The Oxygen Transport System in Three Species of the Boreal Fish Family Gadidae
MOLECULAR PHYLOGENY OF HEMOGLOBIN*[S]

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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 heterotropic 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 Antarctic 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)[2] 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 Antarctic and Arctic 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

Supplemental Material can be found at:
http://www.jbc.org/cgi/content/full/M513080200/DC1

(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 ($\sim$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 *Arctogadus glacialis*, 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 sulfhydryl 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 C_{18} column (0.39 $\times$ 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
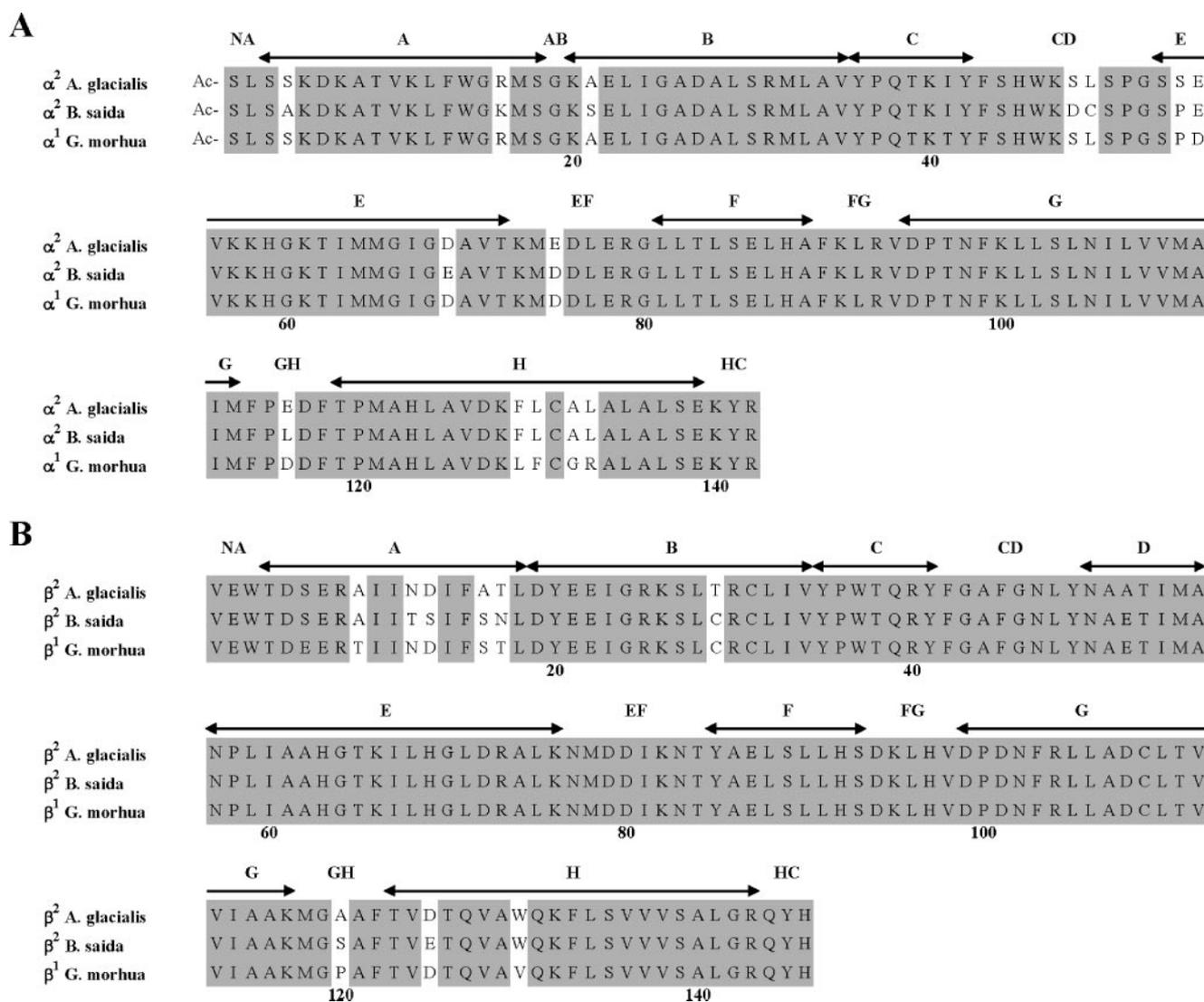
FIGURE 1. **Amino acid sequence of the α (A) and β chains (B) of Hb 3 of the three gadids.** Identical residues are in *gray boxes*. The helical (*A–H*) and nonhelical (*NA, A, CD, EF, FG, GH,* and *HC*) regions, as established for mammalian Hb, are indicated; in α chains, helix D is lacking.

