The Functionally Distinct Hemoglobins of the Arctic Spotted Wolffish *Anarhichas minor**

Cinzia Verde, Vito Carratore, Antonio Riccio, Maurizio Tamburrini, Elio Parisi, and Guido di Prisco‡

From the Institute of Protein Biochemistry, Consiglio Nazionale delle Ricerche, Via Marconi 12, I-80125 Naples, Italy

The Arctic fish *Anarhichas minor,* a benthic sedentary species, displays high hemoglobin multiplicity. The three major hemoglobins (Hb 1, Hb 2, and Hb 3) show important functional differences in pH and organophosphate regulation, subunit cooperativity, and response of oxygen binding to temperature. Hb 1 and Hb 2 display a low, effector-enhanced Bohr effect and no Root effect. In contrast, Hb 3 displays pronounced Bohr and Root effects, accompanied by strong organophosphate regulation. Hb 1 has the β (β^1) chain in common with Hb 2; Hb 3 and Hb 2 share the α (α^2) chain. The amino acid sequences have been established. Several substitutions in crucial positions were observed, such as Cys in place of C-terminal His in the β^1 chain of Hb 1 and Hb 2. In Hb 3, Val E11 of the β^2 chain is replaced by Ile. Homology modeling revealed an unusual structure of the Hb 3 binding site of inositol hexakisphoshate. Phylogenetic analysis indicated that only Hb 2 displays higher overall similarity with the major Antarctic hemoglobins. The oxygen transport system of *A. minor* differs remarkably from those of Antarctic Notothenioidei, indicating distinct evolutionary pathways in the regulatory mechanisms of the fish respiratory system in the two polar environments.

Oxygen carriers are one of the most interesting systems for studying the relationship between environmental conditions and adaptation. Hb, being a direct link between the exterior and body requirements, has experienced a major evolutionary pressure to adapt and modify its functional features. The capacity of fish to colonize a large variety of habitats appears to have evolved in parallel with suitable modulation of their Hb systems at the molecular/functional level.

Hbs generally exhibit marked cooperativity between the oxygen-binding sites of their subunits, which enables maximum oxygen unloading at relatively high oxygen tension. According

to the two-state model (1), cooperativity of oxygen binding arises from a concerted transition between the T and R states. These two extreme conformational states are also involved in modulation of the oxygen affinity brought about by protons, organophosphates, chloride, and carbon dioxide (2). Within the framework of this common mechanism, the respiratory proteins of polar organisms have acquired adaptive mechanisms to meet special needs.

Organisms living in extreme environments, such as the Arctic and Antarctic regions, are exposed to strong constraints, among which temperature is often a driving factor. The northern and southern polar oceans have very different characteristics, and the climatic features of the Antarctic waters are more extreme than those of the Arctic. Due to an oceanographic system, *i.e.* the Antarctic Polar Front, the ichthyofauna is virtually isolated.

The evolutionary trend of Antarctic fish, in the process of cold adaptation, has led to unique specializations, including modification of the hematological characteristics, *e.g.* Hb multiplicity. The vast majority of species of the largely endemic suborder Notothenioidei are bottom dwellers and have a single major Hb (Hb 1), sometimes accompanied by a second, functionally indistinguishable minor component (Hb 2, about 5% of the total) (3–6). Only three notothenioids of the family Nototheniidae, namely *Trematomus newnesi* (7), *Pagothenia borchgrevinki* (8), and *Pleuragramma antarcticum* (9), have high multiplicity of functionally distinct Hbs. The former two species are active and cryopelagic; the latter is pelagic, sluggish but migratory. Thus, the lifestyle of each of these species differs from that of the sluggish species, which spend most of their life on the sea floor, and this is reflected in three specific oxygen-transport systems.

In the Arctic, isolation is less stringent; the range of temperature variations is wider, both in the ocean and in the surrounding lands, which are directly linked to temperate areas, facilitating migration and redistribution of the ichthyofauna. Being an intermediate system, the Arctic thus provides the connection between the most extreme, simpler Antarctic system and the very complex temperate and tropical systems. For this reason, our investigations on the molecular bases of fish cold adaptation have been extended to the Arctic environment. It should be noted that the structure and function of Arctic fish Hbs are virtually unknown.

This study reports the first molecular characterization of the oxygen transport system of an Arctic fish species that has an important role in fishery and aquaculture. The blood of the spotted wolffish *Anarhichas minor* (a benthic, sedentary fish of the family Anarhichadidae, suborder Zoarcoidei, superorder Acanthopterygii) was found to contain three components (Hb 1, Hb 2, and Hb 3). Their amino acid sequences, oxygen binding properties, and thermodynamic features were investigated. Im-

portant characteristics in the primary structures are likely to be related to the marked functional differences shown by these Hbs in pH/organophosphate regulation and thermodynamic behavior. Molecular modeling revealed that Hb 3 (the only component that shows very strong Bohr and Root effects, accompanied by strong organophosphate regulation) has distinct binding sites for ATP (the physiological ligand) and inositol hexakisphosphate (IHP).[1]

The ensemble of results suggests that, in the two polar environments, the evolution of the fish respiratory systems has followed diversified pathways.

EXPERIMENTAL PROCEDURES

Materials—Toyopearl Super Q-650S was from TosoHaas, trypsin (EC 3.4.21.4) treated with L-1-tosylamide-2-phenylethylchloromethylketone was from Cooper Biomedical, dithiothreitol was from Fluka, sequanal-grade reagents were from Applied Biosystems, and HPLC-grade acetonitrile was from Lab-Scan Analytical. All other reagents were of the highest purity commercially available.

Hb Purification—Blood samples were drawn from the caudal vein by means of heparinized syringes. Hemolysates were prepared as described previously (10). Separation of Hbs was achieved by FPLC anion-exchange chromatography on a Toyopearl Super Q-650S column. 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. Hb solutions were stored in small aliquots at −80 °C until use.

Amino Acid Sequencing—Alkylation of the sulfhydryl groups with 4-vinylpyridine, deacetylation of the α chain N terminus, tryptic digestions, and CNBr cleavage were carried out as described previously (9, 11, 12).

Tryptic and CNBr-cleaved peptides were purified by reverse-phase HPLC on a μBondapak-C$_{18}$ column (Waters; 0.39 × 30 cm) as described previously (11, 13). 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 (14). Asp-Pro-cleaved globins were treated with *o*-phthalaldehyde before sequencing (15) 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.

Mass Spectrometry—The molecular mass of the S-pyridylethylated α and β chains and of peptides (<10 kDa) was measured by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager-DE Biospectrometry Work station. Analyses were performed on premixed solutions prepared by diluting samples (final concentration, 5 pmol·μl^{-1}) in 4 volumes of matrix, namely, 10 mg·ml^{-1} sinapinic acid in 30% acetonitrile containing 0.3% trifluoroacetic acid (globins), and 10 mg·ml^{-1} α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% trifluoroacetic acid (peptides).

Oxygen Binding—Hemolysate stripping was carried out as described previously (16). Oxygen equilibria were measured in 100 mM HEPES in the pH range 6.0–8.5 at 6 °C and 10 °C (keeping the pH variation as a function of temperature in due account) at a final Hb concentration of 0.5–1.0 mM on a heme basis. An average S.D. 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 with cascaded Wösthoff pumps for mixing pure nitrogen with air (17). pH values were measured with a Radiometer BMS Mk2 thermostated electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP and 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_{50}) were calculated from the linearized Hill plot of log S/(1 − S) *versus* log pO_2 at half saturation; S = fractional oxygen saturation.

The overall oxygenation enthalpy change ΔH (in kcal·mol^{-1}; 1 kcal = 4.184 kJ), corrected for the heat of oxygen solubilization (−3 kcal·mol^{-1}), was calculated by the integrated van't Hoff equation ΔH = $-4.574[(T_1 \times T_2)/(T_1 - T_2)]$ Δlog p_{50}/1000.

Molecular Modeling—Because phosphate ions preferentially bind to the deoxy T state, molecular models of Hb 2 and Hb 3 were built using the crystallographic structure of deoxy-Hb of the Antarctic fish *Trema-*

tomus bernacchii (Protein Data Bank code 1hbh) (18) as template.

