The Oxygen Transport System in Three Species of the Boreal Fish Family Gadidae

MOLECULAR PHYLOGENY OF HEMOGLOBIN

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The Arctic and Antarctic marine faunas differ by age and isolation. Fishes of the two polar regions have undergone different regional histories that have driven the physiological diversities. Antarctic fish are highly stenothermal, in keeping with stable water temperatures, whereas Arctic fish, being exposed to seasonal temperature variations, exhibit higher physiological plasticity. This study reports the characterization of the oxygen transport system of three Arctic species of the family Gadidae, namely the Arctic cod Arctogadus glacialis, the polar cod Boreogadus saida, and the Atlantic cod Gadus morhua. Unlike Antarctic notothenioids, the blood displays high multiplicity, i.e. it has three hemoglobins, similar to many other acanthomorph teleosts. In the most abundant hemoglobin, oxygen binding is modulated by heterotrophic effectors, with marked Bohr and Root effects. Remarkably, in two species (A. glacialis and B. saida), the Hill coefficient is very close to one in the whole pH range, indicating the apparent absence of cooperativity. The amino acid sequences have been used to gain insight into the evolution history of globins of polar fish. The results indicate that Arctic and Antarctic globins have different phylogenies and lead us to suggest that the selective pressure of environment stability allows the phylogenetic signal to be maintained in the Antarctic sequences, whereas environmental variability would tend to disrupt this signal in the Gadidae sequences.

The main differences between the Arctic and the Antarctic are the older age and longer isolation of the latter (1). The Arctic has been isolated and cold longer than the Arctic, with ice-sheet development preceding that in the Arctic by at least 10 million years. In a recent review, Eastman (2) wrote the following, “The Antarctic experienced a slow and discontinuous transition from a warm-water system in the early Tertiary (15 °C) to the cold-water system of today (−1.87 °C). The first glaciation, including shelf-ice formation, had occurred 36 million years ago (mya) in the early Oligocene. About 25 mya, the formation of the Polar Front, which followed the opening of the Drake passage, further isolated the Antarctic marine fauna from the world ocean. Although Europe began to separate from Greenland in the late Cretaceous, the exchange of Atlantic and Arctic waters through the passage between Greenland and the Svalbard islands was not possible until 27 mya. The Arctic region was in a high latitude position by the early Tertiary, but the climate remained temperate with water temperatures of 10–15 °C. Arctic land masses reached their present positions, and temperatures dropped below freezing during the Miocene about 10–15 mya.”

The Arctic and Antarctic fish faunas differ in age, endemism (denoting species native to a given geographical area), and taxonomic diversity. In the southern ocean, the fauna includes 322 species grouped in 50 families (3), whereas the Arctic fauna has 416 species of 96 families. In the Antarctic, endemism reaches 88% and rises to 97% when only the dominant suborder Notothenioidei is considered. In comparison, endemism in the Arctic is 25% for marine fish (2).

The Arctic fauna has no endemic higher taxonomic category equivalent to Antarctic Notothenioidei, and there has been no comparable adaptive radiation of any fish group. The North Atlantic and North Pacific character of the marine fauna reflects the continuity of shelf areas between the Arctic and boreal regions (2). Some of the marine components are representative of groups, including the cosmopolitan fauna of the late Cretaceous period. Gadiforms, for example, may have originated in boreal Atlantic waters during the Cretaceous period.

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The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the following accession numbers: P84602 (A. glacialis α1 chain); P84603 (A. glacialis α2 chain); P84604 (A. glacialis α3 chain); P84605 (B. saida α1 chain); P84606 (B. saida α2 chain); P84607 (B. saida α3 chain); P84608 (B. saida β1 chain); P84609 (G. morhua α1 chain); 041425 (G. morhua α2 chain); P84610 (G. morhua β1 chain); P84611 (G. morhua β2 chain).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) DQ125474, DQ125475, DQ125476, DQ125470, DQ125471, DQ125472, and DQ125473.

1 The on-line version of this article (available at http://www.jbc.org) contains Figs. 15–35 and Table 15.
2 The abbreviations used are: mya, million years ago; FPLC, fast protein liquid chromatography; RP-HPLC, reverse-phase high performance liquid chromatography; IHP, inositol hexaphosphate; NJ, Neighbor Joining; BP, bootstrap proportion; AFGP, antifreeze glycoprotein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MES, 4-morpholineethanesulfonic acid.
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(4), and a fossil gadiform of the Paleocene period was found in Greenland (5).

The importance of the Arctic in contributing to the knowledge of the overall ensemble of adaptive processes influencing the evolution of marine organisms prompted investigations on adaptations of the main biological systems of Arctic fish. Oxygen carriers provide particularly interesting systems for studying the relationship between environmental conditions and molecular evolution, because the capacity of fish to colonize a large variety of habitats appears strictly correlated to the molecular and functional differences encountered in their Hb systems (6).

A wealth of knowledge is available on the oxygen transport system of fish inhabiting Antarctic waters, but very little is known on the structure and function of Hbs of fish of the other polar marine environment, where the physicochemical features are significantly different. The vast majority of species of the dominant suborder Notothenioidei has a single Hb, sometimes accompanied by a minor, functionally similar component (~5% of the total), which has one of the two globins in common (7). The study of the structure and function of Arctic fish Hbs was initiated only recently when the first molecular characterization of the oxygen transport system of an Arctic zoarcoid species (Anarhichas minor) was reported (8).

