The Unique Hemoglobin System of *Pleuragramma antarcticum*, an Antarctic Migratory Teleost

**STRUCTURE AND FUNCTION OF THE THREE COMPONENTS**

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*Pleuragramma antarcticum* (suborder Notothenioidei, family Nototheniidae) is the most abundant fish in the Antarctic shelf. This pelagic species has a circum-antarctic distribution and is characterized by spawning migration. This species displays the highest multiplicity of major hemoglobins (three); the other notothenioids have a single one (except one species, having two) with relatively low oxygen affinity regulated by pH and organophosphates. The hemoglobins of *P. antarcticum* display strong Bohr and Root effects; however, they reveal important functional differences in subunit cooperativity and organophosphate regulation and, above all, in the response of oxygenation to temperature. Despite the substitution Val\(^{E11} \rightarrow \) Ile found in Hb 2, which decreases the affinity in human mutants, the hemoglobins have similar oxygen affinity, higher than that of the other notothenioids. Hb 1 has the α chain in common with Hb 2 and the β in common with Hb 3. The amino acid sequence of all four chains has been established. Thus the hematological features of *P. antarcticum* differ remarkably from those ofantarctic notothenioids. This unique and sophisticated oxygen transport system may adequately meet the requirements of the unusual mode of life of this fish.

The high-antarctic shelf waters are characterized by low and constant temperatures (−1.6 °C to −2.1 °C). Confined within the antarctic convergence, fish have developed mechanisms of adaptation to extreme life conditions. Blood has a reduced number of erythrocytes, counterbalancing the viscosity increase due to low temperature, and a lower hemoglobin (Hb)\(^1\) content (Everson and Ralph, 1968; Hureau et al., 1977; Wells et al., 1980). The number of Hb components is also reduced. Red-blooded families of the suborder Notothenioidei generally have only a single Hb (Hb 1), accounting for 95–99% of the total content, and often a minor component, Hb 2 (di Prisco and D’Avino, 1989; di Prisco et al., 1990). Species belonging to the family Channichthyidae are devoid of Hb (Ruud, 1984), and the blood contains a small number of erythrocyte-like cells.

Our studies on the biochemistry of oxygen transport in antarctic fish have focused on the molecular structure and the oxygen binding properties of Hbs in search of correlations with life style and evolution of Notothenioidei (di Prisco and Tamburrini, 1992). This suborder is 97% endemic and comprises 95 of the 274 antarctic species so far identified (Andriashev, 1987), which are grouped into the families Bovichtidae, Nototheniidae, Bathyrhacidae, Harpagiferidae, Artedidraconidae, and Channichthyidae (recent evidence suggests that Bovichtidae should be divided into Pseudaphritidae and Bovichtidae). Many Hbs of species belonging to the five (or six) red-blooded families have been functionally characterized, and their amino acid sequence has been established (di Prisco et al., 1991).

Pelagic *Pleuragramma antarcticum* (family Nototheniidae) is overwhelmingly dominant, by number and biomass, in high-antarctic shelf areas. With a key role in the ecosystem and food web of the shelf, this ecologically important species has a circum-antarctic distribution and migrates across different water masses (Andersen, 1984; Hubold, 1985; Kunzmann, 1990). The reproductive habits (*P. antarcticum* spawns pelagic eggs) reflect adaptation for pelagic life.

The studies on adaptation to extreme conditions have been extended to *P. antarcticum* in view of its ecological importance and unusual mode of life. This paper reports a thorough study on the oxygen transport system of this species. Among Notothenioidi, *P. antarcticum* is the only species having three major Hbs. Their amino acid sequence, oxygen binding properties, and thermodynamic features have been investigated.

**EXPERIMENTAL PROCEDURES**

DEAE-cellulose (DE 52) was from Whatman; trypsin (EC 3.4.21.4) treated with l-1-tosylamide-2-phenylethylchloromethylketone was from Cooper Biomedical; Tris and bisTris were from Sigma; dithiothreitol was from Fluka; sequanal-grade reagents were from Applied Biosystems; and HPLC grade acetonitrile from Lab-Scan Analytical. All other reagents were of the highest purity commercially available.

