structural models were built by homology modelling, relying on crystal co-ordinates of closely related Hbs. A three-dimensional model of the structure of both Hbs was built on the basis of homology with four X-ray structures of Hb from the PDB, namely the 1.6 Å resolution structures of Hb of the temperate skate *D. akajei*, the 2.0 Å resolution structure of Hb of the shark *M. griseus*, the 2.2 Å resolution structure of Hb of the Antarctic teleost *T. bernacchii* and the 1.74 Å resolution structure of human HbA.

A second model was built based on the single structure of *D. akajei* Hb; these proteins share approx. 60 % sequence identity (Table 1), and, in the case of *B. eatonii* Hb 1, there are no insertions or deletions in the alignment. Sequence alignments are shown in Figures 2 and 3. From the three models based on the multiple alignment and the three models based on *D. akajei* Hb, a single representative model was chosen for each, in accordance with the MODELER objective function and PROCHECK stereochemical assessment. The stereochemistry of the selected models was acceptable in both cases. The models show no residues within disallowed regions, and only one or two residues in the generously allowed regions of the Ramachandran plot, indicating correct stereochemistry.

The overall quality of the structure was also determined by parameters that assess residue environments and atomic contacts. As shown in Figures 7(A) and 7(B), the three/one-dimensional scores of the models generated by Verify3D for α - and β -chains respectively are always positive and are similar to those obtained with the template structure of temperate *D. akajei* (PDB code 1CG5).

Table 2 Oxygen affinity values (P_{50}) for polar and temperate cartilaginous fish

The reported values were obtained from equilibrium experiments (from the present study and [13,14]).

(a)

	NaCl (100 mM)	ATP (3 mM)	pH . . .	P_{50}					
				8.71	8.11	7.46	7.17	6.62	6.29
<i>B. eatonii</i> (10 °C)	+	—		42.64	35.50	28.92	28.63	33.55	30.44
	+	+		35.90	35.05	33.53	33.96	35.06	40.19
<i>R. hyperborea</i> (10 °C)	+	—		29.19	27.83	24.39	19.20	24.92	21.97
	+	+		26.04	22.35	19.87	19.77	24.57	24.42
<i>B. eatonii</i> (2 °C)	+	—		16.82	15.45	14.54	15.20	16.57	16.80
	+	+		15.17	14.12	13.12	16.61	18.57	22.18
<i>R. hyperborea</i> (2 °C)	+	—		18.78	13.51	12.86	12.41	14.11	12.68
	+	+		17.23	16.61	13.22	16.37	13.54	13.18

(b)

	NaCl (100 mM)	ATP (3 mM)	pH . . .	P_{50}		
				8.5	7.4	6.5
<i>D. akajei</i> (25 °C)	+	—		4.20	11.80	27.90
	+	+		4.30	18.70	61.60
<i>M. griseus</i> (25 °C)	+	—		3.35	5.90	8.05
	+	+		3.99	10.57	20.12

Figure 6 Overall oxygenation enthalpy change of *B. eatonii* (A) and *R. hyperborea* (B) Hb 1

ΔH values (in kcal/mol) were calculated from the experiments shown in Figures 4 and 5. Symbols are the same as in Figures 4 and 5.

The reliability of the selected models was also tested by the ProsaII program, which calculates the $C\beta$ - $C\beta$ pair interaction energy for each residue in the sequence, producing smooth energy plots with negative values for correctly folded proteins. The ProsaII trace for candidate models of α - and β -chains of *B. eatonii* Hb 1 had no positive regions, indicating no misfolding, with a so-called Z-score or normalized energy values of -6.89 and -6.72 (calculated for α -chains from multiple and single alignment respectively), and -6.64 and -6.74 (calculated for β -chains

from multiple and single alignment respectively), comparing favourably with those of *D. akajei* Hb (-5.67 and -6.04 for α - and β -chains respectively). Analogously, the Z-score values for *R. hyperborea* were comparable to those of Hb crystals of the temperate skate (-6.63 and -6.53 for α -chains from multiple and single alignment respectively, and -6.23 and -6.21 for β -chains from multiple and single alignment respectively).

The haem environment

Analysis of the predicted models of *B. eatonii* and *R. hyperborea* Hb 1 showed no significant mutations in the haem region of both α - and β -chains compared with *D. akajei* Hb and human HbA. It is worth noting that removal of D helix in β -subunits has been reported not to affect significantly the conformation of the distal pocket [38].

In the proximal side of both α - and β -haems, the replacement of leucine F7 of HbA with lysine in *D. akajei*, *B. eatonii* and *R. hyperborea* Hb 1 (Figure 1) is worth mentioning.