In an attempt to link polar environmental conditions with the evolution of Hb structure and molecular adaptation, this study investigated the oxygen transport system of three species of the boreal family Gadidae (order Gadiformes), namely the Arctic cod (Arctic cod Glacialias, the polar cod (Boreogadus saida), and the Atlantic cod (Gadus morhua). These species have identical Hb multiplicity, consisting of three components.

The sequences of the globins that constitute all nine Hbs were elucidated for phylogenetic purposes. These gadids are widely distributed not only along the shelf areas in the Arctic basin but also at higher latitudes (9). Based on the catch biomass composition, A. glacialis appeared to be a sedentary fish, caught in the Greenland fjords. B. saida and G. morhua are migratory. B. saida is a pelagic species typically confined to polar seas and also widely distributed south of the ice-covered zone; G. morhua is distributed in a variety of habitats, but the Arcto-Norwegian stock spends most of the year in the Barents Sea and migrates seasonally to the Norwegian coast for spawning (9).

The oxygen affinity and cooperative behavior in the hemolysate and in the only component obtained in pure form (Hb 3) are modulated by heterotropic allosteric effectors. The largest Bohr effect was observed in A. glacialis. In this species there was no apparent enhancement of the Root effect by organophosphates, because oxygenation was dramatically lowered by pH also in the absence of the effectors. Remarkably, in A. glacialis and B. saida Hb 3, the Hill coefficient is close to one in the whole pH range, indicating apparent low cooperative oxygen binding.

As clearly shown by phylogenetic analysis, under the constant physicochemical conditions of marine habitats, we are able to recover teleostean phylogeny (zoarcoids with notothenioids, gadids as sister-group to both) in globins of fish from the Antarctic, whereas the variability typical of the Arctic ocean seems to correspond to high sequence variation in gadiform β-globins.

MATERIALS AND METHODS

Collection of Specimens—Adult A. glacialis, B. saida and G. morhua were collected by bottom and midwater trawling from the research vessel Jan Mayen near the coasts of Greenland and Norway. Blood was taken by heparinized syringes from the caudal vein. Saline-washed erythrocytes were frozen at −80 °C until use.

Hb and Globin Purification—Hemolysates were prepared as described (10). Separation of Hbs was achieved by fast protein liquid chromatography on a Mono Q anion-exchange column (Amersham Biosciences). The Hb-containing pooled fractions were dialyzed against 10 mM HEPES, pH 7.7. All steps were carried out at 0−5 °C. No oxidation was spectrophotometrically detectable during the time needed for functional experiments. Hb solutions were stored in small aliquots at −80 °C until use. Separation of globins was carried out by reverse-phase high performance liquid chromatography (RP-HPLC) as described (8).

Amino Acid Sequencing—Alkylation of sulphhydryl groups with 4-vinylpyridine, deacetylation of the α chain N terminus, tryptic digestions, and CNBr cleavage were carried out as described previously (11−13). Tryptic and CNBr-cleaved peptides were purified by RP-HPLC on a µBondapak C18 column (0.39 × 30 cm; Waters Associates) as described (14). Cleavage of Asp-Pro bonds was performed on Polybrene-coated glass-fiber filters in 70% (v/v) formic acid for 24 h at 42 °C (15). Asp-Pro-cleaved globins were treated with o-phthalaldehyde before sequencing (16) in order to block the non-Pro N terminus and reduce the background. Sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids.

Cloning and Sequence Analysis of Globin cDNAs—Total RNA was isolated from B. saida and A. glacialis spleen using TRI Reagent® (Sigma), as described (17). 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 at the N-terminal regions as direct primers and at the adaptor primer as the reverse primer. Amplifications were performed as described (18). Primer sequences are available from the authors upon request. Amplified cDNA was purified and ligated in the pDrive vector (Qiagen). Escherichia coli cells (strain DH5α) were transformed with the ligation mixtures. Standard molecular biology techniques (19) were used in the isolation, restriction, and sequence analysis of plasmid DNA. Both strands of the cloned cDNA fragments underwent automated sequencing.

Mass Spectrometry—The molecular masses of the S-pyridylethylated α and β chains and of peptides (less than 10 kDa) were measured by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager-DE biospectrometry work station, as described (8).

Oxygen Binding—Hemolysate stripping was carried out as described (20). Oxygen equilibria were measured in 100 mm
MES/HEPES in the pH range 6.3–8.7, at 5 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 heme basis. An average standard deviation of $\frac{1}{3\%}$ for values of $p_{50}$ was calculated; experiments were performed in duplicate. To obtain stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wo¨sthoff pumps for mixing pure nitrogen with air (21). Values of pH were measured with a radiometer BMS Mk2 thermostatted electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP and inositol hexaphosphate (IHP) were measured at a final ligand concentration of 3 mM, namely a large excess over tetrameric Hb concentration. Oxygen affinity ($p_{50}$) and cooperativity ($n_{Hill}$) were calculated from the linearized Hill plot of $\log \frac{S}{(1 - S)}$ versus $\log pO_2$ at half-saturation ($S = \text{fractional oxygen saturation}$). The formation of met-Hb after each measurement was less than 2%.