The fish were caught in the Weddell Sea by benthopelagic trawl. Blood samples were drawn from the caudal vein by means of heparinized syringes. Hemolysates were prepared as described (D’Avino and di Prisco, 1988).

Cellulose acetate electrophoresis of the hemolyse and of the purified Hb components and SDS-polycrylamide gel electrophoresis of the purified globin chains were carried out as described (D’Avino and di Prisco, 1989; Laemmli, 1970).

In each globin, alkylation of the sulphydryl groups with 4-vinylpyridine, deacetylation of the α-chain N terminus, tryptic digestions, and cleavage of Asp-Pro bonds were carried out as described (D’Avino et al., 1989; Tamburrini et al., 1992).

Tryptic peptides were purified by reverse-phase HPLC of the hydrolysates on a μBondapak-C\(_{18}\) column (Waters, 0.39 × 30 cm), equilibrated with eluent A (0.1% trifluoroacetic acid) and eluted with a multistep gradient of eluent B (acetonitrile, containing 0.08% trifluoroacetic acid).
Amino acid analyses were performed with an Applied Biosystems automatic derivatizer model 420A, equipped with the hydrolysis option and on-line detection of phenylthiocarbamyl amino acids.

Amino acid sequencing was performed with an Applied Biosystems automatic sequenator model 477A, equipped with on-line detection of phenylthiohydantoin amino acids. Sequencing of Asp-Pro-cleaved globins was performed after treatment with p-phenylalanine (Brauer et al., 1984), which blocked the non-Pro N terminus and reduced the background.

Oxygen saturation experiments were carried out at 2 °C, as described (D’Avinio and di Prisco, 1989). Oxygen equilibrium curves were determined tonometrically (Giardina and Amiconi, 1981) at 2 and 10 °C, in the pH range 6.5–8.0. The overall oxygenation enthalpy change (ΔH (kcal/mol; 1 kcal = 4.184 kJ) corrected for the heat of oxygen solubilization (−3 kcal/mol) was calculated by the integrated van’t Hoff equation:

\[ \Delta H = -4.574 \frac{(T1 \cdot T2)/(T1 - T2)}{\Delta \log P_{\text{O}_2}/1000} \]  

(1)

**RESULTS**

**Purification of Hbs and Globin Chains—** Electrophoretic analysis on cellulose acetate of the hemolysate of *P. antarcticum* showed three major Hb components (Hb 1, Hb 2, and Hb 3). These were purified by ion-exchange chromatography on DE 52 (Fig. 1). Elution was carried out at pH 7.6 at 50, 80, and 200 mM Tris-HCl, respectively. Trace amounts of a fourth component coeluted with Hb 3.

The globin chains were isolated by reverse-phase chromatography of the purified Hbs, according to a previously described procedure (D’Avinio and di Prisco, 1989). Their elution time, amino acid composition, and migration in SDS-polyacrylamide gel electrophoresis indicated that Hb 1 has the α chain in common with Hb 2 and the β chain in common with Hb 3. Hb 2 and Hb 3 have no chain in common. These observations were subsequently confirmed by the amino acid sequence analysis. Thus, the Hb system of *P. antarcticum* is made of two α and two β chains.

**Amino Acid Sequence of the α Chains—** The N terminus of the α chain in common between Hb 1 and Hb 2 (α*) and of the α chain of Hb 3 (α*') was not available to Edman degradation. Fast atom bombardment mass spectrometry of the N-terminal tryptic peptide of the α* and α*’ chains revealed that the blocking group was acetyl, similar to all teleost Hbs sequenced to date.

An internal region became accessible in both α chains after cleavage of an Asp-Pro bond. After treatment with p-phenylalaldehyde, sequencing proceeded from Pro36 to Val134 in the α* chain, and from Pro36 to Val122 in the α*’ chain.

Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the chromatographic patterns of α* (panel A) and α*’ (panel B) chains.

In α*, all peptides were identified and sequenced except T2.