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 ±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 Wösthoff 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 (measured as p_{50}) and cooperativity (n_{Hill}) were calculated from the linearized Hill plot of log $S/(1 - S)$ *versus* log pO_2 at half-saturation (S = fractional oxygen saturation). The formation of met-Hb after each measurement was less than 2%.

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

Phylogenetic Analysis—Multiple alignments of the amino acid sequences of α- and β-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-

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. 2S*A*) and of purified Hb 3 (see supplemental Fig. 2S*B*). 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 β^1) and differ by the α chain (α^1 and α^2), (ii) Hb 3 differs from Hb 2 only by the β chain (β^2), 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 $\alpha^1_2\beta^1_2$, $\alpha^2_2\beta^1_2$, and $\alpha^2_2\beta^2_2$, 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 $\alpha^1_2\beta^1_2$, $\alpha^2_2\beta^2_2$, and $\alpha^1_2\beta^2_2$, 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* β^1 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 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 $\alpha_1\beta_2$ "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 teleostean 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.

TABLE 1

Residues in the $\alpha_1\beta_2$ switch region

Position	Human HbA	Hb 1	Hb 2	Hb 3
A. glacialis				
β97(FG4)	His	His	His	His
α44(CD2)	Pro	Ser	Ser	Ser
α38(C3)	Thr	Gln	Gln	Gln
α41(C6)	Thr	Ser	Ile	Ile
B. saida				
β97(FG4)	His	His	His	His
α44(CD2)	Pro	Ala	Ser	Ser
α38(C3)	Thr	Gln	Gln	Gln
α41(C6)	Thr	Ser	Ile	Ile
G. morhua				
β97(FG4)	His	His	His	His
α44(CD2)	Pro	Ser	Ser	Ser
α38(C3)	Thr	Gln	Gln	Gln
α41(C6)	Thr	Thr	Ser	Thr

TABLE 2

Oxygen-binding parameters of Hb 3 of the three boreal Gadidae, in comparison with Hb 1 of a selection of notothenioids. Absence (−) and presence (+) of the physiological effectors (100 mM NaCl and 3 mM organophosphate)

Species	$\log p_{50}$[a]		n_{Hill}[b]		φ[c]		RE (%)[d]		Ref.
	−	+	−	+	−	+	−	+	
Gadidae									
A. glacialis	1.5	2.5	1.1	1.1	−0.8	−1.7	40	40	
B. saida	1.2	1.5	0.9	1.1	−0.3	−1.1	50	25	
G. morhua	1.2	1.8	1.3	1.5	−0.3	−1.3	55	35	
Notothenioidei									
N. coriiceps[e]	1.2	1.8	3.0	1.2	−0.5	−0.7	40	30	11
G. gibberifrons[e]	1.3	1.8	1.8	1.2	−0.7	−1.0	75	60	24, 25
T. newnesi[e]	1.5	1.5	2.0	2.0	−0.1	−0.2	90	85	26
C. mawsoni[e]	1.3	1.7	2.4	2.4	−0.4	−0.6	50	40	27
A. orianae[e]	0.6	0.8	1.0	1.0	−0.3	−0.4	100	80	14
P. urvillii[f]	0.0	0.2	1.4	1.6	−1.2	−1.5	80	50	28

[a] The oxygen affinity is expressed as $\log p_{50}$ (mm Hg) at pH 7.5.
[b] The highest values (pH range 8.0–8.5) of subunit cooperativity (n_{Hill}) are reported.
[c] $\varphi = \Delta\log p_{50}/\Delta$pH denotes the average number of protons bound upon heme oxygenation.
[d] The Root effect (RE) is expressed as percentage of oxygen saturation at pH 6.25.
[e] Antarctic notothenioids.
[f] Temperate notothenioid *Pseudaphritis urvillii*.