The oxygenation-enthalpy change, $\Delta H$, corrected for the heat of oxygen solubilization ($\sim 3 \text{ kcal mol}^{-1}$), was calculated by the integrated van’t Hoff equation $\Delta H = -4.574 \left[ \frac{(T_1 \times T_2)}{(T_1 - T_2)} \right] \Delta \log p_{50}/1000$.

Phylogenetic Analysis—Multiple alignments of the amino acid sequences of $\alpha$- and $\beta$-globins were performed with the program ClustalX (22). These multiple alignments are available upon request to the authors. Phenetic trees of globin sequences were inferred by using the Neighbor Joining (NJ) method implemented in the program MEGA 2 (23). The genetic distances were measured according to the $p$-distance model. Robustness of the NJ trees was assessed by bootstrap analysis with 10,000 replications.

RESULTS

Purification of Hbs and Separation of Globins—Anion-exchange chromatography of the hemolysate showed three components in $A$. glacialis, $B$. saida, and $G$. morhua, indicated as Hb 1, Hb 2, and Hb 3. Supplemental Fig. 1S refers to $A$. glacialis. The chromatography of the hemolysates of $B$. saida and $G$. morhua yielded similar elution patterns (data not shown). Cel-
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lulose-acetate electrophoresis (11) of the hemolysates showed one broad band, indicative of unresolved Hbs. Peak 3 corresponded to homogenous Hb 3, which is also present in large amounts in peaks 1 and 2, and is thus considered to be the main component. Several procedures were attempted in order to purify the other components Hb 1 and Hb 2 to homogeneity, but they were unsuccessful. The only achievement was the removal of Hb 3. We infer the existence of concentration-dependent equilibria between dimers (particularly in Hb 1 and Hb 2), similar to that observed in the Antarctic notothenioid Notothenia coriiceps (11). Based on the recoveries from the chromatography steps, the hemolysates contain ~50% of Hb 3, the remaining 50% being accounted for by the other two components.

All globins were separated by RP-HPLC. Supplemental Fig. 2S reports the separation of the globins in A. glacialis hemolysate (see supplemental Fig. 2SA) and of purified Hb 3 (see supplemental Fig. 2SB). RP-HPLC of B. saida and G. morhua hemolysates and Hb 3 yielded similar elution patterns (data not shown).

Following complete removal of Hb 3, the globin analysis showed that peaks 1 and 2 contained mixtures of Hb 1 and Hb 2, each one being the predominant component of peaks 1 and 2, respectively. The relative amounts of globins and sequencing of the purified globins indicated that in A. glacialis and B. saida (i) Hb 1 and Hb 2 have identical β chains (indicated as β1) and differ by the α chain (α1 and α2), (ii) Hb 3 differs from Hb 2 only by the β chain (β3), and (iii) Hb 1 and Hb 3 have no chain in common. Thus in A. glacialis and B. saida the chain compositions of Hb 1, Hb 2, and Hb 3 are α1β1β2, α2β1β2, and α2β2β2, respectively. In G. morhua, Hb 1 and Hb 2 have no chain in common, whereas Hb 3 differs from Hb 1 only by the β chain and from Hb 2 by the α chain; the chain compositions of G. morhua Hb 1, Hb 2, and Hb 3 are α1β1β2, α2β2β2, and α1β2β2, respectively.

**Primary Structure**—The amino acid sequences of the α and β chains (142 and 146 residues, respectively) constituting Hb 1, Hb 2, and Hb 3 of A. glacialis, B. saida, and G. morhua are reported in supplemental Fig. 3S. Fig. 1 highlights the sequences of the α and β chains of Hb 3 of A. glacialis, B. saida, and G. morhua. The sequences were established by alignment of tryptic and CNBr peptides (not shown). In cases when it was not possible to obtain a peptide in pure form, DNA sequencing was utilized. Because of insolubility, the primary structure of the A. glacialis β1 chain was obtained by cDNA sequencing, except for the 35 residues at the N terminus. The α chain N termini were not available for Edman degradation. MALDI-TOF mass spectrometry of the N-terminal tryptic peptides revealed acetyl to be the blocking group, similar to teleost Hbs revealed acetyl to be the blocking group, similar to teleost Hbs sequenced to date. The molecular mass values of the 12 globins constituting the 9 Hbs are reported in supplemental Table 1S. These values are in agreement with those determined by MALDI-TOF mass spectrometry (data not shown).

In each Hb 3, some of the side chains forming the αβ2 “dovetailed” switch region in human HbA (Pro αCD2, Thr αC3, Thr αC6, and His βFG4) are replaced (Table 1). His βFG4 is conserved in each Hb 3, whereas Pro αCD2 is replaced by Ser, and Thr αC3 is replaced by Gln. Thr αC6 is replaced by Ile in A. glacialis and B. saida but is conserved in G. morhua. Interestingly, Val βE4, considered to be invariant in vertebrates, including most teleost fish, is replaced by Ile in each Hb 3.

**Oxygen Equilibria, Subunit Cooperativity, and Root Effect**—Detailed functional studies are essentially described on Hb 3, the only component obtained in pure form.