In the α -haem distal cavity, glycine E3 of human HbA and *D. akajei* Hb is replaced by proline in *B. eatonii* Hb 1, and by histidine in *R. hyperborea* Hb 1, thus modifying the hydrogen-binding interaction pattern of distal histidine. In the β -chains, it is worth noting that two polar residues (histidine FG4 and lysine E3), involved in hydrogen-bonding with proximal and distal histidine of HbA respectively, are replaced by glycine in *D. akajei* Hb, *B. eatonii* and *R. hyperborea* Hb 1 (Figure 1).

Organophosphate-binding site

The lack of effector modulation displayed by Hb 1 of polar skates can be explained by a molecular modelling study predicting the favourable binding sites for ATP. In both polar species, lacking organophosphate regulation, the ATP-binding site was absent. A model for the binding site of ATP in bony fish Hbs has been proposed [39]. In this model, the same residues [lysine β (EF6), histidine β (H21), histidine β (NA2) and β N-terminal α -amino group] which bind 2,3-BPG (2,3-biphosphoglycerate) in HbA take part in the binding of ATP. In bony fish Hb, glutamate or aspartate replaces histidine at NA2 and arginine replaces histidine at H21, but the former residue can be modelled to accept a

Figure 7 Verify3D plots

Obtained by the Verify3D program using (A) the α -chain co-ordinates of *B. eatonii* Hb 1 (grey lines) and *R. hyperborea* Hb 1 (black lines) molecular models, multiple (continuous lines) and pairwise (broken lines) alignments respectively, and *D. akajei* Hb ($\square$) crystallographic structures; (B) the β -chain co-ordinates of *B. eatonii* Hb 1 (grey lines) and *R. hyperborea* Hb 1 (black lines) molecular models, multiple (continuous lines) and pairwise (broken lines) alignments respectively and *D. akajei* Hb ($\square$) crystallographic structures. The x-axis numbering corresponds to the amino acid numbering of Hbs. The y-axis gives the average three/one-dimensional scores for residues in a 21-residue sliding window.

Figure 8 Organophosphate-binding site in *D. akajei* Hb

The predicted interaction between ATP and *D. akajei* Hb, colour-coded by atom type (C, green; O, red; N, blue; P, magenta), is shown. A ribbon diagram representation of helices G and H is displayed using InsightII (Accelrys). Violet-coloured leucine 130 β , which is present in both *B. eatonii* and *R. hyperborea* Hbs and which disallows the binding within the central cavity of Hb, is superimposed.

hydrogen bond from adenine and the latter to participate in the binding of phosphate. With respect to bony fish Hb, in *D. akajei*, *B. eatonii* and *R. hyperborea* Hbs, lysine β (EF6) is replaced by an acidic residue and arginine β (H21) is replaced by serine in *B. eatonii* Hb 1 and by alanine in *R. hyperborea* Hb 1; in β NA2, there is lysine residue in *B. eatonii* and a histidine residue in *R. hyperborea*. Thus skate Hbs cannot bind ATP with the same interaction pattern characteristic of bony fish Hbs. However, in *D. akajei* Hb, Chong et al. [13] suggested, as a candidate binding site for ATP, the region of arginine β (H13) and lysine β (G6) in the central cavity just inside the 2,3-BPG-binding site of human HbA.

By using the program GRID, we succeeded in demonstrating that ATP is indeed bound to *D. akajei* Hb as hypothesized [13], whereas the ATP site is absent in the polar skate Hb 1, since the replacement of Arg¹³⁰ β (H13) with leucine in both Hbs does prevent organophosphate binding. Figure 8 shows the predicted binding site for ATP in *D. akajei* Leu¹³⁰ β (H13) of *B. eatonii* and *R. hyperborea* Hbs, which disallows the binding within the protein cavity, is superimposed.

Bohr effect

The replacement of Asp⁹⁴ β (FG1) of HbA with leucine in *B. eatonii* Hb and threonine in *R. hyperborea* Hb (Figure 1) causes the loss of salt bridges which are essential for the Bohr effect in human HbA. *D. akajei* Hb compensates the effects of the substitution of glutamate for Asp⁹⁴ β (FG1), with the hydrogen bond between His¹⁴¹ β (HC3) and Asn¹³⁹ β (HC1) likely to be responsible for part of the Bohr effect displayed by Hb of the temperate skate [13]. In contrast, in *B. eatonii* and *R. hyperborea* Hb, the presence of glycine in HC1 gives rise to an arrangement which does not allow hydrogen bonding formation.