FIGURE 2. **Oxygen equilibrium isotherms (Bohr effect), subunit cooperativity, and oxygen saturation at atmospheric pressure (Root effect) as a function of pH of *A. glacialis* Hb 3 (*A–C*).** 100 mM HEPES at 10 °C in the absence of effectors is shown by the *filled circles*, in the presence of 100 mM NaCl by the *open circles*, 100 mM NaCl, 3 mM ATP by the *filled triangles*, and 100 mM NaCl, 3 mM IHP by the *open triangles*.
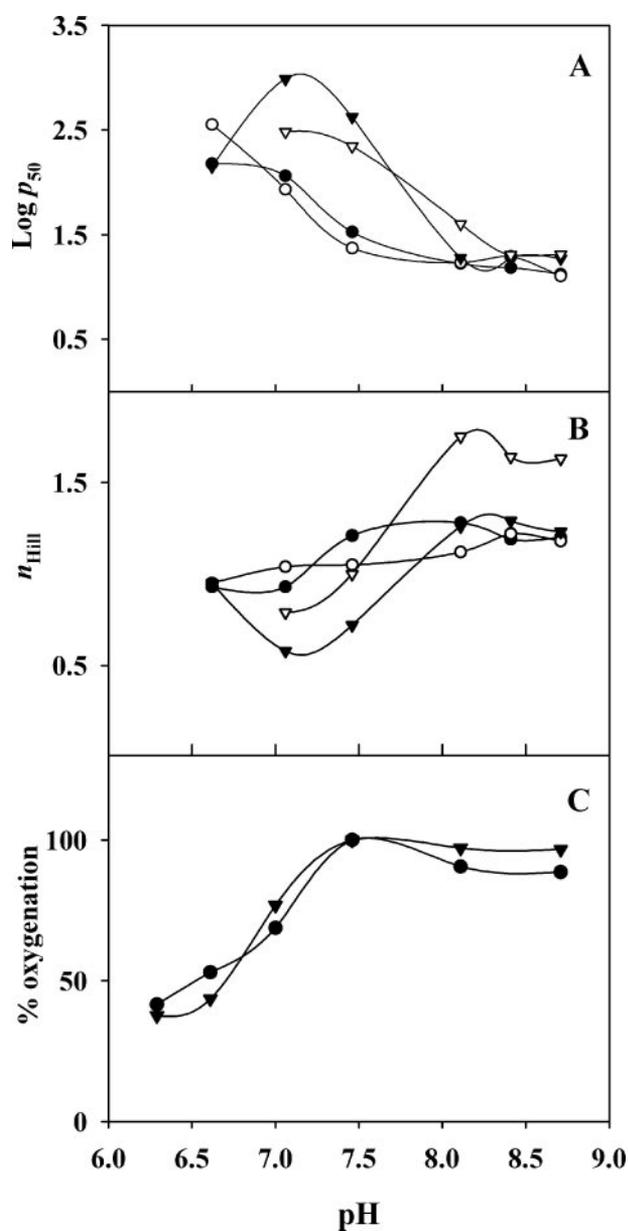

FIGURE 3. **Oxygen equilibrium isotherms (Bohr effect), subunit cooperativity, and oxygen saturation at atmospheric pressure (Root effect) as a function of pH of *B. saida* Hb 3 (*A–C*).** Other details are as in Fig. 2.