Amino acid residues identical to those of the template in the same sequence positions were assumed to maintain the same conformations; replacements were manually modeled in, using the low-energy rotamer conformation (19). To ensure the stability of the models, a 5-Å water shell was subsequently added around both Hbs. Hydrogen atoms were added to the models at pH 8.0, partial charges were assigned to all atoms, and refinement was performed by the most commonly used methods (20). pH 8.0 was chosen because at this value Hb 3, unlike Hb 2, showed maximal difference in oxygen affinity in the presence of IHP. After minimization of the models, the ATP and IHP were added by automated docking to the classical phosphate binding site of both Hbs in the central cavity located between the two β chains of the deoxy form (21, 22).

Automated docking was carried out with the Affinity package (Biosym/Molecular Simulation Inc.). This program finds the best binding structures of the ligand based on the energy of the ligand·receptor complex, with the ligand and the binding site of the receptor flexible during the search. The ligand/receptor system was partitioned into two subsets, *i.e.* bulk and movable atoms. Bulk atoms are defined as the atoms of the receptor that are not located in the binding site, and they were held rigid during the docking search. Movable atoms include those located in the binding region and can thus move freely, except for atoms of the binding site close to restrained bulk atoms.

The molecular model of IHP was built using cyclohexane as template (23); the phosphate groups were considered to have a total charge of −9.0 at pH 8.0 (24); the model was identical to that reported in the crystallographic structure of the human deoxy-Hb·IHP complex (21). The molecular model of ATP was built using the crystallographic structure of ATPase as template (25), the total charge was −4.0 at pH 8.0. In the starting models of the automated docking, ATP and IHP sit in the central cavity formed by the two β chains, and the movable subset is formed by the residues within 12 Å from the organophosphate.

The consistent valence force field parameters were used in all calculations with a distance-dependent dielectric constant $\epsilon = 1xR$ and a 12-Å cutoff distance for the treatment of the electrostatic and nonbonded interactions.

The pK_a values of Glu-2 of the two β chains of Hb 3 were established by the Delphi package (Biosym/Molecular Simulation Inc.), using a solvent dielectric constant $\epsilon = 80$, a solute dielectric constant $\epsilon = 4$, an ionic concentration of 0.1 M, and the linear Poisson-Boltzmann equation for calculating the electrostatic potential.

Computer graphics, structural manipulations, and energy minimization calculations were carried out in a Silicon Graphics R12000 work station using the Insight II software package (Biosym/Molecular Simulation Inc.). The Discover-3 package (Biosym/Molecular Simulation Inc.) was used for all energy refinement procedures.

Phylogenetic Analysis—Multiple alignments of globin amino acid sequences were performed with the program Clustal X. Phylogenetic trees of globin sequences were inferred using maximum parsimony and neighbor joining methods implemented in the programs PAUP*4b10 (26) and MEGA 2 (27), respectively. Substitution model and settings maximizing the likelihood function were estimated by the program Treepuzzle (28) and used as parameters in the construction of the neighbor joining topology.

RESULTS

Purification of Hbs and Separation of Globins—Ion-exchange chromatography of the hemolysate showed three components, indicated as Hb 1, Hb 2, and Hb 3. Purification was achieved by Tris-HCl gradient elution (Fig. 1); Hb 1 emerged with the equilibration buffer as a discrete peak.

Globins were separated by reverse-phase HPLC. Fig. 2 reports the elution profile of the globins found in the hemolysate. The elution time and partial sequencing of the globins of each purified Hb indicated that Hb 1 and Hb 2 have identical β chains (indicated as β^1) and differ by the α chain (α^1 and α^2), Hb 3 differs from Hb 2 only by the β chain (β^2), and Hb 1 and Hb 3 have no chain in common. Thus the chain composition of Hb 1, Hb 2, and Hb 3 is $\alpha_2^1\beta_2^1$, $\alpha_2^2\beta_2^1$, and $\alpha_2^2\beta_2^2$, respectively.

Primary Structure and Phylogeny—The sequences of the two α and of the two β chains (142 and 146 residues, respectively) constituting the three Hbs of *A. minor* are reported in Fig. 3. They were established by alignment of tryptic and CNBr peptides (data not shown) and on the basis of the strong sequence

---

[1] The abbreviations used are: IHP, inositol hexakisphosphate; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
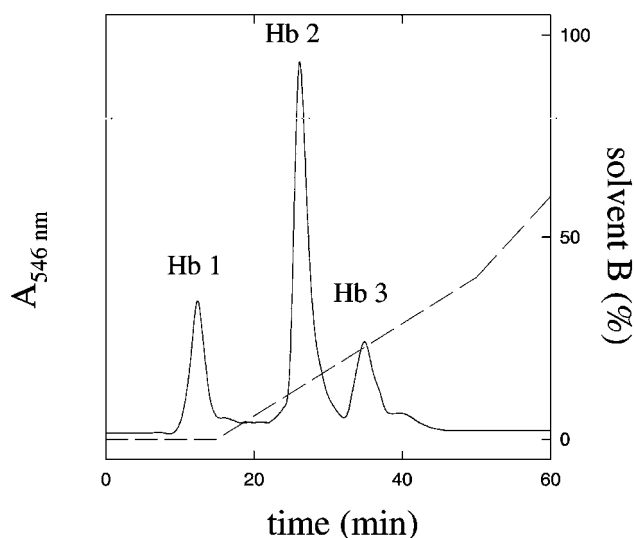
FIG. 1. **Ion-exchange chromatography of *A. minor* hemolysate.** A Toyopearl column (1 × 24 cm) was equilibrated in 10 mM Tris-HCl, pH 7.6. Hb 1, Hb 2, and Hb 3 were eluted at 10, 20, and 30 mM buffer, respectively, at a flow rate of 1.5 ml/min.
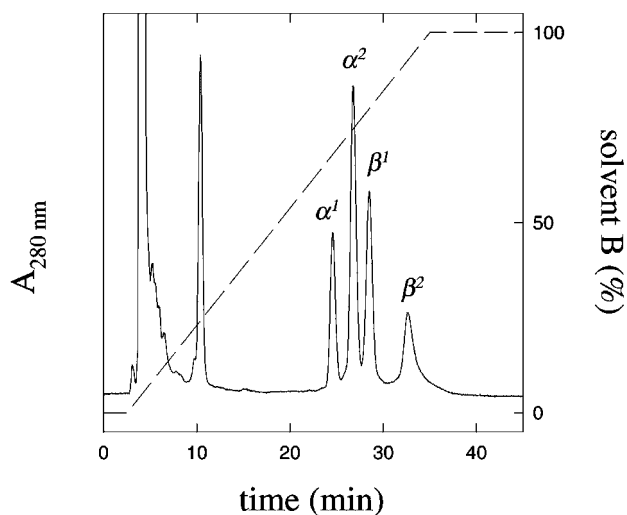

FIG. 2. **Reverse-phase HPLC of *A. minor* hemolysate.** Details are given under "Experimental Procedures."

identity with other Hbs. The N terminus of the α chains was not available to 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 of the four globins is 15,738 and 15,567 Da for α^1 and α^2 (including the acetyl group) and 16,049 and 16,290 Da for β^1 and β^2, respectively. These values are in agreement with the mass of the four globins determined by MALDI-TOF mass spectrometry.

Among the functionally important amino acid residues suggested by Perutz and Brunori (29) to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs, in the β^1 chain of Hb 1 and Hb 2, Ser F9, Glu FG1, and Gln HC1 are conserved, whereas His HC3 is replaced by Cys. In the β^2 chain of Hb 3, all four residues are conserved. The functional implications will be discussed below.

Val E11, usually present at the distal side of heme, is replaced by Ile in the β^2 chain of Hb 3. This substitution may produce functional subunit heterogeneity, as reported for Hb of

the temperate fish *Chelidonichthys kumu* (30). In Hb A 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 state (31) and R state (32); in fact, in deoxy human Hb A, Val E11 overlaps the ligand binding site and is considered to play a key role in controlling the oxygen affinity (33).