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**Table 1**

| Residues in the αβ2 switch region | Position | Human HbA | Hb 1 | Hb 2 | Hb 3 |
|-----------------------------------|----------|-----------|------|------|------|
| A. glacialis βFG4                 | His      | His       | His  | His  |      |
| αCD2                             | Pro      | Ser       | Ser  | Ser  | Ser  |
| αC3                              | Thr      | Gln       | Gln  | Gln  | Gln  |
| αC6                              | Thr      | Ser       | Ile  | Ile  | Ile  |
| B. saida βFG4                    | His      | His       | His  | His  |      |
| αCD2                             | Pro      | Ala       | Ser  | Ser  | Ser  |
| αC3                              | Thr      | Gln       | Gln  | Gln  | Gln  |
| αC6                              | Thr      | Ser       | Ile  | Ile  | Ile  |
| G. morhua βFG4                   | His      | His       | His  | His  |      |
| αCD2                             | Pro      | Ser       | Ser  | Ser  | Ser  |
| αC3                              | Thr      | Gln       | Gln  | Gln  | Gln  |
| αC6                              | Thr      | Thr       | Ser  | Thr  | Thr  |

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**Table 2**

| Species            | logP50 (mm Hg) | H1mll | ϕ | RE (%) |
|--------------------|----------------|-------|---|--------|
| G. morhua          | 1.5            | 1.1   | -0.8 | 40     |
| A. glacialis       | 1.2            | 1.1   | -0.3 | 50     |
| B. saida           | 1.2            | 1.2   | -0.3 | 55     |

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* The oxygen affinity is expressed as logP50 (mm Hg) at pH 7.5.
* The highest values (pH range 8.0–8.5) of subunit cooperativity (H1mll) are reported.
* ϕ = 𝛿logP50/𝛿pH denotes the average number of protons bound upon heme oxygenation.
* The Root effect (RE) is expressed as percentage of oxygen saturation at pH 6.25.
* Antarctic notothenioids.

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Bohr effects of different magnitudes were observed in each Hb 3 (Table 2) (24–28). The enhancement by organophosphates was consistently high. Figs. 2–4 illustrate the oxygen-binding properties of *A. glacialis*, *B. saida*, and *G. morhua* Hb 3. *A. glacialis* Hb 3 (Fig. 2A) displayed the highest Bohr coefficient ($\varphi = \Delta \log p_{50}/\Delta \mathrm{pH}$), which denotes the average number of protons bound upon heme oxygenation. The number of oxygen-linked protons bound by tetrameric Hb in the deoxygenated state was increased by ATP from 3.36 (in stripped Hb 3) to 6.64. The magnitude of the Bohr effect was lower in the other two species. In *B. saida* Hb 3 (Fig. 3A), the number of oxygen-linked protons was increased by ATP from 1.2 to 4.4. In *G. morhua* (Fig. 4A), ATP increased the number of protons from 1.36 to 5.04. Chloride alone had no effect on the oxygen affinities.

At pH 7.5, in *A. glacialis* and *B. saida* Hb 3, the values of $p_{50}$ in the presence of NaCl and IHP, correspond to an even lower affinity than that observed at pH 6.5 in the absence of effectors. IHP possesses additional negative charges and displays a larger effect (29). The exceptional affinity decrease brought about by IHP at alkaline pH in Hb 3 of the three species suggests that this nonphysiological ligand is able to lock the proteins in the low affinity T state also at high pH values.

In the whole pH range, in Hb 3 of *A. glacialis* and *B. saida* the Hill coefficient ($n_{\text{Hill}}$) was close to 1, reflecting very low levels or apparent lack of subunit cooperativity (Figs. 2B and 3B and Table 2). In *G. morhua* Hb 3, cooperativity is slightly but significantly higher (Fig. 4B and Table 2) in the alkaline pH range; only when the oxygen affinity reaches the highest $p_{50}$ values is
the apparent cooperativity abolished at the maximal allosteric constraint.

Absence of cooperativity implies that the oxygen dissociation curve of Hb is hyperbolic rather than sigmoidal. The oxygen dissociation curves of Hb 3 of the three gadids are shown in Fig. 5. In Hb 3 of *A. glacialis* and *B. saida* the curves are hyperbolic, whereas the slightly higher cooperativity of *G. morhua* Hb 3 leads to the appearance of the sigmoidal pattern. The markedly sigmoid curve of Hb 1 of a temperate nototheniid, *Bovichtus diacanthus*, whose $n_{III}$ is $\sim 2.7$ is also reported.3

In all species, Hb 3 exhibited a marked Root effect (Figs. 2C, 3C, and 4C and Table 2), enhanced by ATP except in *A. glacialis*, where it was very high also in the absence of the ligand. The functionally important residues suggested to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs are all conserved (30).

The regulation of the oxygen affinity by temperature was investigated in the range 5–10 °C (Table 3). Thermodynamic analysis showed that the oxygenation-enthalpy change in Hb 3 of the three gadids is low when compared with temperate fish and very similar to the values seen in Antarctic fish Hbs (31). Among the three species, the oxygenation of *G. morhua* Hb 3 is more exothermic, somewhat resembling temperate Hbs. The values of *A. glacialis* Hb 3 are comparatively higher at alkaline pH than the other two Hb 3. In *B. saida* Hb 3, the value at low pH in the presence of chloride and ATP is endothermic.