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. 2*A*) displayed the highest Bohr coefficient ($\varphi = \Delta\log p_{50}/\Delta\text{pH}$), 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. 3*A*), the number of oxygen-linked protons was increased by ATP from 1.2 to 4.4. In *G. morhua* (Fig. 4*A*), 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_{Hill}) was close to 1, reflecting very low levels or apparent lack of subunit cooperativity (Figs. 2*B* and 3*B* and Table 2). In *G. morhua* Hb 3, cooperativity is slightly but significantly higher (Fig. 4*B* and Table 2) in the alkaline pH range; only when the oxygen affinity reaches the highest p_{50} values is
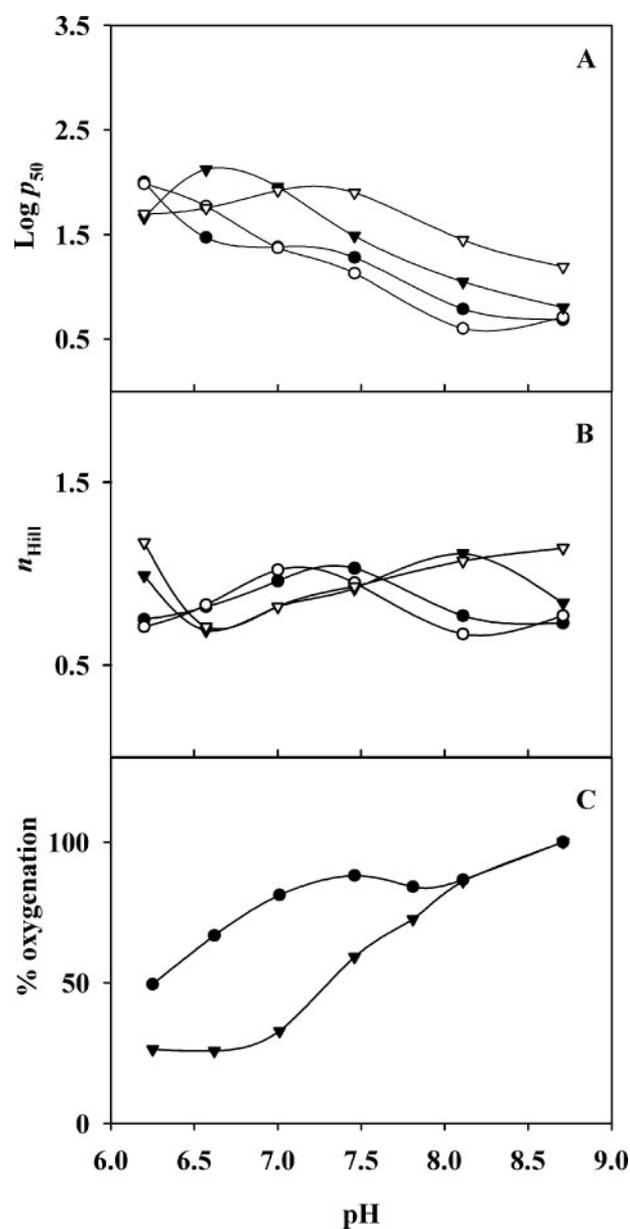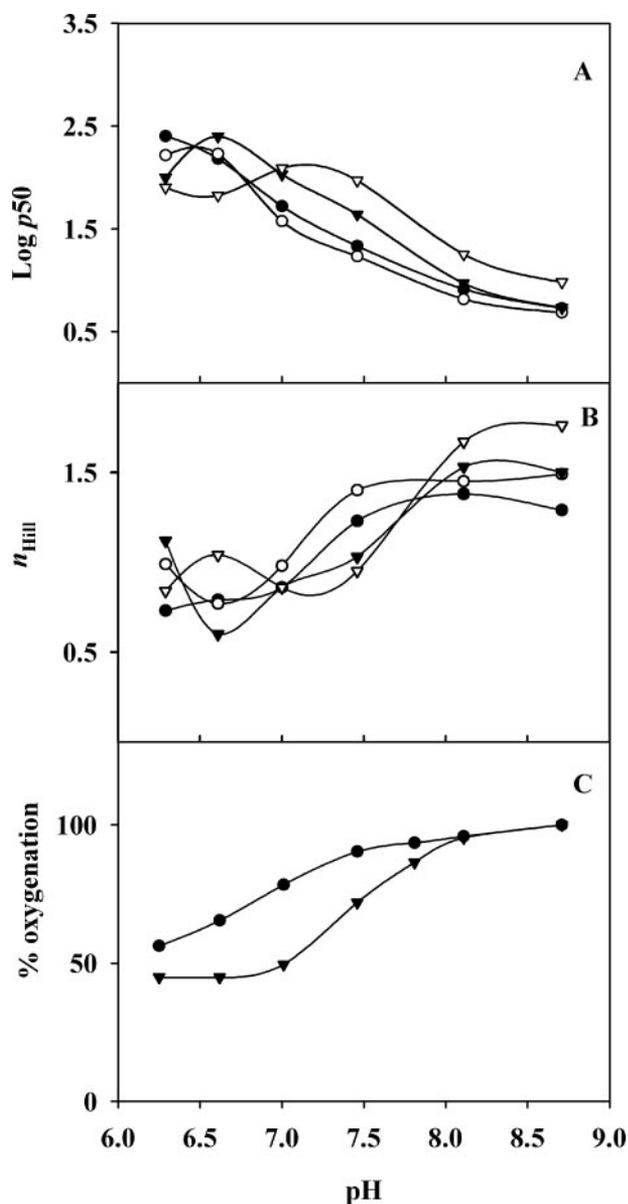
FIGURE 4. **Oxygen equilibrium isotherms (Bohr effect), subunit cooperativity, and oxygen saturation at atmospheric pressure (Root effect) as a function of pH of *G. morhua* Hb 3 (*A–C*).** Other details are as in Fig. 2.

