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# 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$\beta$E11 → 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 $\alpha$ chain in common with Hb 2 and the $\beta$ 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 of antarctic 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, 1954), 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, Bathydraconidae, 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 Notothenioidei, *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 hemolysate and of the purified Hb components and SDS-polyacrylamide 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 sulfhydryl groups with 4-vinylpyridine, deacetylation of the $\alpha$-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 $\mu$Bondapak-$C_{18}$ 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).

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[1] The abbreviations used are: Hb, hemoglobin; HPLC, high performance liquid chromatography; bisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

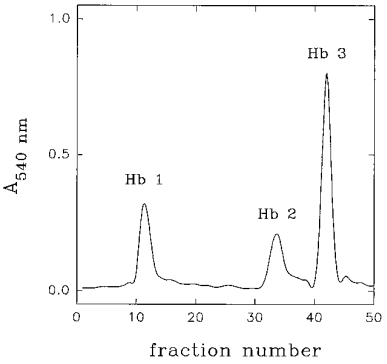


FIG. 1. **Ion-exchange chromatography of the hemolysate of *P. antarcticum*.** A DE 52 column (1 × 20 cm) was equilibrated in 20 mM Tris-HCl, pH 7.6. Hb 1, Hb 2, and Hb 3 were eluted with 50, 80, and 200 mM buffer, respectively.

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 sequencer model 477A, equipped with on-line detection of phenylthiohydantoin amino acids. Sequencing of Asp-Pro-cleaved globins was performed after treatment with *o*-phthalaldehyde (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'Avino 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 $\Delta H$ (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:

$$\Delta H = -4.574[(T1 \cdot T2)/(T1 - T2)]\Delta \log P_{50}/1000 \qquad \text{(Eq. 1)}$$

## 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'Avino and di Prisco, 1989). Their elution times, amino acid composition, and migration in SDS-polyacrylamide gel electrophoresis indicated that Hb 1 has the $\alpha$ chain in common with Hb 2 and the $\beta$ 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 $\alpha$ and two $\beta$ chains.

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

An internal region became accessible in both $\alpha$ chains after cleavage of an Asp-Pro bond. After treatment with *o*-phthalaldehyde, sequencing proceeded from $Pro^{96}$ to $Val^{124}$ in the $\alpha^a$ chain, and from $Pro^{96}$ to $Val^{122}$ in the $\alpha^b$ chain.

Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the chromatographic patterns of $\alpha^a$ (*panel A*) and $\alpha^b$ (*panel B*) chains.

In $\alpha^a$, all peptides were identified and sequenced except T2.

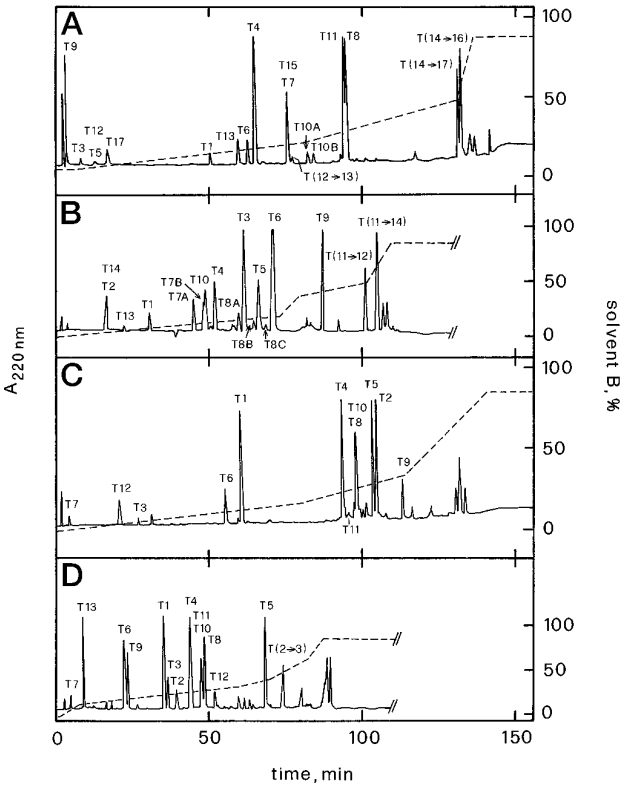


FIG. 2. **Reverse-phase HPLC of the tryptic peptides of $\alpha^a$, $\alpha^b$, $\beta^a$, and $\beta^b$ chains (*panels A, B, C,* and *D,* respectively).** Details are given under "Experimental Procedures."