Oxygen-binding properties were also investigated in the mixture of Hb 1 and Hb 2 (data not shown). In comparison with Hb 3, the oxygen-binding features of the mixture appeared somewhat different. For example, in *A. glacialis*, the Root effect of the mixture, in the absence of organophosphates, seemed lower; in *G. morhua*, unlike in Hb 3, the cooperativity was absent; a dramatic decrease in affinity was observed with IHP at alkaline pH, to a much higher extent than that observed in each Hb 3. However, because we are dealing with mixtures of Hb 1 and Hb 2, any conclusion would be too speculative.

**Molecular Phylogeny**—Table 4 lists the species examined in this study and the accession numbers of $\alpha$-globin and $\beta$-globin sequences used in the phylogenetic analysis (UniProtKB/Swiss-
TABLE 4
Species and globin sequences investigated

| Order and species                   | Subunit | UniProt Knowledgebase/Swiss-Prot Accession no./Ref. |
|-------------------------------------|---------|-----------------------------------------------------|
| Coelacanthiformes                   |         |                                                     |
| Latimeria chalumnae	extsuperscript{(outgroup)} | α, β    | P23740, P23741                                     |
| Scorpaeniformes                     |         |                                                     |
| C. kuni	extsuperscript{(a)}        | α, β    | P80270, P80271                                     |
| Scombroidei perciforms              |         |                                                     |
| Thunnus thynnus	extsuperscript{(b)}| α, β    | P11748, P11749                                     |
| Zoarcoid perciforms                 |         |                                                     |
| A. minor	extsuperscript{(b)}       | α (Hb 1), α (Hb 2, Hb 3) | P63270, P83271                                    |
|                                    | β (Hb 1, Hb 2), β (Hb 3) | P63272, P83273                                    |
| Sparoid perciforms                  |         |                                                     |
| Chrysophyrs auratus	extsuperscript{(c)} | α, β (Hb 4) | 32                                                  |
| Notothenioidei perciforms           |         |                                                     |
| N. coriceps	extsuperscript{(a)}    | major α (Hb 1) | P10777                                              |
|                                    | minor α (Hb 2) | P16308                                             |
| Notothenia angustata	extsuperscript{(a)} | α (Hb 1) | P29624                                              |
|                                    | minor α (Hb 2) | P16308                                             |
| Pleuragramma antarcticus	extsuperscript{(c)} | α (Hb 1, Hb 2) | 32                                                  |
|                                    | β (Hb 1, Hb 3) | 32                                                  |
| Pagothecia borchgrevinki	extsuperscript{(a)} | α (Hb 1, Hb 0) | P63244                                             |
|                                    | minor α (Hb 1) | P63246                                             |
| Gobionotothen giberifrons	extsuperscript{(a)} | α, β (Hb 2) | 32                                                  |
| Aethotaxis mitopteryx	extsuperscript{(a)} | α, β | 32                                                  |
| T. nevnesi	extsuperscript{(a)}     | major α, β (Hb 1) | P45718, P45720                                    |
|                                    | minor α (Hb 2, Hb C) | P45719, P45721                                    |
| T. bernacchii	extsuperscript{(a)}  | major α, β (Hb 1) | P80043, P80044                                    |
|                                    | minor β (Hb C) | P45722                                             |
| Cygnodraco mawsoni	extsuperscript{(a)} | α (Hb 1, Hb 2) | P23016                                             |
|                                    | major β (Hb 1) | P23017                                             |
|                                    | minor β (Hb 2) | P23018                                             |
| Gymnodraco acuticeps	extsuperscript{(a)} | α, β | 32                                                  |
| Bathydraco mawsoni	extsuperscript{(c)} | α, β | 32                                                  |
| Pogonoryphene scotti	extsuperscript{(a)} | α, β | 32                                                  |
| A. oriana	extsuperscript{(c)}      | α, β    | 32                                                  |
| Salmoniformes                       |         |                                                     |
| Salmo salar	extsuperscript{(a)}    | α       | P11251                                             |
| O. mykiss	extsuperscript{(a)}      | α, β (Hb I) | P02019, P02142                                    |
|                                    | α, β (Hb IV) | P14527, P2141                                     |
| Gadiformes                          |         |                                                     |
| A. glacialis	extsuperscript{(a)}   | α	extsuperscript{1} (Hb 1), α	extsuperscript{2} (Hb 2, Hb 3) | This study                                        |
|                                    | β	extsuperscript{1} (Hb 1, Hb 2), β	extsuperscript{2} (Hb 2, Hb 3) | This study                                        |
|                                    | α	extsuperscript{1} (Hb 1), α	extsuperscript{2} (Hb 2, Hb 3) | This study                                        |
| B. saida	extsuperscript{(a)}       | α	extsuperscript{1} (Hb 1, Hb 2), β	extsuperscript{1} (Hb 2, Hb 3) | This study                                        |
|                                    | β	extsuperscript{1} (Hb 1, Hb 2), β	extsuperscript{2} (Hb 2, Hb 3) | This study                                        |
|                                    | α	extsuperscript{1} (Hb 1, Hb 2), α	extsuperscript{2} (Hb 2, Hb 3) | This study                                        |
|                                    | β	extsuperscript{1} (Hb 1, β' (Hb 2, Hb 3) | This study                                        |
|                                    | (additional chain) | O13077                                             |
| Anguilliformes                      |         |                                                     |
| Anguilla anguilla	extsuperscript{(a)} | α, β (Hb C) | P80726, P80727                                    |
|                                    | α, β (Hb A) | P80495, P80496                                    |
| Gymnotiformes                      |         |                                                     |
| Electrophorus electricus	extsuperscript{(a)} | α, β | P14520, P14521                                    |
| Siluriformes                        |         |                                                     |
| Hoplosternum littorale	extsuperscript{(a)} | α, β (Hb C) | P82315, P82316                                    |
| Cypriniformes                       |         |                                                     |
| Cyprinus carpio	extsuperscript{(a)} | α, β | P02016, P02139                                     |
| C. auratus	extsuperscript{(c)}     | α, β    | P02018, P02140                                     |
| Catostomus clarki	extsuperscript{(c)} | α | P02017                                             |