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 notothenioid, *Bovichtus diacanthus*, whose n_{Hill} is ~2.7 is also reported.[3]

In all species, Hb 3 exhibited a marked Root effect (Figs. 2*C*, 3*C*, and 4*C* and Table 2), enhanced by ATP except in *A. glacialis*, where it was very high also in the absence of the ligand. The

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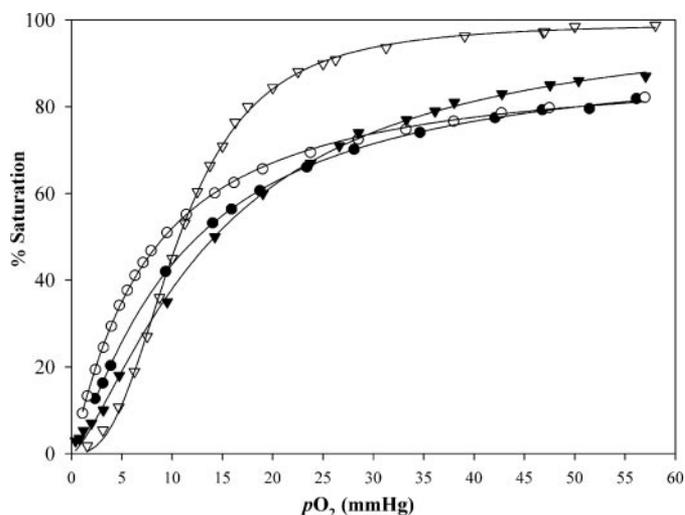[3] C. Verde, L. Grassi, D. Giordano, and G. di Prisco, unpublished results.

FIGURE 5. **Oxygen dissociation curves of *A. glacialis* Hb 3 (*filled circles*), *B. saida* Hb 3 (*open circles*), *G. morhua* Hb 3 (*filled triangles*), and *B. diacanthus* (*open triangles*);** 100 mM HEPES, pH 8.1, 10 °C.

TABLE 3
Overall oxygenation enthalpy change (Δ*H*)
Values are in kcal·mol^{-1} (1 kcal = 4.184 kJ).

Species	Hb	100 mM NaCl	3 mM ATP	pH 6.5	pH 8.7
A. glacialis	Hb 3	−	−	−2.5	−10.8
		+	−	−4.3	−10.2
		+	+	−4.4	−12.0
B. saida	Hb 3	−	−	−5.0	−3.30
		+	−	−13.1	−5.9
		+	+	+0.4	+8.4
G. morhua	Hb 3	−	−	−9.6	−5.7
		+	−	−13.4	−9.9
		+	+	−8.0	−10.4

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 α-globin and β-globin sequences used in the phylogenetic analysis (UniProtKB/Swiss-