Hb 3 displays other potentially important substitutions with respect to human Hb A, namely, in β^2, Lys E3 and Lys E10, close to distal His E7, are replaced by Asn and Ile, respectively, producing large electrostatic variations in the distal portion of the heme pocket.

At the $\alpha_1\beta_2$ "dovetailed" switch interface, several substitutions are observed when compared with human Hb A. In all three Hbs, two of the residues forming the $\alpha_1\beta_2$ switch region in Hb A (His βFG4 and Thr αC6) are conserved, whereas in both α^1 and α^2, Thr αC3 and Pro αCD2 are replaced by Gln and Ser, respectively.

In comparison with Antarctic Hbs, α^1 and β^2 of *A. minor* display higher identity with the corresponding chains of minor Hbs (Hb 2 and Hb C), whereas the α^2 and β^1 chains have higher identity with the chains of major Hbs (Table I). It follows that Hb 2 is the only component of *A. minor* displaying overall higher identity with the major Antarctic fish Hbs. In all cases, the identities are consistently higher than those with Hbs of temperate species (34). Whether these differences are evolutionarily significant is an important open question.

To gain further insight, phylogenetic analysis was undertaken. Phylogenetic trees for both α and β globin sequences inferred with the neighbor joining method are shown in Fig. 4. The polytomies concerning some groups, including the Antarctic group, must be ascribed to the low variability among vertebrate globins at the protein level. However, some general conclusions can be drawn. In both trees, the globin sequences of freshwater fish are grouped in distinct clades with respect to the clade formed by the marine species. The latter clade includes two subclades, one containing the major chains of Antarctic Notothenioidei, and the other containing the minor chains of Antarctic Notothenioidei. Temperate marine species (*Thunnus thynnus* and *C. kumu*) occupy intermediate positions between the two Antarctic globin families. In Fig. 4*A*, the α^2 chain shared by *A. minor* Hb 2 and Hb 3 is close to the major Antarctic globins but also to the two temperate globins, whereas α^1 of Hb 1 appears more closely related to minor Antarctic globins. In the tree of Fig. 4*B*, relative to the β chains, the position of the *A. minor* β^1 chain shared by Hb 1 and Hb 2 is placed in the group of the major Antarctic globins, whereas the Hb 3 β^2 chain is close to the *C. kumu* globin, and both appear well separated from the subclades of major and minor Antarctic globins.

Analogous results were obtained by applying the maximum parsimony method.

Oxygen Binding Properties—*A. minor* Hb 1 and Hb 2 displayed a modest Bohr effect that was slightly enhanced by the physiological ligand ATP (data not shown) and, in a similar way, by IHP. IHP, both alone and in association with chloride, decreased the affinity, but chloride alone had no effect. The effect of IHP was investigated because, as an alternative to some physiological ligands (2,3-diphosphoglycerate and ATP), this organophosphate has often been used to study the functional modulation of Hb (35, 36) because it possesses additional negative charges and displays a larger effect. Fig. 5*A* reports the curves of Hb 2 (the most abundant component), omitting those of Hb 1, which are almost identical, in agreement with the log p_{50} values reported in Table II. In both Hbs, the Bohr coefficient ($\phi = \Delta\log p_{50}/\Delta$pH) was close to -0.35 and to -0.43
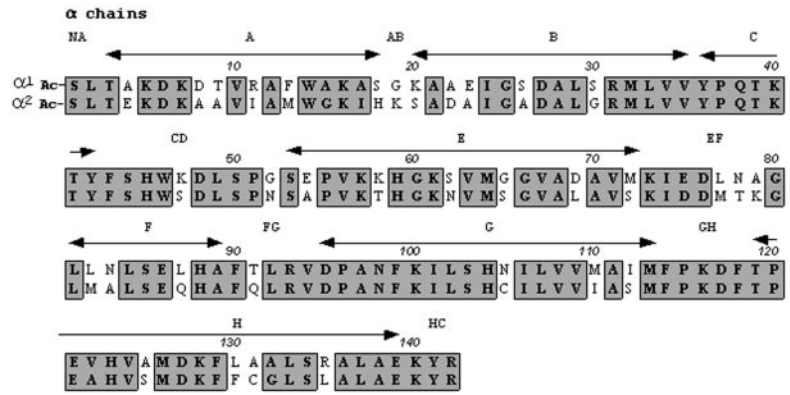
α chains

```
        NA              A                AB              B              C
        10              20               30             40
α1 Ac- S L T A K D K D T V R A F W A K A S G K A A E I G S D A L S R M L V V Y P Q T K
α2 Ac- S L T E K D K A A V I A M W G K I H K S A D A I G A D A L G R M L V V Y P Q T K

            CD           50           E          60            70         EF    80
       T Y F S H W K D L S P G S E P V K K H G K S V M G G V A D A V M K I E D L N A G
       T Y F S H W S D L S P N S A P V K T H G K N V M S G V A L A V S K I D D M T K G

             F           90     FG      100       G          110      GH        120
       L L N L S E L H A F T L R V D P A N F K I L S H N I L V V M A I M F P K D F T P
       L M A L S E Q H A F Q L R V D P A N F K I L S H C I L V V I A S M F P K D F T P

              H            130          140 HC
       E V H V A M D K F L A A L S R A L A E K Y R
       E A H V S M D K F F C G L S L A L A E K Y R
```

FIG. 3. **Amino acid sequences of the α and β chains of *A. minor* Hbs.** α^1, α chain of Hb 1; α^2, α chain of Hb 2 and Hb 3; β^1, β chain of Hb 1 and Hb 2; β^2, β chain of Hb 3. *Shading* denotes residue identity.

β chains

```
        NA               A             10          20        B        30         C    40
β1      V K W S D K E R A V I I S I F A G L D Y E D I G P K A L S R C L I V Y P W T Q R
β2      V E W T D F E R A T I Q D I F S K M D Y E V V G P A A L S R C L I V Y P W T Q R

            CD           D        50            60         E          70        EF    80
       Y F G S F G N L S T P A A I M G N P K I A A H G I K V L H G L D R G V K N M D N
       Y F G S F G N L Y N A A A I M G N P N V A K H G T I I L H G L D R G V K N M D N

             F          90       FG       100       G         110        GH       120
       I K D A Y T E L S I L H S E T L H V D P D N F K L L A D C L T I V V A A K M G C
       I K E T Y A E L S V L H S E K L H V D P D N F K L I S D C L T V V V A A Q F G K

             H           130          140        HC
       A F T P D T Q L A F Q K F L A V V V S A L G K Q Y C
       A F T G E V Q A A F Q K F M A V V V S A L G R Q Y H
```
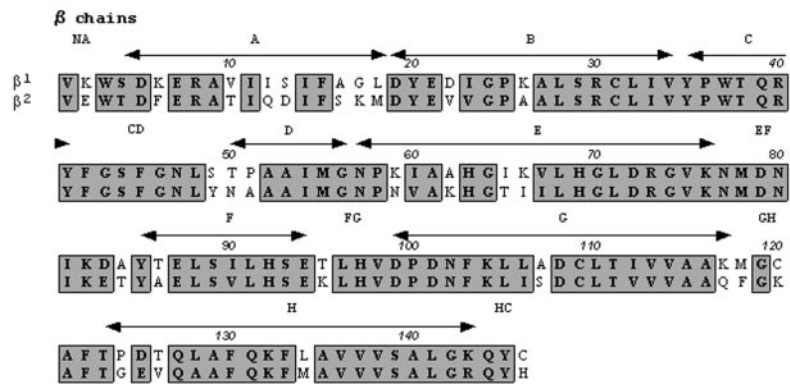
TABLE I

Percentage of sequence identity between α and β globin chains of A. minor Hbs and those of Antarctic and temperate fish Hbs[a]

A. minor globins	Antarctic fish major Hbs (Hb 1)	Antarctic fish minor Hbs (Hb 2/Hb C)	Temperate fish Hbs
α^1 (Hb 1)	62–68%	75–80%	58–68%
α^2 (Hb 2/Hb 3)	73–77%	65–68%	56–64%
β^1 (Hb 1/Hb 2)	74–80%	62–70%	60–66%
β^2 (Hb 3)	70–77%	78–84%	58–65%

[a] Besides those described in this paper, all sequences are from Ref. 34.

in the absence and presence of effectors, respectively. Cooperativity was relatively low, as shown by the decrease of n_H from about 1.7 at alkaline pH to values close to 1 at lower pH (Fig. 5*B*). The Root effect was absent in both the absence and presence of the effectors (Fig. 5*C*).