In α*’, all peptides were identified and sequenced except T2.
Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the chromatographic patterns of \( \alpha \) (panel C) and \( \beta \) (panel D) chains. All peptides were identified and sequenced. In \( \beta \), due to incomplete cleavage at Lys17, peptide T(2+3), from Ala9 to Arg30, was also isolated; moreover, T10 and T11 coeluted.

**Fig. 3. Amino acid sequences of the \( \alpha \) and \( \beta \) chains of \( P. \) antarcticum Hbs.** \( \alpha \) is identical in Hb 1 and Hb 2; \( \alpha \) is the \( \alpha \) chain of Hb 3. \( \beta \) is identical in Hb 1 and Hb 3; \( \beta \) is the \( \beta \) chain of Hb 2. The tryptic peptides (T) and the sequence portions elucidated by automated Edman degradation from the N terminus and after cleavage of an Asp-Pro bond are indicated.

| \( \alpha \) | NA | A | AB | B | C | CD | E |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the chromatographic patterns of \( \beta \) (panel C) and \( \beta \) (panel D) chains. All peptides were identified and sequenced. In \( \beta \), due to incomplete cleavage at Lys17, peptide T(2+3), from Ala9 to Arg30, was also isolated; moreover, T10 and T11 coeluted. |
Sequencing from the N terminus provided overlap from T1 to T4 in \( \beta^a \) and from T1 to T3 in \( \beta^b \). The sequences obtained after Asp-Pro cleavage provided overlap between T9 and T10 in \( \beta^a \) and from T9 to T11 in \( \beta^b \).

The complete sequence of the two \( \alpha \) and two \( \beta \) chains (142 and 146 residues, respectively) constituting the three Hbs of \( \textit{P. antarcticum} \) is reported in Fig. 3. Alignment of the tryptic peptides was obtained by the described overlaps and by homology with other known fish Hb sequences. The derived chain composition of Hb 1, Hb 2, and Hb 3 is, respectively, \( \alpha^a \beta^a_2 \), \( \alpha^b \beta^b_2 \), and \( \alpha^b \beta^b_2 \). A fourth component, isolated in trace amounts, differed from Hb 3 only in having Glu instead of Gln at position 94 of the \( \beta \) chain.

Tables I and II summarize the degree of sequence identity among antarctic and non-antarctic fish Hbs. It is worth noting that the chains of Hb 2 and Hb 3 that are not in common with Hb 1 have a high degree of identity with those of minor Hbs from antarctic fish; in particular, the \( \alpha^a \) chain of Hb 3 has 90–92% identity with \( \alpha \) chains of Hb 2 of the other antarctic species, and the \( \beta^b \) chain of Hb 2 has 86 and 91% identity with the \( \beta \) chain of \( \textit{Cygnodraco mawsoni} \) Hb 2 (Caruso et al., 1991) and \( \textit{Trematomus newnesi} \) Hb C (D’Avino et al., 1994), respectively.

### Table I

| Species | Non-Notothenioids | Notothenioids |
|---------|------------------|--------------|
|         | \( \textit{Cyprinus carpio} \) | \( \textit{O. mykiss} \) | \( \textit{N. coriiceps} \) | \( \textit{P. antarcticum} \) | \( \textit{P. bernacchii} \) | \( \textit{A. mitopteryx} \) | \( \textit{T. newnesi} \) | \( \textit{P. urvillii} \) |
| Hb 1   | 59               | 57           | 55           | 63           | 63           | 61           | 69           | 80           | 99           | 95           | 82           | 83           | 89           | 87           |

### Table II

| Species | Non-Notothenioids | Notothenioids |
|---------|------------------|--------------|
|         | \( \textit{Cyprinus carpio} \) | \( \textit{O. mykiss} \) | \( \textit{N. coriiceps} \) | \( \textit{P. antarcticum} \) | \( \textit{P. bernacchii} \) | \( \textit{A. mitopteryx} \) | \( \textit{T. newnesi} \) | \( \textit{P. urvillii} \) |
| Hb 2   | 57               | 63           | 53           | 70           | 65           | 72           | 70           | 70           | 77           | 93           | 86           | 80           | 88           | 88           | 90           | 86           |

