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

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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(1) chain in common with Hb 2; Hb 3 and Hb 2 share the 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(2) 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 hexakisphosphate. 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 Notothenidae, namely Trematomus nevnesi (7), Pagothenia borchgerovinki (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 4-650S was from Tosohaas, trypsin (EC 3.4.21.4) treated with 1:1-tosylamide-2-phenylethylchloromethylketone was from Cooper Biomedical, diithiothreitol was from Fluka, sequenase-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 4-650S column. The hemolysate was passed through a heparinized syringe. Hemolysates were prepared as described previously (10). Separation of Hbs was achieved by FPLC anion-exchange chromatography on a Toyopearl Super Q 4-650S column. The Hb-concentrated pooled fractions were dialyzed against 10 mM HEPES, pH 7.7. All steps were carried out at 0–5 °C. No oxidation was spectroscopically 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- C₈ 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 ω-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 phenylthiohydroxolantoin 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).

**Hb 3** differs from Hb 2 only by the β chain (indicated as Hb 1 and Hb 2), and Hb 1 and Hb 3 differ by the β chain. The purification of the β chain(s) of Hb 3 was established by the Delphi package (BioSym/Molecular Simulation Inc.), using a solvent dielectric constant ε = 80, a solute dielectric constant ε = 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* (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) and differ by the α chain (α¹ and α²), Hb 3 differs from Hb 2 only by the chain β², and Hb 1 and Hb 3 have no chain component. Thus the chain composition of Hb 1, Hb 2, and Hb 3 is α²β, α¹β, and α¹β², 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 similarity.
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 α1 and α2 (including the acetyl group) and 16,049 and 16,290 Da for β1 and β2, 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 β1 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 β2 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 β2 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 β2, 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 α1β2 "dovetailed" switch interface, several substitutions are observed when compared with human Hb A. In all three Hbs, two of the residues forming the α1β2 switch region in Hb A (His βFG4 and Thr αC6) are conserved, whereas in both α1 and α2, Thr αC3 and Pro αCD2 are replaced by Gln and Ser, respectively.

In comparison with Antarctic Hbs, α1 and β2 of A. minor display higher identity with the corresponding chains of minor Hbs (Hb 2 and Hb C), whereas the α2 and β1 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. 4A, the α2 chain shared by A. minor Hb 2 and Hb 3 is close to the major Antarctic globins, whereas α1 of Hb 1 appears more closely related to minor Antarctic globins. In the tree of Fig. 4B, relative to the β chains, the position of the A. minor β1 chain shared by Hb 1 and Hb 2 is placed in the group of the major Antarctic globins, whereas the Hb 3 β2 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. 5A reports the curves of Hb 2 (the most abundant component), omitting those of Hb 1, which are almost identical, in agreement with the log p50 values reported in Table II. In both Hbs, the Bohr coefficient (φ = ∆log p50/∆pH) was close to −0.35 and to −0.43.
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. 5B). The Root effect was absent in both the absence and presence of the effectors (Fig. 5C).

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, $n_H = 0.63$ 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. 5D). 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. 5E). Hb 3 showed a strong, effector-enhanced Root effect (Fig. 5F).

The stripped hemolysate had intermediate oxygen binding features, reflecting the mixture of the three Hbs (Fig. 6); $n_H$ was 1.7–2.0 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, $\Delta H$, 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, $\Delta H$ 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 $\Delta H$ 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, $\Delta H$ 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, $\Delta H$ 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.
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.

\[ \text{Hb} \]

Table II: Values of log \( p_{50} \) of Hb 1 and Hb 2 at 6°C

| Hb component | 100 mM NaCl | 3 mM HEPES | pH 6.0 | 7.0 | 8.0 |
|--------------|-------------|------------|--------|-----|-----|
| Hb 1         | +           | +          | 0.97   | 0.59| 0.32|
| Hb 2         | +           | +          | 1.04   | 0.71| 0.49|

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.

\( \Delta H \) was also measured in the unstripped hemolysate, con-
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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. 7C), 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. 7D), 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 pKᵈ 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 characteristics 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 β¹ chain of Hb 1 and Hb 2, the replacement of C-
terminal His by Cys conceivably accounts for the decreased alkaline Bohr effect. In contrast, the \( \beta^2 \) 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 and C-terminal His, which respectively account for about 30% and 50–65% of Bohr effect (38). In \( \alpha NA1 \), fish Hbs have acetyl-Ser, thus the modest Bohr effect of \( A. \ minor \) Hb 1 and Hb 2 is due to the His→Cys substitution. The role of the 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. The \( \alpha \) of \( A. \ minor \) Hb 1 and Hb 2 is close to the latter two species. In contrast, \( \alpha^1 \) 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.

In the \( \beta^2 \) 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 \( \alpha \beta_2 \) “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 \( \alpha^2 \) 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, \( \alpha^1 \) 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 \( \beta^3 \) chain shared by Hb 1 and Hb 2 clusters with the major Antarctic globins and is well separated from all the others. Similar to \( \alpha^1 \), \( \beta^3 \) of Hb 3 is closer to the subclades of the minor Antarctic globins; however, unlike \( \alpha^1 \), it is also close to the \( \alpha \) chain of temperate \( C. \ kumu \). Interestingly, both \( C. \ kumu \) Hb and \( A. \ minor \) Hb 3 display the Root effect, similar to most

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**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 \( \beta_1 \) and \( \beta_2 \) 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.
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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 organophosphate 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 surprisingly similar to that observed in some species whose evolution has often favored evolutionary development has often favored 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 surprisingly similar to that observed in some species whose evolution has often favored evolutionary development.

The H values may thus 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 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.

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 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.
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 between the high-affinity R state and the liganded low-affinity T state of Hb.

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