In comparison with Hb 1 and Hb 2, the oxygen binding features of Hb 3 were quite different. The oxygen affinity was very strongly regulated by protons and organophosphates. The Bohr effect was strong, $\phi = -0.63$ with very high affinity at pH 8.5 ($p_{50} = 1.65$ mmHg); at pH 6.0, the affinity became much lower ($p_{50} = 29.51$ mmHg). Chloride had a modest effect, whereas organophosphates, both alone (data not shown) and in association with chloride, produced a large affinity decrease. This suggests that the binding sites of the two ligands do not overlap or that they overlap only partially.

The effect of IHP differed from that of ATP both quantitatively and qualitatively. In fact, a dramatic decrease in affinity was observed with IHP already at alkaline pH; at pH 8.0, $p_{50} = 55.95$ mmHg, namely, a value corresponding to even lower affinity than that at pH 6.0 in the absence of effectors (Fig. 5*D*). Subunit cooperativity was decreased at lower pH values (n_H was 1.7–2.0 at pH 8.0 in the absence of organophosphates) and

abolished by ATP and IHP (Fig. 5*E*). Hb 3 showed a strong, effector-enhanced Root effect (Fig. 5*F*).

The stripped hemolysate had intermediate oxygen binding features, reflecting the mixture of the three Hbs (Fig. 6); n_H was 1.7–1.8 at pH 8.0 in the absence of organophosphates. The Bohr and Root effects were enhanced by ATP and, to a larger extent, by IHP. Whole blood and intact erythrocytes were assayed in the absence of added effectors and also showed strong Bohr and Root effects (data not shown); at alkaline pH values, subunit cooperativity was lower than that of the stripped hemolysate in the absence of effectors, due to the presence of endogenous organophosphates.

Thermodynamics of Oxygen Binding—The temperature dependence of oxygen binding equilibria was investigated in the 6–10 °C range (Table III).

In human Hb A, the apparent overall oxygenation enthalpy change, ΔH, is more exothermic at alkaline pH values, where the Bohr effect is not operative, and the contribution of the Bohr protons (endothermic) is abolished. In *A. minor* Hb 1, in the absence of effectors, ΔH reaches a minimum in absolute value at pH 7.0 and tends to become more exothermic at higher or lower pH. This behavior is qualitatively similar to that of Hb 2 only when both effectors are present. In the absence of effectors, in Hb 2 ΔH maintains a rather constant absolute value, much lower than that in Hb 1 at pH 6.0 and 8.0. Similar to Hb A, in Hb 3 the oxygenation process, also in the presence of chloride, is more exothermic at alkaline pH; as pH falls, ΔH becomes less exothermic, due to the increasing contribution of the Bohr protons, which cancel some of the heat released upon oxygen binding (37). However, when organophosphates are also present, ΔH becomes more exothermic at pH 6.0 and 8.0. This is in contrast with Hb 1 and Hb 2. Thus organophosphates have a large, but different, influence on the thermodynamic behavior of the three Hbs.
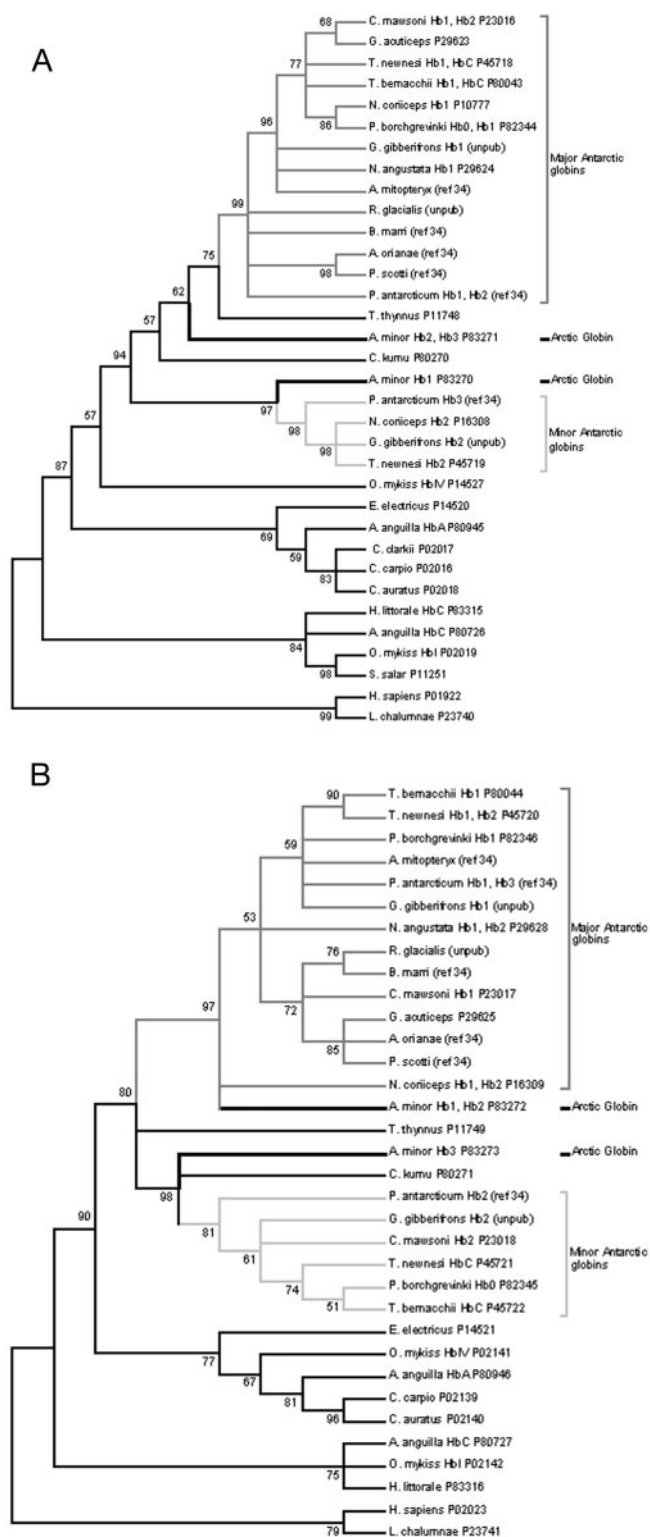
A

B

FIG. 4. **Condensed phylogenetic trees of (A) α-globin and (B) β-globin sequences of *A. minor*, temperate, and Antarctic fish.** The phylogeny was inferred by the neighbor joining method implemented in the program MEGA 2, assuming a γ function distribution of the substitution rates among sites. The value of the shape parameter was set to 1, according to the results of maximum likelihood analyses performed with the quartet maximum likelihood method implemented in the program Treepuzzle. The *numbers* on the *branch nodes*, obtained by the internal branch test implemented in MEGA 2, correspond to confidence probability that the branch length is significantly higher than zero (after 10,000 replicates). The *numbers* near the *taxon names* are the Swiss-Prot accession numbers of the globin sequences. Addi-
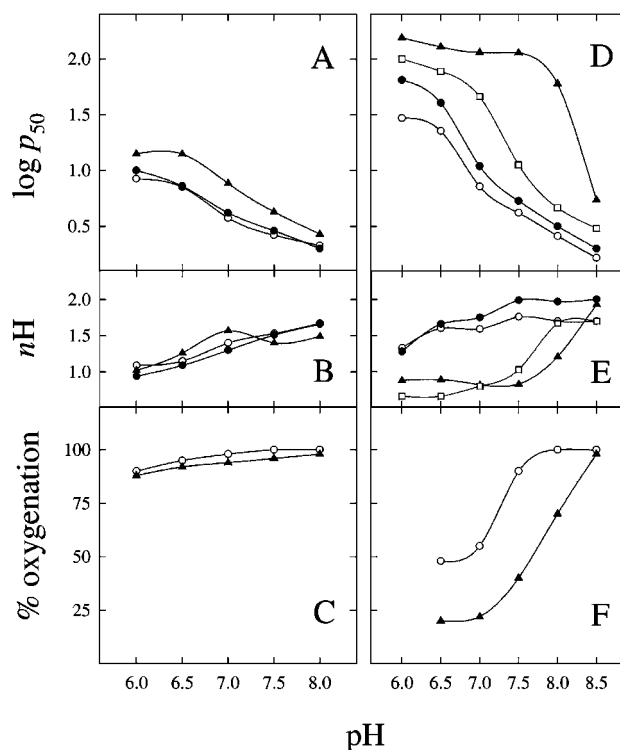