The latter was sequenced directly from the N terminus after deacetylation by acid treatment. This procedure provided the amino acid sequence of T1 and T2 and overlap from T1 to T3. Due to the presence of Lys in both positions 61 and 62, tryptic digestion produced T10A and T10B, both ending at $Lys^{73}$. T12 and T13 were isolated as individual peptides and in the uncleaved form T(12+13), due to cleavage failure at $Arg^{93}$. A single peptide was isolated containing the C-terminal region from $Ile^{101}$ to $Arg^{142}$ (T14 to T17), as well as another peptide containing residues from $Ile^{101}$ to $Arg^{140}$ (T14 to T16). T15 and T17 were also identified. The sequence obtained after Asp-Pro cleavage provided overlap from T13 to T15.

In $\alpha^b$, all peptides were identified and sequenced. T1 was sequenced after partial deacetylation of N-terminal Ser. T2 contained two lysyl residues, because trypsin failed to cleave at $Lys^7$. Due to the sequence $Arg^{57}$-$Lys^{58}$, T7A and T7B were obtained, both starting with $Glu^{48}$. Three forms of peptide T8 were isolated due to a deamidation site ($Asn^{65}$-$Gly^{66}$) in the native peptide (T8B). Partially deamidated T8 (T8C), having Asp instead of Asn at position 65, was also sequenced; moreover, sequencing of another form of T8 (T8A) stopped at $Met^{64}$, suggesting the presence of an isoaspartyl residue at position 65 as a result of the deamidation mechanism (Galletti *et al.*, 1988). Due to partial cleavage failure at $Lys^{128}$, $Arg^{135}$, and $Lys^{140}$, C-terminal T(11+12+13+14) from $Ile^{101}$ to $Arg^{142}$ and T(11+12) from $Ile^{101}$ to $Arg^{135}$ were also isolated. The sequence obtained after Asp-Pro cleavage provided overlap between T10 and T11.

*Amino Acid Sequence of the β Chains*—Direct sequencing from the N terminus proceeded for 34 residues in the $\beta$ chain in common between Hb 1 and Hb 3 ($\beta^a$), and for 30 residues in the $\beta$ chain of Hb 2 ($\beta^b$).

After cleavage of an internal Asp-Pro bond and treatment with *o*-phthalaldehyde, sequencing proceeded from $Pro^{100}$ to $Glu^{125}$ in $\beta^a$ and from $Pro^{100}$ to $Asn^{120}$ in $\beta^b$.