*Non-antarctic species.*
The Three Hemoglobins of *P. antarcticum*

**DISCUSSION**

Most Notothenioidei have a single Hb (Hb 1), often accompanied by a minor component (Hb 2). Hb 1 and Hb 2 have identical β chains (di Prisco, 1988; di Prisco and D’Avino, 1989; di Prisco et al., 1991), with the exception of the bathydraconid *C. mawsoni*, whose Hbs have the α chain in common (Caruso et al., 1991). A caddis Hb (Hb C), with the α chain in common with Hb 1, is present in trace amounts, except in *T. newnesi*, in which it accounts for 20–25% of the total (di Prisco et al., 1991).

The hemolysate of *P. antarcticum* (a high Antarctic fish with circum-Antarctic distribution characterized by spawning migrations) contains three major Hbs. Similar to other Antarctic species, there is low sequence identity between the chains of *P. antarcticum* Hb 1 and those of major Hbs from Antartic fish and low identity with non-Antarctic fish globins.

The oxygen affinity of the three Hbs is much higher than that of the other notothenioids. In Hb 2, the substitution ValE11 → Ile, reported to be responsible for lower affinity in engineered human Hb at alkaline pH (Nagai et al., 1987; Mathews et al., 1989), does not decrease the affinity significantly in comparison with Hb 1 (in which the α chain is identical). Furthermore, the substitution GluFG1 → Gin in the βα chain, identical in Hb 1 and Hb 3, does not hinder a strong Root effect. Thus, in these Hbs, the Root effect must be due to other mechanisms than formation of a salt bridge between GluFG1 and HisβHC3, as suggested by Ito et al. (1995) for the Root effect Hb of the antarctic teleost *Pagothenia bernacchii*, because no such salt bridge was found in the T state. In *P. bernacchii* AspG1, AspG3, and AspβG1 interact with each other, and half of the Root effect has been ascribed to these interactions (Ito et al., 1995); these residues are also found in the Hbs of *P. antarcticum*. Within the positive charge cluster recently proposed by Mylavanam et al. (1996) to be responsible for the other half of the Root effect in Spot HbCO, ValβNA1, LysβH21, and HisβHC3 are conserved, but LysβEF6 is not, similar to all antarctic Hbs except in one minor component (di Prisco et al., 1991). The constraints that stabilize the positive charge cluster once again cannot include the bond between GluβHC1 and GluβFG1 in Hb 1 and Hb 3, because in FG1 Glu is replaced by Gin.

Among the other amino acid residues suggested by Perutz and Brunori (1982) to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs, LysαC5, SerβP9, GinβHC1, and HisβHC3 are conserved in the three Hbs. In the phosphate binding sites, AspβNA2 is conservatively replaced by Glu; in the βα chain, ArgβH21 is conservatively replaced by Lys, similar to all other Hbs of Antarctic fish (di Prisco et al., 1991), and LysβEF6 is replaced by Ala and Thr in the βα and ββ chain, respectively. The substitution of LysβEF6 with non-polar or neutral residues is frequently found in the Hbs of Antarctic fish and does not decrease the effect of organophosphates on the oxygen affinity of Bohr and Root effect Hbs (di Prisco et al., 1991).

The three Hbs of *P. antarcticum* are similar in several oxygen binding features, e.g. they all display the Bohr and Root effects, but they show differences and peculiarities that deserve some comments. Organophosphates enhance cooperativity at all pH values higher than 6.5 and lower the oxygen affinity also at pH 8.0 in Hb 3 only, indicating strong interaction with the binding site even under alkaline conditions. However, attention should mainly be addressed to the thermodynamic differentiation of the three components.

Hb 1 and Hb 3 show a very strong enthalpy change at pH 8.0 further enhanced by chloride and organophosphates in the former but drastically decreased in the latter; the heat of oxygenation of Hb 2 in the presence and absence of effectors is much lower. A dramatic decrease is observed at lower pH in Hb 3 and also in Hb 2 (ΔH approaches zero in both); in contrast, Hb 1 retains high oxygenation enthalpy (slightly lower in the presence of effectors). These observations clearly indicate a stronger Bohr effect at physiological temperatures in Hb 1 (in the presence of effectors) and Hb 3 (in their absence). In addition, the moderate effect of temperature on Hb 2 in the pH range 7.0–8.0 and on Hb 3 at pH 7.0 is indicative of energy-saving mechanisms of oxygen loading and unloading. The ensemble of thermodynamic features of the three components is likely to reflect highly refined molecular mechanisms of adaptation to a pelagic life style.