TABLE 4

Species and globin sequences investigated

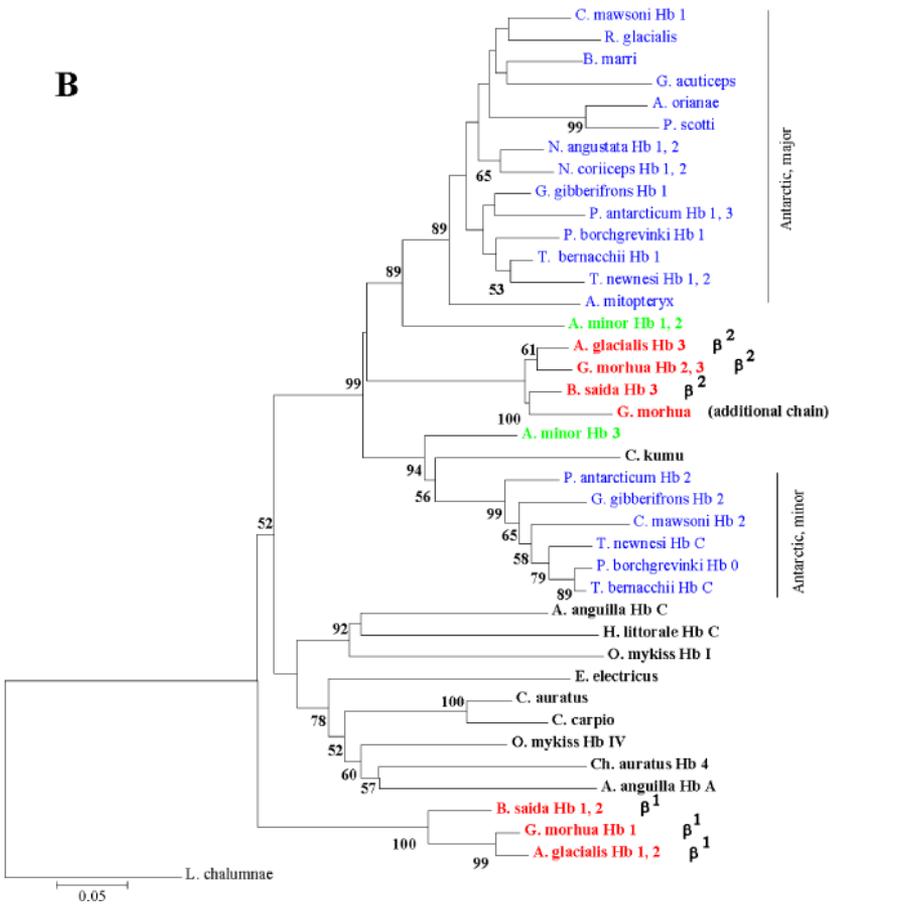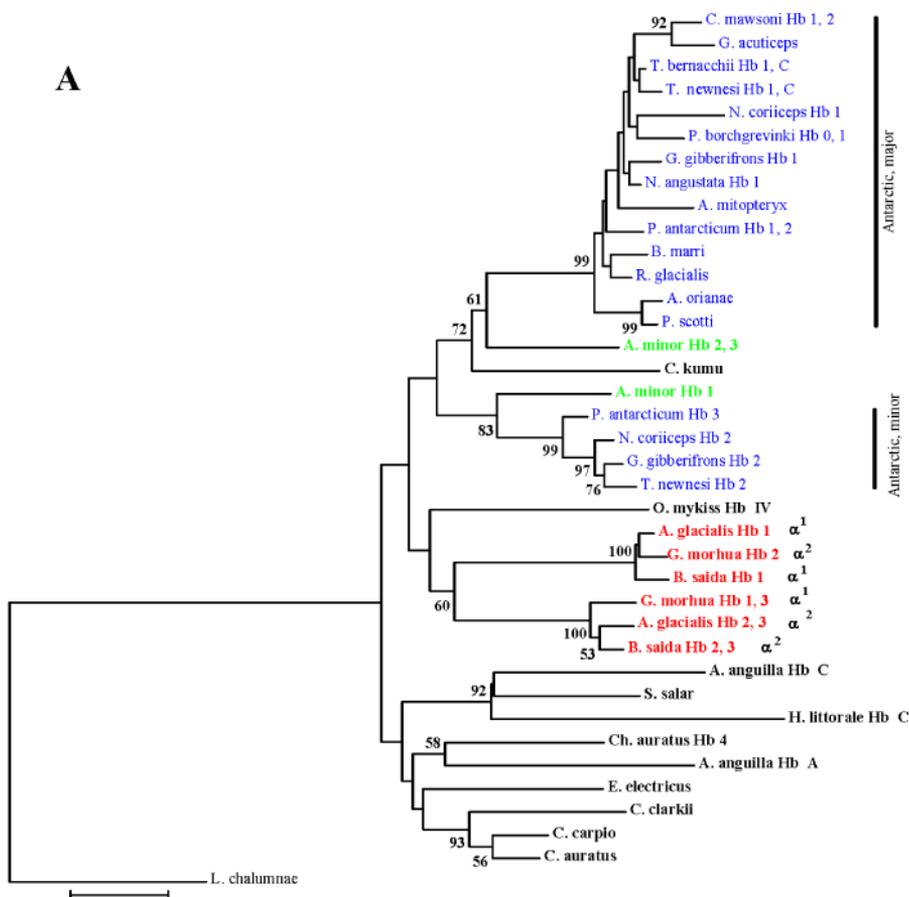| Order and species	Subunit	UniProt Knowledgebase/ Swiss-Prot Accession no./Ref.
Coelacanthiformes (outgroup)		
Latimeria chalumnae[a]	α, β	P23740, P23741
Scorpaeniformes		
C. kumu[a]	α, β	P80270, P80271
Scombroid perciforms		
Thunnus thynnus[a]	α, β	P11748, P11749
Zoarcoid perciforms		
A. minor[b]	α (Hb 1), α (Hb 2, Hb 3)	P83270, P83271
	β (Hb 1, Hb 2), β (Hb 3)	P83272, P83273
Sparoid perciforms		
Chrysophrys auratus[a]	α, β (Hb 4)	32
Notothenioid perciforms		
N. coriiceps[c]	major α (Hb 1)	P10777
	minor α (Hb 2)	P16308
	β (Hb 1, Hb 2)	P16309
Notothenia angustata[d]	major α (Hb 1)	P29624
	minor α (Hb 2)	P16308
	β (Hb 1, Hb 2)	P29628
Pleuragramma antarcticum[c]	α (Hb 1, Hb 2)	32
	β (Hb 1, Hb 3)	32
	minor α (Hb 3), β (Hb 2)	32
Pagothenia borchgrevinki[c]	α (Hb 1, Hb 0)	P82344
	major β (Hb 1)	P82346
	minor β (Hb 0)	P83245
Gobionotothen gibberifrons[c]	major α, β (Hb 1)	P83611, P83612
	minor α, β (Hb 2)	P83613, P83614
Aethotaxis mitopteryx[c]	α, β	32
T. newnesi[c]	major α, β (Hb 1)	P45718, P45720