FIG. 5. **Oxygen equilibrium isotherms (Bohr effect), subunit cooperativity, and oxygen saturation at atmospheric pressure (Root effect) as a function of pH of Hb 2 (A−C, respectively) and Hb 3 (D−F, respectively).** Experiments were carried out in 100 mM HEPES at 6 °C in the absence of effectors (○) or in the presence of 100 mM NaCl (●), 100 mM NaCl and 3 mM ATP (□), or 100 mM NaCl and 3 mM IHP (▲).

TABLE II
Values of log p_{50} of Hb 1 and Hb 2 at 6 °C

Hb component	100 mM NaCl	3 mM IHP	pH		
			6.0	7.0	8.0
Hb 1	−	−	0.97	0.59	0.32
	+	−	1.04	0.71	0.40
	+	+	1.30	1.02	0.55
Hb 2	−	−	0.92	0.57	0.33
	+	−	1.00	0.62	0.30
	+	+	1.15	0.88	0.43

In the stripped hemolysate, the combined presence of the three Hbs produced a progressively increasingly exothermic character of oxygen binding in the absence of the effectors as pH is lowered, despite the increasing concentration of the Bohr protons. This thermodynamic behavior is opposite to that of Hb A. This feature could be of great significance because it could well be related to the need of the fish to remove metabolically released heat. It is worth noting that oxygenation becomes endothermic in the stripped hemolysate (pH 8.0) and Hb 3 (pH 6.0) in the presence of chloride.

ΔH was also measured in the unstripped hemolysate, con-

tional sequences are from Ref. 34. The species used are *Cygnodraco mawsoni*, *Gymnodraco acuticeps*, *T. newnesi*, *T. bernacchii*, *Notothenia coriiceps*, *Notothenia angustata*, *P. borchgrevinki*, *Gobionotothen gibberifrons*, *Aethotaxis mitopteryx*, *Racovitzia glacialis*, *Bathydraco marri*, *Artedidraco orianae*, *Pogonophryne scotti*, *P. antarcticum*, *T. thynnus*, *C. kumu*, *Oncorhynchus mykiss*, *Electrophorus electricus*, *Anguilla anguilla*, *Catostomus clarkii*, *Cyprinus carpio*, *Carassius auratus*, *Hoplosternum littorale*, *Salmo salar*, and *Latimeria chalumnae*.
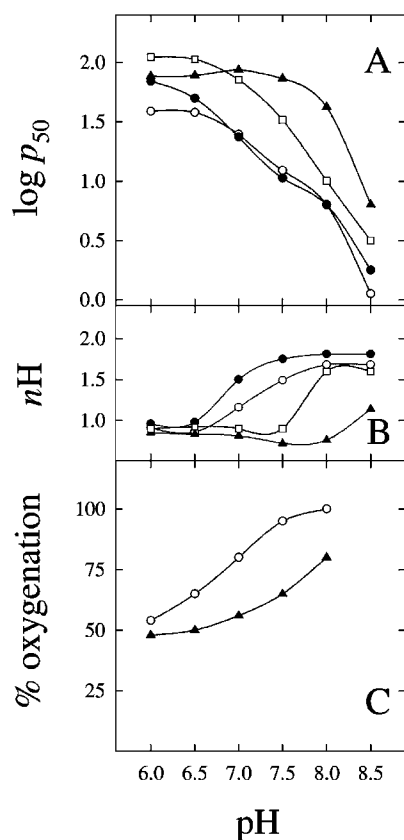
FIG. 6. **Bohr effect (*A*), subunit cooperativity (*B*), and Root effect (*C*) of the stripped hemolysate.** See Fig. 5 legend for experimental conditions.

TABLE III

Overall oxygenation enthalpy change (ΔH)[a] of A. minor hemolysate and Hbs

Hb component	100 mM NaCl	3 mM IHP	pH		
			6.0	7.0	8.0
Unstripped hemolysate	−	−	−1.3	−5.2	6.1
Stripped hemolysate	−	−	−13.4	−9.1	−2.9
	+	−	−0.2	−2.4	5.5
	+	+	−1.3	2.9	−11.0
Hb 1	−	−	−14.2	−6.4	−9.5
	+	−	1.0	−8.8	−2.9
	+	+	−7.5	−16.1	−7.2
Hb 2	−	−	−7.5	−7.4	−3.4
	+	−	−7.2	−11.5	−9.6
	+	+	−12.2	−1.5	−7.2
Hb 3	−	−	−0.3	−10.6	−7.6
	+	−	5.3	−8.4	−11.6
	+	+	−15.8	−4.4	−18.1

[a] ΔH values are in kcal·mol^{-1} (1 kcal = 4.184 kJ).

taining the physiological ATP concentration. As expected, the values were much less negative than those of mammalian Hbs.

Molecular Modeling—The quality of the minimized models of the complexes of *A. minor* Hbs with ATP and IHP was assessed by analyzing their stereochemical properties and by comparison with the crystallographic structure of template *T. bernacchii* Hb. The sequence of the α^2 chain of Hb 2 and Hb 3 shows 74% identity with that of *T. bernacchii* Hb, whereas the β chains of Hb 2 and Hb 3 have 75% and 76% identity, respectively. The identity between the two β chains of *A. minor* Hbs is 72%.

The minimized models of Hb 2 and Hb 3 retained all the α-helices responsible for Hb folding; the Ramachandran plot was very similar to that of *T. bernacchii* Hb in the T state (data

not shown). The root mean square deviations were 0.61 and 0.54, respectively, between the backbone of Hb 2 and Hb 3 and *T. bernacchii* Hb and 0.25 between the two *A. minor* Hbs, suggesting the reliability of the minimized models.

As evidenced by the crystallographic structure of the complexes of Hb A with IHP and 2,3-diphosphoglycerate (21, 22), the classical phosphate binding site is made of charged residues (Val-1, His-2, Lys-82, and His-143) belonging to both β chains that are in direct contact with phosphate. For the considerations that follow, it should be recalled that *A. minor* Hb 1 and Hb 2 have the β chain in common.

In position 2 of the β chain, Hb 2 has a substitution unusual in fish, namely Lys replaces Glu (or Asp); but, as in most fish Hbs, Hb 3 does retain Glu. In the Hb 2-ATP and Hb 2·IHP complexes, automated docking showed that the phosphate binding site of this Hb appears very similar to the binding region of human Hb A (21, 22). In fact, in the β chains of Hb 2, as in human Hb A, four charged residues of both chains, *i.e.* Val-1, Lys-2 (His-2 in Hb A), Lys-82, and Lys-143, are bound to ATP and IHP with salt bridges (Fig. 7, *A* and *B*). Thus, IHP and ATP are centered in a region characterized by eight positively charged residues but in a position exposed to the solvent.

In the Hb 3·ATP complex (Fig. 7*C*), the docking procedure suggested that ATP provides stronger pH regulation by sitting in a more internal position relative to the classical phosphate binding site formed by the β chains. The ligand is bound to Lys-82, Arg-143, and the side chains of Glu-2, placed outside the β cleft.