Concluding remarks

Globins of higher vertebrates have eight helices, designated A–H from the N-terminus, but all known vertebrate α -globins lack helix D. The absence of helix D in α -subunits is considered to be the most distinct conserved feature which distinguishes modern α - and β -globins. The widespread distribution of helix-D-containing globins throughout plants, bacteria and ancestral vertebrates suggests that helix D was present in the very early ancestral protein, but was lost in the α -globin of vertebrates.

A feature which distinguishes cartilaginous from teleost Hbs is the lack of helix D in the β subunits of the former. In β -globins, it was shown previously that the presence or absence of helix D does not affect assembly into co-operative tetramers [40]. Since recombinant human Hbs engineered with β -subunits without helix D and α -subunits having helix D show a small change in oxygen affinity, the lack of helix D in the β -subunit is thought not to exert a large functional effect. *B. eatonii* and *R. hyperborea* Hb 1 do not have helix D in either α - or β -subunits, as well as the sharks *S. acanthias* [34], *H. portusjacksoni* [35,36] and *M. griseus* [14], and the temperate skate *D. akajei* [13].

The very high sequence similarity observed in *B. eatonii* and *R. hyperborea* Hbs may reflect a common origin of polar skates, but may also suggest that the primary structure in polar fish may be related to the development of cold adaptation.

Oxygen delivery to the tissues of *B. eatonii* and *R. hyperborea* occurs in the virtual absence of pH or phosphate-linked reductions in Hb 1 affinity.

These features resemble those of ancestral oxygen carriers, and also of Hbs of several amphibians, of trout Hb I and of abnormal Hiroshima Hb (histidine β HC3 replaced by aspartate), but are not unique to polar elasmobranchs. In fact, previous studies on temperate elasmobranch Hbs have shown that their functional

properties do not always include well-developed co-operativity or proton and organophosphate regulation. For instance, in *Torpedo marmorata* Hb, the replacement of aspartate β FG4 with lysine [41] causes the loss of the salt bridge crucial for the Bohr effect in human HbA [39]; this mutation may be responsible for the lack of Bohr effect, as the Hb of *Torpedo nobiliana* binds oxygen with low co-operativity ($h < 1$), and shows no appreciable pH-dependence or response to organophosphates [42]. Elasmobranchs may have diverged from the mammalian line before stabilization of the homotropic and heterotropic interactions typical of mammalian Hbs.

In contrast, some cartilaginous fish (e.g. *D. akajei* and *M. griseus*) display a modest Bohr effect and significant ATP regulation. Such regulation originated in the common ancestor of the subclass Elasmobranchii and all other jawed vertebrates; ATP was the first organophosphate regulator of Hb function [43]. The switch from ATP to 2,3-BPG regulation may have been a consequence of curtailed oxidative phosphorylation in erythrocytes of higher vertebrates such as mammals.

In summary, Hbs of polar skates appear functionally different from those of several temperate cartilaginous fish. On the other hand, the close functional similarity (also reflected in the very high sequence identity) in the two species, each living in one of the polar environments, is of interest. The Hb systems of teleosts thriving at the two poles are far from showing the sequence identities found in skates. Antarctic notothenioids, with very few exceptions, are sedentary bottom dwellers, and have a single major Hb usually displaying strong Bohr and Root effects. Among Arctic teleosts, characterized by higher biodiversity, the number of pelagic and migratory species is instead very abundant, the Hb multiplicity is higher, and the various components appear to be functionally distinct [44,45]. Although both are cold, the Arctic and Antarctic habitats differ in many aspects, e.g., in the Arctic, the range of temperature variations is wider and isolation is less pronounced. These differences appear to be minimized in the case of two benthic sedentary skates, unlikely to disperse across wide latitude and temperature gradients and living in microhabitats with similar temperatures; this similar lifestyle may have channelled the Hb functional mechanisms (Bohr effect and ATP regulation) towards coinciding evolutionary pathways. Conversely, it is conceivable that the wide environmental differences between temperate and polar waters have given rise to far more extensive differences in physiological adaptations to the respective environments than those caused by the two polar environments, which do differ from each other, but to a lesser extent.

Physiological studies provide tantalizing insights into the possible mechanisms used by polar fish in response to low temperature. The comparison of structure and function of proteins from cold-adapted and non-cold-adapted species is an additional powerful tool. In fact, it will allow us to gain insights into the extent to which strategies of cold-adaptation are similar or vary in different phylogenies, and to what extent extreme environments require specific adaptations or simply select for more generalist or phenotypically plastic lifestyles.

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