	extsuperscript{a} Temperate freshwater and marine species.  
	extsuperscript{b} Arctic species.  
	extsuperscript{c} Antarctic Notothenioidei.  
	extsuperscript{d} Non-Antarctic Notothenioidei.  
	extsuperscript{e} M. Tamburrini and G. di Prisco, unpublished results.

The Hbs of the Boreal Fish Family Gadidae—Ectotherms organisms living in the polar regions are exposed to strong environmental constraints. Polar fish needed to acquire sophisticated molecular mechanisms of physiological/biochemical adaptations to deal with variations in water conditions, e.g. oxy-
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gen level, temperature, and salinity. In the two environments, adaptive responses were achieved to different extents. In comparison with Antarctic Notothenioidei, boreal Gadidae exhibit lesser specialization of organ systems to achieve suitable adjustments in response to differences and fluctuations in the physicochemical features of Arctic waters, much larger than in the Antarctic.

In the Arctic species multiplicity of Hbs is higher, and in the phetic trees the globins are highly dispersed, losing the memory of the species phylogeny (see Fig. 6). In contrast, because notothenioids are always found monophyletic for a given ortholog, the record of species phylogeny is maintained. The search for an explanation for this discrepancy led to the hypothesis that in thermostable environments such as the Arctic the need for multiplicity may be reduced, and the rate of change in the primary structure may be constant and similar for all taxa, causing no variation in the mutational space among these. Higher Hb multiplicity and strong sequence variability, typical of temperate and tropical environments, but observed also in the Arctic (to somewhat lower extent), might be linked to the variations in physicochemical features (essentially temperature and oxygen availability) characterizing these environments. Of course, in pelagic, migratory fish, the dynamic life style may well be a driving factor for high Hb multiplicity.

Hbs are highly sensitive to temperature; therefore, their structural and functional properties in part mirror the thermal conditions encountered by species during their evolutionary histories. Rather than relying on a single respiratory protein, many organisms, including teleosts, express multiple oxygen carriers with different oxygen-binding properties in order to meet oxygen demand under changing environmental conditions or metabolic challenges. Pertinent examples are common in species with active life styles, e.g. Trematomus novemini (26) in the Antarctic (one of the few notothenioids with multiple Hbs) and O. mykiss (35) in cool temperate habitats. For instance, a single Hb with Bohr/Root effects would make oxygen delivery to tissues quite difficult under conditions of acidosis. In the latter fish (36), it has been found that variable temperatures, oxygen availability, and photoperiod regulate the relative abundance of the individual Hbs. This plesiomorphic system has been inherited by boreal gadids and is used in a cold, variable context, such as the Arctic. However, polymorphism may often have no visible phenotypic effect and no obvious correlation with environmental conditions and/or life style.

On the other hand, Antarctic notothenioids lost globin variability and concentration/multiplicity of Hb, and the erythrocyte number became reduced, probably because of environmental stability (notably of temperature). This adaptive reduction counterbalances the increase in blood viscosity produced by subzero seawater temperature (37) with potentially negative physiological effects (i.e. higher demand of energy needed for circulation).

Collectively, A. glacialis, B. saida, and G. morhua have a geographic distribution extending from high Arctic to temperate latitudes. A. glacialis is caught in the ice-covered fjords of Greenland and is a relatively sedentary fish, seldom found below 70°N. The other two species are highly migratory; B. saida is normally found above 60°N, whereas G. morhua reaches much lower latitudes. It is reasonable to expect that the temperature gradient covered by species of the same family would merely correspond to small (but significant) adjustments in the molecular adaptations of their Hb systems in response to local environmental changes. Indeed, some differences were found in the features of Hb 3 of each species. Preliminary results clearly indicate that A. glacialis has the lowest hematorit (similar to the erythrocyte levels of cold-adapted Antarctic fish) and G. morhua the highest, in keeping with their thermal distribution. Also the lower oxygen affinity seen in the species of highest northern latitudes, A. glacialis, is typical of the most cold-adapted species. On the other hand, the Bohr coefficient (although correlation with the temperature gradient cannot be ruled out) may be preferentially related to life style, higher in the sedentary species and lower in the two migratory species because of the need to easily release oxygen to tissues also during bursts of activity.