In the Hb 3·IHP complex (Fig. 7*D*), although the starting position of IHP is very similar to that in Hb 2, the docking procedure suggested a different binding site. In comparison with Hb 2, IHP is bound with salt bridges to Lys-82, Lys-104, Lys-132, and Arg-143, and binding is followed by loss of symmetry around the dyad axis along the central cavity because IHP becomes H-bonded with Ser-139 of one of the β chains. The negative charge of Glu-2 in the β chain causes migration of IHP (more spherical and less bulky than ATP) to a more internal region of the central cavity between the β chains, which (unlike that in Hb 2) is not easily accessible to the solvent, with consequent stabilization of the T state. In fact, even if the high negative charge of IHP can shift the pK_a values of Glu-2 of the two β chains to 5.3 and 4.9, only about 1‰ of the two residues would not be charged at pH 8.0.

DISCUSSION

The importance of the Arctic in contributing to the overall ensemble of adaptive processes influencing the evolution of marine organisms calls for investigations on adaptations of the main biological systems (*e.g.* respiration) of Arctic fish. A wealth of knowledge is available on the oxygen transport system of fish inhabiting the Antarctic sea water, but very little is known regarding the structure and function of Hbs of fish of the other polar marine environment, where the physico-chemical features are so different. In this light, it may be assumed that the main characteristic of the Hb system of *A. minor* is again the response to the need to optimally adapt to the Arctic waters, where the low temperature shows larger differences and fluctuations than in the Antarctic.

The study of the structure/function relationship in the three Hbs of *A. minor* has revealed several important features.

Primary Structure and Functional Properties—The identification of functionally significant changes in the primary structure of vertebrate Hbs helps to shed light on the evolution of protein function. Compared with other fish Hbs and human Hb A, several substitutions occur in crucial positions of the amino acid sequence.

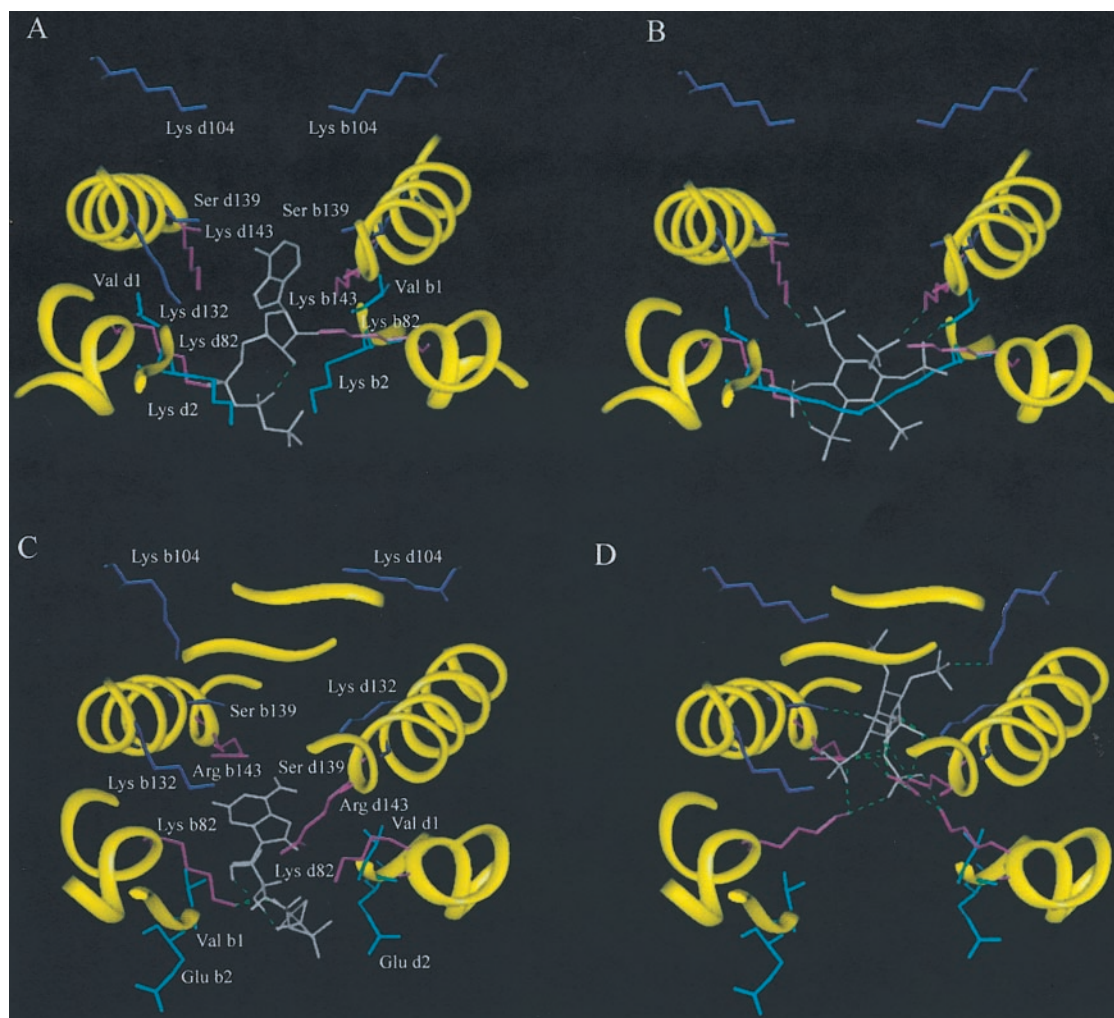In the β^1 chain of Hb 1 and Hb 2, the replacement of C-

FIG. 7. **Phosphate binding site of Hb 2 (*A and B*) and Hb 3 (*C and D*).** ATP (*A and C*) and IHP (*B and D*) are in *white*. The H-bonds established between phosphate and Hb are indicated by *green dashed lines*. The residues indicated with *b* and *d* belong to the β_1 and β_2 chains, respectively. The residues forming the novel binding region are in *blue*, the residues shared by the novel and classical sites are in *pink*, and the residues forming the classical site and not belonging to the novel site are in *cyan*. The ribbon of residues forming the site wall is in *yellow*.

terminal His by Cys conceivably accounts for the decreased alkaline Bohr effect. In contrast, the β^2 chain of Bohr and Root effect Hb 3 has His at the C terminus. In human Hb A, the main Bohr groups are N-terminal Val $\alpha 1$ NA1 and C-terminal His $\beta 146$ HC3, which respectively account for about 30% and 50–65% of Bohr effect (38). In α NA1, fish Hbs have acetyl-Ser, thus the modest Bohr effect of *A. minor* Hb 1 and Hb 2 is due to the His→Cys $\beta 146$ substitution. The role of the β C terminus is supported by the observation that His, normally present in Bohr and Root effect Hbs, is replaced by Phe in trout Hb I (39) and eel cathodic Hb (40), whose oxygen affinities are essentially pH-insensitive. The Root effect is an exaggerated Bohr effect, and although several mechanisms have been proposed, the molecular basis for the overstabilization of the T state in Root effect Hbs is not yet fully understood. *T. newnesi* Hb 1 (41) has no Root effect, despite 95% sequence identity with *T. bernacchii* Root effect Hb (42) and the presence of His at the β C terminus in both Hbs. It is becoming increasingly clear that there is no single molecular explanation of the Root effect.

In Hb 3, Val βE11 is replaced by Ile. In the distal portion of the heme pocket, Val βE11 has been proposed to play a key role in controlling the oxygen affinity (32, 43). Therefore, in *A. minor* Hb 3, this substitution may facilitate the observed decrease in oxygen affinity (44). It is worth recalling that helix E plays a relevant role in the regulation of equilibrium and kinetic properties of ligand binding.

In the β^2 chain of Hb 3, replacement of Lys E3 and Lys E10 by Asn and Ile produces potentially important changes in electric charge. Two replacements in the $\alpha_1\beta_2$ "dovetailed" switch region (which has a primary role in the cooperative, quaternary R-T transition) are unlikely to have functional importance because they occurred in all three Hbs.