Among the investigated species of Nototheniidae and of the other red-blooded families of the suborder Notothenioidei, *P. antarcticum* is the only one having such a high multiplicity of major components. *T. newnesi* also has three Hbs: Hb C, Hb 1, and Hb 2 (D’Avino et al., 1994). However, the latter is a minor component, and only one (Hb C) of the two major components

**TABLE III**

| Heat of oxygenation of *P. antarcticum* Hbs | 100 mM NaCl, 3 mM ATP | ΔH (kcal/mol O2) |
|--------------------------------------------|-----------------------|-----------------|
|                                            | pH 7.0                | pH 8.0          |
| Hb 1                                       | –                     | –12.8           | –15.3           |
|                                            | +                     | –8.6            | –17.4           |
| Hb 2                                       | –                     | –3.6            | –6.4            |
|                                            | +                     | –1.8            | –8.1            |
| Hb 3                                       | –                     | –0.1            | –16.5           |
|                                            | +                     | –4.1            | –7.6            |

**FIG. 4.** Oxygen equilibrium isotherms (panels A, B, and C) and subunit cooperativity (panels D, E, F) as a function of pH, of Hb 1 (A and D), Hb 2 (B and E) and Hb 3 (C and F). Experiments were carried out in 100 mM Tris-HCl or bisTris-HCl, at 2°C, in the absence (○) and the presence (●) of 100 mM NaCl and 3 mM ATP.

**FIG. 5.** Oxygen saturation at atmospheric pressure as a function of pH of Hb 1, Hb 2, and Hb 3 (panels A, B, and C, respectively). Experiments were carried out at 2°C in 100 mM Tris-HCl or bisTris-HCl in the absence (○) and presence (●) of 3 mM ATP.
displays the Bohr and Root effects. The mode of life of these two notothenioids is widely different. The Hb system of T. newnesi (an active, cryopelagic fish) must conceivably ensure oxygen delivery to tissues also in conditions of acidosis. In turn, P. antarcticum, albeit a migratory, pelagic species, is considered sluggish (see Eastman (1993), p. 218). The main adaptive feature of the Hb system of this fish should conceivably be the response to the need to save energy during migration across water regions where the low temperature is likely to show significant differences and fluctuations. A single Hb (or none at all) appears sufficient to the other notothenioids, all sedentary bottom feeders. P. antarcticum can instead rely on three major Hbs, which differ in subunit cooperativity, phosphate regulation, and, above all, overall heat of oxygenation and influence of pH on temperature regulation of oxygen affinity. It is tempting to speculate that during evolution the oxygen transport system of P. antarcticum has developed physiological and biochemical adaptations suitable to allow optimal energy savings during the oxygenation-deoxygenation cycle under different and extreme environmental conditions, producing Hbs differing in thermodynamic behavior rather than in pH and organophosphate regulation.

From this standpoint and on the basis of their relative amounts, the three components of P. antarcticum, unlike the minor components found in the benthic notothenioids, cannot be considered as evolutionary remnants devoid of physiological significance (di Prisco et al., 1991), even though the sequence data reveal high phylogenetic distance between Hb 1 and the globins of Hb 2 and Hb 3 that are not in common (see Table I and Table II). In fact, as in T. newnesi, the selective advantage offered by multiple Hb genes appears clearly. The expression of multiple genes remains high in these two species also in the adult stage, in closer similarity with juveniles (di Prisco et al., unpublished), suggesting refined mechanisms of regulation within the gene family. It is of interest noting the loss of Hb expression in Hb-less Channichthyidae, in which retention in the genome of inactive α-globin-related sequences has been demonstrated (Cocca et al., 1995).

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