Val(E11), usually present in both chains at the distal side of the heme, is replaced by Ile in A. glacialis, B. saida, and G. morhua Hb 3. In HbA mutants it has been shown that the bulky side chain of Ile(E11) blocks the access of oxygen to the β chain, significantly lowering the association (and equilibrium) constant both in the T (38) and R state (39). In deoxy-HbA, Val BE11 overlaps the ligand-binding site and is considered to play a key role in controlling the oxygen affinity (40). It has also been shown that the replacement of Val BE11 with Ile affects the kinetic parameters of oxygen binding (41), because of the larger sec-butyl group that sterically hinders access of ligands to the heme iron. The contact surface area between the β-heme and Ile(E11) is 59 Å² in T. newnesi Hb C (displaying the substitution Val BE11 → Ile BE11 at the distal side of the heme pocket), whereas it is 50 Å² in Trematomus bernacchii Hb, which has Val (42). These data indicate that steric hindrance is an effective means of regulating ligand affinity; interestingly, all minor Antarctic Hbs display this substitution.

In the primary structures, the replacements in the switch region are of special interest. This region, which has a primary role in the cooperative, quaternary transition T → R, is highly conserved in vertebrate Hbs. Side chain packing at this interface is likely to be the major reason for the larger rotation of the two dimers in the R state in Hb of T. bernacchii compared with human HbA (43). Moreover, a different αβ2 interface in fish Hbs is consistent with the lower tendency to dissociate into dimers than human HbA (44). The lack of cooperativity observed in A. glacialis and B. saida Hb 3 may be due to a less constrained T state produced by looser side chain packing at the switch region. The slightly higher cooperativity of G. morhua Hb 3 may be linked to retention of Thr αC6.

![FIGURE 7. Phenetic trees of amino acid sequences of α chains (A) and of β chains (B) of Arctic, Antarctic, and temperate fish Hbs showing the degree of similarity among sequences. BP (percentage of 10,000 replicates) are given at the nodes. Globin sequences of notothenioids are in blue, zoarcooids in green, gadids in red, and non-acanthomorph fishes in black. Colors are relevant with regard to Fig. 6.](image-url)
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According to the concerted two-state model of Monod et al. (45), cooperativity arises from a transition between the T and R states. The quaternary transition to R state occurs when the \( \alpha_1\beta_1 \) and \( \alpha_2\beta_2 \) dimers have at least one ligated subunit, impairing stability of the \( \alpha_1\beta_2 \) interface in the T state. In contrast, in Root-effect Hbs, which bind oxygen noncooperatively at lower pH values, the interface remains stable upon oxygenation in the T state, where a large subunit functional heterogeneity is observed (46, 47), although its structural basis has not been established unequivocally (43). In cases where cooperativity is impaired also at alkaline or neutral pH, such as the Gadidae, Anguilla anguilla (48) and Chelidonichthys kumu, subunit heterogeneity is probably more marked (47).

The lack of cooperativity in Hb 3 of the two species thriving at higher latitudes resembles the findings on some Antarctic notothenioids of the family Arctedraconidae; the single Hb of Artedidraco orianae also displays lack of cooperativity, and hyperbolic oxygen dissociation curves were obtained (14). In comparison with Hb of A. orianae, Hb 3 of the gadids displays lower oxygen affinity and higher Bohr/Root effects. Moreover, A. orianae is sluggish, whereas two of the gadids actively perform migrations. Thus, lack of cooperativity may occur in Hbs that are functionally quite different and are found in fish with a wide range of modes of life. In cold-adapted polar fish with very low metabolic rates and decreased dependence on Hb-mediated oxygen transport (7), lack of cooperativity might be correlated to a less critical role of Hb, having the mere physiological role of an “oxygen store” for anoxic conditions. Although this hypothesis cannot account for all instances, interesting questions on the mode of function of a multisubunit molecule remain open. For instance, do these Hbs undergo the T \( \rightarrow \) R conformational transition upon going from the deoxy to the oxy state?

Relying upon Hbs with reduced \( \Delta H \) values seems a frequent evolutionary strategy of cold-adapted fish, indicating that oxygen release in the tissues is favored by low temperature. The reduced thermal sensitivity of Hbs of polar fish is a most elegant strategy adopted during evolution to solve the problem of oxygen transport to tissues. These Hbs do not require a significant amount of energy during both oxygenation at the gills and deoxygenation in the tissues (31).

Phylogeny—p-Distances and NJ trees were used for the following reason. Pure phenetic approaches to sequence comparisons, i.e. with uncorrected distances, provide trees that exhibit degrees of similarity. When evolutionary pressures and rates of change are the same across taxa, similarity is proportional to phylogeny, and in that case a phenetic tree (NJ tree used with p-distances) reflects the phylogenetic tree. We chose p-distances to evaluate to what extent the resulting tree differed from the expected among-species interrelationships (i.e. gene copies diversified from an ancestral speciation). Differences found robust enough, BP >70%, are supposed to reflect changes in selective pressures or rates in those sequences placed at an unexpected position. Using corrected distances or likelihood methods that incorporate models of sequence evolution would have “buffered” effects from variation in selective pressures, making results very difficult to interpret.