The *A. minor* Hb amino acid sequences clearly show low identity levels with temperate fish species, which may imply some degree of correlation with cold adaptation. These observations are in keeping with the results of phylogenetic analysis, which provide additional indications. The α^2 chain shared by Hb 2 and Hb 3 clusters close to the major Antarctic Hbs but is also close to the marine temperate species (*T. thynnus* and *C. kumu*); however, the resolution level of this branch does not allow us to draw a definitive conclusion concerning the position of *A. minor* with respect to the latter two species. In contrast, α^1 of Hb 1 clusters with the minor Antarctic globins and is well separated (with good confidence probability value) from the group that comprises the major Antarctic globins, as well as the globins of marine temperate species and *A. minor* Hb 2/Hb 3.

The β^1 chain shared by Hb 1 and Hb 2 clusters with the major Antarctic globins and is well separated from all the others. Similar to α^1, β^2 of Hb 3 is closer to the subclades of the minor Antarctic globins; however, unlike α^1, it is also close to the α chain of temperate *C. kumu*. Interestingly, both *C. kumu* Hb and *A. minor* Hb 3 display the Root effect, similar to most

minor Antarctic Hbs. However, a close correlation between phylogeny and Root effect cannot be drawn, because Root effect Hbs are also present in other clades of the tree.

The phylogenetic analysis emphasizes that the major component Hb 2 is the only Hb of *A. minor* displaying higher overall similarity with the major Antarctic Hbs, whose globins are preferentially closer to both of those of Hb 2.

Different Effects and Binding Sites of Organophosphates— One of the functional differences, *i.e.* the organophoshate specificity, is conceivably not related to cold adaptation; nonetheless, it is of general importance in the chemistry of allosteric proteins and therefore calls for a few comments.

The oxygen equilibria and affinity of Hb 3 are strongly influenced by organophosphates in a wide range of physiological pH values. Important differences have been observed in the oxygen affinity regulation of Hb 3 with ATP *versus* IHP. Molecular modeling has suggested differently structured phosphate binding sites in Hb 1 and Hb 2 on one hand and Hb 3 on the other, which, at the molecular level, may explain these findings. As in human Hb A, the site of Hb 1/Hb 2 is composed of four residues belonging to both β chains and binds both phosphate ligands. In Hb 3, ATP binds to a more internal domain relative to the classical phosphate site of the β cleft, yielding higher pH regulation of oxygen binding.

In contrast, in the model of the Hb 3·IHP complex, because of the replacement of Lys by Glu in the β2 position, IHP migrates along the central cavity, in the inner portion of the site, to a region not exposed to the solvent. This configuration allows strong binding of IHP also to other residues of both β chains (such as Lys-104 and Lys-132, in addition to Lys-82 and Arg-143), thus stabilizing the T structure. Stereochemical hindrance instead impairs binding of ATP in this region. The exceptional affinity decrease already brought about by IHP at alkaline pH (where the affinity is lower than that measured at pH 6.0 in the absence of effectors) and the differently structured IHP binding site strongly suggest that this ligand is able to lock the protein in the low-affinity T state also at high pH values. Thus the molecular structure of *A. minor* Hb 3, under suitable conditions, is able to retain the liganded low-affinity noncooperative T state throughout the whole physiological pH range. Adequate exploitation of this remarkable feature, with the general aim to gain further insight into the R-T allosteric transition, calls for establishing the crystallographic structure of the Hb 3·IHP complex at pH 8.0.

Thermodynamic Analysis—The detailed thermodynamic analysis of each Hb of *A. minor* has revealed further important differences. Temperature dependence, which is governed by the associated overall enthalpy change, is an important feature of the reaction of Hbs with oxygen. Heat absorption and release can be considered physiologically relevant modulating factors, similar to hetero- and homotropic ligands. The affinity and cooperativity of all Hb systems are strictly correlated with the extent of heat absorption accompanying oxygenation. Oxygen binding to mammalian Hbs is generally exothermic (negative ΔH), so that a decrease in temperature induces an increase of oxygen affinity. Thermodynamic analysis has shown that the oxygenation enthalpy change in Antarctic fish species is often very low (45) when compared with fish of temperate waters and is very similar to that of some Hb components of *A. minor*. Relying upon Hbs with reduced ΔH values may thus be a frequent evolutionary strategy of cold-adapted fish. It seems that in polar fish, evolutionary development has often favored a decrease in the temperature sensitivity of Hb oxygen affinity, surprisingly similar to that observed in some species whose lifestyle is very different and that must adapt to large temperature fluctuations, *e.g.* tuna (45).

The ΔH values as a function of pH and ligands show large variations among the three *A. minor* Hbs. Although a simple scheme is difficult to achieve, fine thermodynamic regulation of the oxygenation/deoxygenation cycle, which may play a significant role in keeping the internal temperature constant, may be an important adaptive tool.

Concluding Remarks—Whereas a single Hb appears sufficient to most Antarctic notothenioids, the Hb system of *A. minor* is made of three components.

During the activity linked to fast movements, there is increased demand of oxygen at the gills, production of heat due to the increased rate of the metabolic reactions, and a concomitant pH decrease brought about by lactic acid and/or heat production. At the gills, Hbs may find acidic pH, which may dangerously lower the oxygen affinity if only strong Bohr and Root effect Hbs were present. *A. minor* may have to face acidosis and possibly heat production during fast movements, as well as temperature variations at decreasing latitudes; therefore, there is an obvious evolutionary advantage in relying upon a multiplicity of functionally distinct Hbs, ensuring adequate oxygen binding at the gills (via Hb 1 and Hb 2) and controlled release in the tissues (via Hb 3). *A. minor* appears to be a sedentary benthic species, therefore these functional characteristics are likely to become operative essentially during emergencies. On the other hand, the differences in thermodynamic behavior of the three Hb components may have a crucial adaptive importance, being linked to the range of temperature experienced by *A. minor*. Compared with the fish habitat in the Antarctic, this range is much larger. This is due to the lower isolation, which has allowed this fish to colonize a wide range of latitudes.

There are advantages in using organisms from both poles in evolutionary studies using phyletically unrelated taxa for a comparative approach. One remarkable example is the analysis of antifreeze glycoproteins (46). The Arctic cod (*Boreogadus saida*) is phylogenetically unrelated to Notothenioidei (which belong to different superorder and order); however, its genome contains genes that encode antifreezes nearly identical to those of notothenioids. Although this would suggest a common ancestry, the genes of the two fish groups are not homologous and hence have not followed the same evolutionary pathway. Assuming an endogenous yet unknown genetic origin, the Arctic cod antifreeze genes have evolved from a genomic locus certainly different from that of notothenioids, which is that of trypsinogen. This is an extraordinary example of convergent evolution adopted by Arctic and Antarctic fish leading to an identical adaptation. Another pertinent example, at the protein level, is the object of this investigation, namely the oxygen transport system. It is worth mentioning that establishing whether convergent or parallel evolution has taken place in proteins is indeed a worthwhile goal but requires complex and lengthy procedures, which are currently under lively debate.

The remarkable differences in multiplicity and structure/function of *A. minor* Hbs compared with the majority of the Antarctic Notothenioidei again suggest that the two polar environments have dictated markedly distinct evolutionary pathways in the regulatory mechanisms of the Hb system. Biological diversity is reflected in the molecular aspects of biological processes (*e.g.* respiration and freezing avoidance), and the present findings are helpful for searching new insights into the molecular basis of cold adaptation of polar fish.

In conclusion, evolution of the oxygen transport system of *A. minor* appears to have produced Hbs differing in pH, organophosphate, and temperature regulation, thus developing physiological and biochemical adaptations suitable to reconcile respiration with lifestyle, allowing optimization of Hb function

under the variety of conditions experienced in the Arctic marine environment. The characterization of gene clusters and the study of their organization and expression in the genome and of their regulation mediated by promoters and/or enhancers are important future developments, which will take advantage of the wealth of information available on the globin genes of cold-adapted Antarctic fish (47, 48). In addition to the implications on cold adaptation, this study is offering an excellent model (*i.e.* Hb 3) for further structural investigations on the general theme of allosteric mechanisms, namely, the transition betweeen the high-affinity R state and the liganded low-affinity T state of Hb.