	minor α (Hb 2), β (Hb C)	P45719, P45721
T. bernacchii[c]	major α, β (Hb 1)	P80043, P80044
	minor β (Hb C)	P45722
Cygnodraco mawsoni[c]	α (Hb 1, Hb 2)	P23016
	major β (Hb 1)	P23017
	minor β (Hb 2)	P23018
Gymnodraco acuticeps[c]	α, β	P29623, P29625
Racovitzia glacialis[c]	α, β	[e]
Bathydraco marri[c]	α, β	32
Pogonophryne scotti[c]	α, β	32
A. orianae[c]	α, β	32
Salmoniformes		
Salmo salar[a]	α	P11251
O. mykiss[a]	α, β (Hb I)	P02019, P02142
	α, β (Hb IV)	P14527, P02141
Gadiformes		
A. glacialis[b]	α^1 (Hb 1), α^2 (Hb 2, Hb 3)	This study
	β^1 (Hb 1, Hb 2), β^2 (Hb 3)	This study
B. saida[b]	α^1 (Hb 1), α^2 (Hb 2, Hb 3)	This study
	β^1 (Hb 1, Hb 2), β^2 (Hb 3)	This study
G. morhua[b]	α^1 (Hb 1, Hb 3), α^2 (Hb 2)	This study
	β^1 (Hb 1), β^2 (Hb 2, Hb 3)	This study
	β (additional chain)	O13077
Anguilliformes		
Anguilla anguilla[a]	α, β (Hb C)	P80726, P80727
	α, β (Hb A)	P80945, P80946
Gymnotiformes		
Electrophorus electricus[a]	α, β	P14520, P14521
Siluriformes		
Hoplosternum littorale[a]	α, β (Hb C)	P82315, P82316
Cypriniformes		
Cyprinus carpio[a]	α, β	P02016, P02139
Carassius auratus[a]	α, β	P02018, P02140
Catostomus clarkii[a]	α	P02017