To perform this comparison, there must be some expected relationships among species based on zoological knowledge. Comparison of recent molecular work in higher teleost phylogeny (49–54) shows areas of resolution and areas of ambiguity. The “Clade X” (see Fig. 6) is recovered by multiple recent independent studies (51, 52, 55), even based on a quite different taxonomic sampling. Fig. 6 is also supported by morphology, both recent (see Ref. 56 for example) and classical (57). Even from the anatomical point of view, zoarcoids and notothenioids are “perciforms”; gadiforms are closer to perciforms than to any other fish of the present data set. Departures from these relationships within an ortholog cluster are only considered when robust for adaptive interpretation.

Showing low identity with temperate species, the globin sequences of the Arctic zoarcoid A. minor are consistent with species history, as A. minor consistently appears close to the nototheniid clades (8) as predicted by teleostean phylogenies (50–52, 54). By contrast, Arctic gadiform sequences occupy different positions in the two trees with regard to temperate and Antarctic sequences.

Gene duplication that led to \( \alpha^1 \)- and \( \alpha^2 \)-gene copies in Arctic gadids must have occurred at least some 60 mya before the divergence of clade X (50), which groups notothenioids, zoarcoids, and percoids among others. The deepest nodes of the \( \alpha \)-globin tree are not robust enough to interpret the timing of gene divergence. However, \( \alpha^1 \) and \( \alpha^2 \) copies in gadids are clearly not related to their phylogenetic counterparts in members of clade X (zoarcoids and notothenioids). A specific perciform duplication of \( \alpha^1 \) and \( \alpha^2 \) copies is suggested between the emergence of gadiforms from the acanthomorph bush some 120 mya (52) and the emergence of the perciform clade X some 60 mya. Such duplication is independent from those leading to gadiform copies (see Fig. 6). The alternative scenario would be a common duplication event ancestral to all these \( \alpha^1 \) and \( \alpha^2 \) copies but followed by very strong amino acid convergences in Arctic gadiform sequences because of cold adaptation. Additional sequences from non-Arctic gadiforms will help in deciding which scenario is to be accepted.

For the \( \beta \) chains the response seems clearer. Gene duplication that led to \( \beta^1 \) and \( \beta^2 \) chains must have occurred before 120 mya, before the separation of gadiform ancestors and perciform-like ancestors of clade X, as \( \beta^2 \) chains are grouped with their counterparts of clade X (BP of 99%). It is possible that the clade grouping gadiform \( \beta^1 \) chains is embedded within the basal polytomy, either because of long branch attraction or because the divergence times of the nodes inferred are too old for the level of variability of these globin amino acid sequences. The “long branch attraction” is more precisely formulated herewith. The (too) basal position of Arctic \( \beta^1 \) globins of gadids (they were expected to be the sister group of the clade assemblineng Antarctic minor Hbs and Hb 3 of A. minor) should be considered as an effect of extreme perturbation of the available mutational space in gadid \( \beta^1 \)-globin sequences. This would cause a long branch attraction artifact, more parsimonious than identifying those three sequences as members of a new globin paralog that would have disappeared from all other fishes. As a consequence, this perturbation is difficult to interpret in a context of thermal stability of the environment, but it is more likely
a response to varying oxygen demands in a more variable thermal environment. In changing environments, multiplicity of forms and large sequence changes are advantageous; in contrast, in thermostable environments reduction in multiplicity can occur and is in fact observed in notothenioids of the high Antarctic.

Concluding Remarks—The different selective pressures on Arctic and Antarctic sequences depend on the respective habitats, and correlation with environmental conditions may have had a driving role. Notothenioids acquired a completely different globin genotype with respect to other teleostean groups. For the Arctic ichthyofauna, thriving in a much more complex oceanographic system than the Antarctic one, we can speculate that it may have been advantageous to maintain a multiple-globin system, helping to deal with environmental changes and metabolic demands. As suggested by other authors (6, 58), multiple Hbs may protect against deleterious mutational changes in the globin genes, provide higher total Hb concentration in the erythrocyte (according to the phase rule, in a saturated solution multiple proteins afford higher total concentration than a single protein), and increase the expression rate of the genes. In contrast, the Antarctic ichthyofauna (dominated by a single taxonomically uniform group) lost its globin multiplicity in correlation with temperature stability. In the trees, Antarctic globs form two distinct compact groups, corresponding to major and minor Hbs (33), in agreement with Ref. 59. In contrast, the gadid globs occupy dispersed positions. The differences between the two polar regions, the wide latitudinal range in which the three Gadidae are found, as well as the active, pelagic, and migratory life style of B. saita and G. morhua, offer a possible explanation to the dispersal of the gadid globs in the trees between the Antarctic and non-Antarctic clades. The stability of the environment may allow the “phylogenetic signal” to be maintained in the Antarctic sequences under selective pressure, whereas environmental variations might tend to erase this “signal” in the gadid sequences, where sequence similarity would not reflect species interrelationships anymore. The inclusion of globin sequences of other nonpolar gadiforms and of zoarcoids will be the next step to better discriminate the part of these relationships inherent to fish phylogeny from that due to cold adaptation.

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