REFERENCES

1. Monod, J., Wyman, J., and Changeux, J. P. (1965) *J. Mol. Biol.* **12,** 88–118
2. Imai, K. (1982) *Allosteric Effects in Hemoglobin*, Cambridge University Press, Cambridge, United Kingdom
3. di Prisco, G. (1988) *Comp. Biochem. Physiol.* **90B,** 631–635
4. D'Avino, R., Caruso, C., Camardella, L., Schinina', M. E., Rutigliano, B., Romano, M., Carratore, V., Barra, D., and di Prisco, G. (1991) in *Life under Extreme Conditions* (di Prisco, G., ed), pp. 15–33, Springer-Verlag, Berlin
5. di Prisco, G. (1998) in *Fishes of Antarctica: A Biological Overview* (di Prisco, G., Pisano, E., and Clarke A., eds), pp. 339–353, Springer-Verlag, Berlin
6. di Prisco, G. (2000) *J. Mar. Syst.* **27,** 253–265
7. D'Avino, R., Caruso, C., Tamburrini, M., Romano, M., Rutigliano, B., Polverino de Laureto, P., Camardella, L., Carratore, V., and di Prisco, G. (1994) *J. Biol. Chem.* **269,** 9675–9681
8. Riccio, A., Tamburrini, M., Carratore, V., and di Prisco, G. (2000) *J. Fish Biol.* **57,** 20–32
9. Tamburrini, M., D'Avino, R., Fago, A., Carratore, V., Kunzmann, A., and di Prisco, G. (1996) *J. Biol. Chem.* **271,** 23780–23785
10. D'Avino, R., and di Prisco, G. (1988) *Comp. Biochem. Physiol.* **90B,** 579–584
11. D'Avino, R., and di Prisco, G. (1989) *Eur. J. Biochem.* **179,** 699–705
12. Tamburrini, M., Brancaccio, A., Ippoliti, R., and di Prisco, G. (1992) *Arch. Biochem. Biophys.* **292,** 295–302
13. D'Avino, R., Fago, A., Kunzmann, A., and di Prisco, G. (1992) *Polar Biol.* **12,** 135–140
14. Landon, M. (1977) *Methods Enzymol.* **47,** 145–149
15. Brauer, A. W., Oman, C. L., and Margolies, M. N. (1984) *Anal. Biochem.* **137,** 134–142
16. Tamburrini, M., Condo', S. G., di Prisco, G., and Giardina, B. (1994) *J. Mol. Biol.* **237,** 615–621
17. Weber, R. E., Jensen, F. B., and Cox, R. P. (1987) *J. Comp. Physiol. B* **157,** 145–152
18. Ito, N., Komiyama, N. H., and Fermi, G. (1995) *J. Mol. Biol.* **250,** 648–658
19. Ponder, J. W., and Richards, F. M. (1987) *J. Mol. Biol.* **193,** 775–791
20. Bajorath, J., Stenkamp, R., and Aruffo, A. (1993) *Protein Sci.* **2,** 1798–1810
21. Arnone, A., and Perutz, M. F. (1974) *Nature* **249,** 34–36
22. Richard, V., Dodson, G. G., and Mauguen, Y. (1993) *J. Mol. Biol.* **233,** 270–274
23. Tamburrini, M., Riccio, A., Romano, M., Giardina, B., and di Prisco, G. (2000) *Eur. J. Biochem.* **267,** 6089–6098
24. Zuiderweg, E. R. P., van Beek, G. G. M., and De Bruin, S. H. (1979) *Eur. J. Biochem.* **94,** 297–306
25. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Nat. Struct. Biol.* **7,** 1055–1061
26. Swofford, D. L. (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods)*, Sinauer Associates, Sunderland, MA
27. Kumar, S., Tamura, K., Jakobsen, I., and Nei, M. (2000) *MEGA: Molecular Evolutionary Genetic Analysis, Version 2*, Pennsylvania State University, University Park, PA and Arizona State University, Tempe, AZ
28. Strimmer, K., and von Haeseler, A. (1998) *Mol. Biol. Evol.* **13,** 964–969
29. Perutz, M. F., and Brunori, M. (1982) *Nature* **299,** 421–426
30. Fago, A., Romano, M., Tamburrini, M., Coletta, M., D'Avino, R., and di Prisco, G. (1993) *Eur. J. Biochem.* **218,** 829–835
31. Nagai, K., Luisi, B. F., Shih, D. T.-b., Miyazaki, G., Imai, K, Poyart, C., De Young, A., Kwiatkowski, L., Noble, R. W., Lin, S.-H., and Yu, N.-T. (1987) *Nature* **329,** 858–860
32. Mathews, H. J., Rohlfs, R. J., Olson, J. S., Tame, J., Renaud, J.-P., and Nagai, K. (1989) *J. Biol. Chem.* **264,** 16573–16583
33. Fermi, G., Perutz, M. F., Shaanan, B., and Fourme, R. (1984) *J. Mol. Biol.* **175,** 159–174
34. Stam, W. T., Beintema, J. J., D'Avino, R., Tamburrini, M., and di Prisco, G. (1997) *J. Mol. Evol.* **45,** 437–445
35. Kister, J., Poyart, C., and Edelstein, S. J. (1987) *Biophys. J.* **52,** 527–535
36. Robert, C. H., Fall, L., and Gill, S. J. (1988) *Biochemistry* **27,** 6835–6843
37. Antonini, E., and Brunori, M. (1977) in *Hemoglobin and Myoglobin in Their Reactions with Ligands* (Neuberger, A., and Tatum, E. L., eds), pp. 178–187, North-Holland Publishing Co., Amsterdam
38. Shih, D. T.-b., Luisi, B. F., Myazaki, G., Perutz, M. F., and Nagai, K. (1993) *J. Mol. Biol.* **230,** 1291–1296
39. Barra, D., Petruzzelli, R., Bossa, F., and Brunori, M. (1983) *Biochim. Biophys. Acta* **742,** 72–77
40. Fago, A., Carratore, V., di Prisco, G., Feuerlein, R. J., Sottrup-Jensen, L., and Weber, R. E. (1995) *J. Biol. Chem.* **270,** 18897–18902
41. Mazzarella, L., D'Avino, R., di Prisco, G., Savino, C., Vitagliano, L., Moody, P. C. E., and Zagari, A. (1999) *J. Mol. Biol.* **287,** 897–906
42. Camardella, L., Caruso, C., D'Avino, R., di Prisco, G., Rutigliano, B., Tamburrini, M., Fermi, G., and Perutz, M. (1992) *J. Mol. Biol.* **224,** 449–460
43. Naoi, Y., Chong, K.-T., Yoshimatsu, K., Miyazaki, G., Tame, J.-R.-H, Park, S.-Y., Adachi, S., and Morimoto, H. (2001) *J. Mol. Biol.* **307,** 259–270
44. Coletta, M., Ascenzi, P., D'Avino, R., and di Prisco, G. (1996) *J. Biol. Chem.* **271,** 29859–29864
45. di Prisco, G., Condo', S. G., Tamburrini, M., and Giardina, B. (1991) *Trends Biochem. Sci.* **16,** 471–474
46. Cheng, C. H. (1998) *Curr. Opin. Genet. Dev.* **8,** 715–720
47. Cocca, E., Ratnayake-Lecamwasam, M., Parker, S. K., Camardella, L., Ciaramella, M., di Prisco, G., and Detrich, H. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1817–1821
48. Zhao, Y., Ratnayake-Lecamwasam, M., Parker, S. K., Cocca, E., Camardella, L., di Prisco, G., and Detrich, H. W., III (1998) *J. Biol. Chem.* **273,** 14745–14752

The Functionally Distinct Hemoglobins of the Arctic Spotted Wolffish *Anarhichas minor*

Cinzia Verde, Vito Carratore, Antonio Riccio, Maurizio Tamburrini, Elio Parisi and Guido di Prisco

Access the most updated version of this article at doi: 10.1074/jbc.M202474200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 7 of which can be accessed free at
http://www.jbc.org/content/277/39/36312.full.html#ref-list-1