[a] Temperate freshwater and marine species.
[b] Arctic species.
[c] Antarctic Notothenioidei.
[d] Non-Antarctic Notothenioidei.
[e] M. Tamburrini and G. di Prisco, unpublished results.
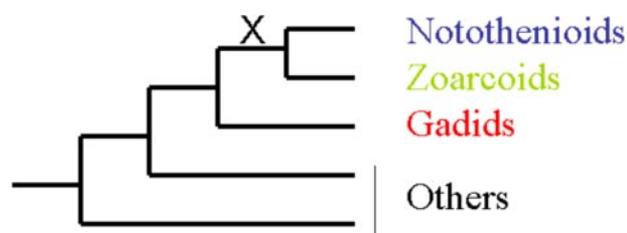
FIGURE 6. **Interrelationships of the acanthomorph fishes of this study as found in the recent phylogenetic literature (50–52, 54), presented to be compared with the phenetic trees of Fig. 7.** *X* denotes clade X, see "Discussion" (51, 52). Note that these relationships are also supported by classical classifications from anatomical knowledge. *Others* means non-acanthomorph sequences of the present sample. For color relevance, see Fig. 7.

Prot data base). The sequences not available in data banks are indicated in Ref. 32. Fig. 6 exhibits the interrelationships we should expect in a given cluster of orthologs among the main three taxonomic components: notothenioids, zoarcoids, and gadids; the rest of the species are external to these three components. The inferred NJ trees for α and β globins are reported in Fig. 7, *A* and *B*. The topologies shown by the two phenetic trees suggest different evolutionary histories for the α and β chains. According to previous results (33), globin paralogs (*e.g.* gene copies originated by duplication in a given genome) currently found in Antarctic fish diverged ~250 mya, *i.e.* at the onset of the Mesozoic period; hence, unlike antifreeze glycoproteins, whose appearance coincided with cooling of the Antarctic continent (34), Hb diversification appears less stringently correlated to changes in the environmental conditions.

Unlike the Antarctic globins, which form two distinct compact groups, the Arctic globins occupy several positions in both trees, suggesting independent evolutionary pressures. The globin sequences of the Arctic zoarcoid *A. minor* follow the track of species history, as *A. minor* consistently appears close to the notothenioid clades as predicted by teleostean phylogenies (interrelationships within notothenioids are not robust enough to be discussed here). By contrast, Arctic gadiform sequences occupy different positions in the two trees with regard to temperate and Antarctic sequences. On one hand, gadiform Arctic α chains appear related to the notothenioid-zoarcoid group. As a result, all α chains of spinous teleosts are clustered (we consider the position of trout *Oncorhynchus mykiss* as unresolved). On the other hand, the β^1 chains of *A. glacialis*, *B. saida,* and *G. morhua* are excluded from the β^2 chains of the same species and from major and minor Antarctic globins with very good bootstrap proportion (BP of 99%). The precise position of the clade of the gadid β^1 chains is not clear because we consider all basal nodes of the β-globin tree as a polytomy. The β^2 chain of the three gadid species and another β chain (possibly belonging to a larval Hb, and whose sequence has been deduced from DNA) of *G. morhua* constitute a clade well separated from the subclades of major and minor Antarctic globins.

DISCUSSION

The Hb System in Gadidae—Ectotherm 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-

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 phenetic 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 Antarctic 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 newnesi* (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[4] clearly indicate that *A. glacialis* has the lowest hematocrit (similar to the erythrocyte levels of cold-adapted Antarctic fish) and *G. morhua* the highest, in keeping with their latitudinal 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 βE11 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 βE11 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 Å^2 in *T. newnesi* Hb C (displaying the substitution Val βE11 → Ile βE11 at the distal side of the heme pocket), whereas it is 50 Å^2 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 $\alpha_1\beta_2$ 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.

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[4] G. di Prisco, unpublished results.

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*, zoarcoids in *green*, gadids in *red*, and non-acanthomorph fishes in *black*. Colors are relevant with regard to Fig. 6.

The Hbs of the Boreal Fish Family Gadidae

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 Artedidraconidae; 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 ΔH 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 (see Fig. 6) in a given cluster of orthologs (*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 notothenioid 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 α^1- and α^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 α-globin tree are not robust enough to interpret the timing of gene divergence. However, α^1 and α^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 α^1 and α^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 α^1 and α^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 β chains the response seems clearer. Gene duplication that led to β^1 and β^2 chains must have occurred before 120 mya, before the separation of gadiform ancestors and perciform-like ancestors of clade X, as β^2 chains are grouped with their counterparts of clade X (BP of 99%). It is possible that the clade grouping gadiform β^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 β^1 globins of gadids (they were expected to be the sister group of the clade assembling 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 β^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 globins form two distinct compact groups, corresponding to major and minor Hbs (33), in agreement with Ref. 59. In contrast, the gadid globins 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. saida* and *G. morhua*, offer a possible explanation to the dispersal of the gadid globins 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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