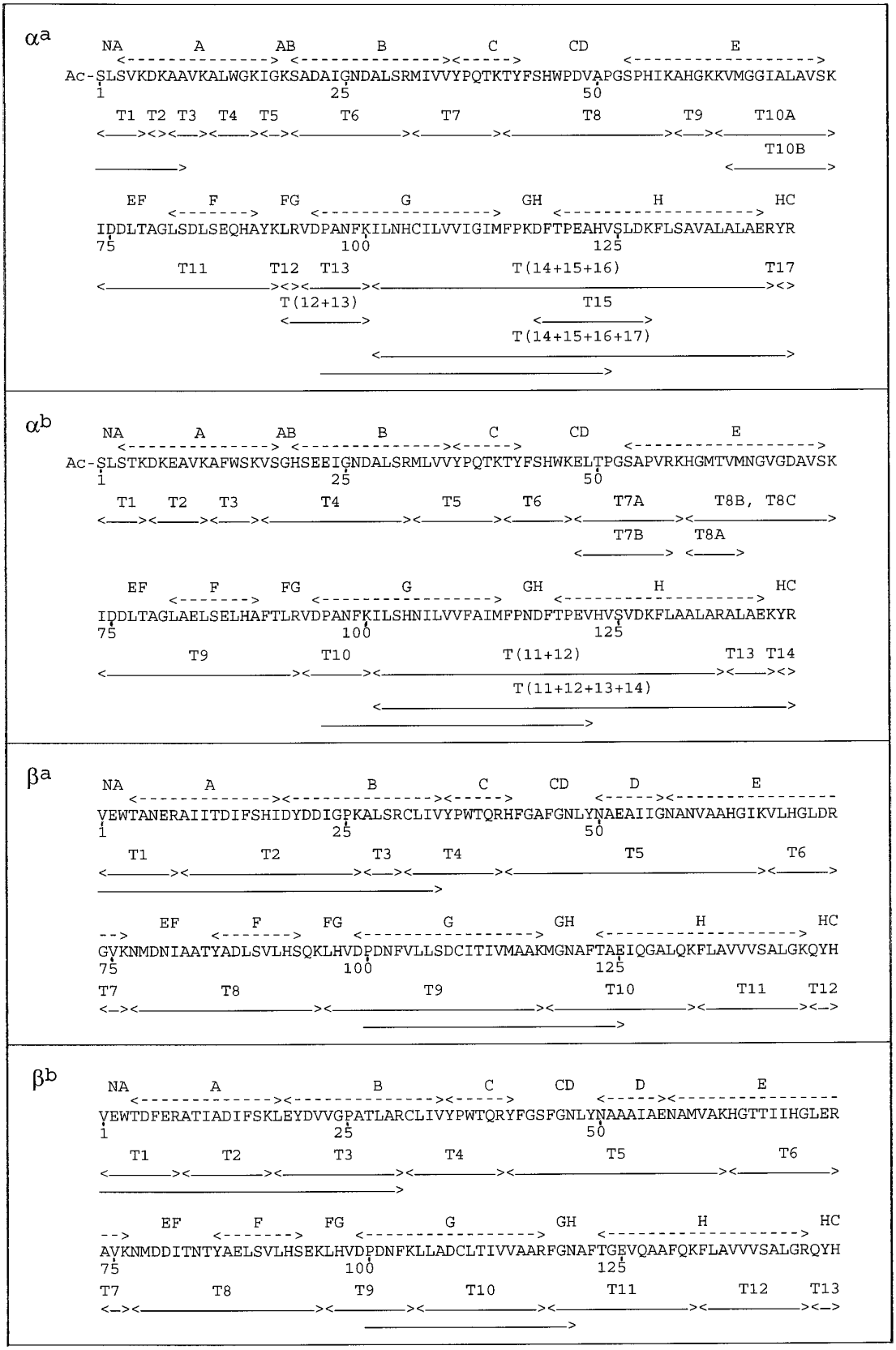


FIG. 3. **Amino acid sequences of the α and β chains of *P. antarcticum* Hbs.** $\alpha^a$ is identical in Hb 1 and Hb 2; $\alpha^b$ is the α chain of Hb 3. $\beta^a$ is identical in Hb 1 and Hb 3; $\beta^b$ is the β chain of Hb 2. The tryptic peptides (*T*) and the sequence portions elucidated by automated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

Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the chromatographic patterns of $\beta^a$ (*panel C*) and $\beta^b$ (*panel D*) chains. All peptides were identified and sequenced. In $\beta^b$, due to incomplete cleavage at $Lys^{17}$, peptide T(2+3), from $Ala^9$ to $Arg^{30}$, was also isolated; moreover, T10 and T11 coeluted.

TABLE I
*Sequence identity (%) in α chains of fish hemoglobins*

| Species | Non-Notothenioidei | | | Notothenioidei | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Cyprinus carpio*[a] | *O. mykiss* Hb IV[a] | *O. mykiss* Hb I[a] | *N. angustata* Hb 2[a] | *N. coriiceps* Hb 2 | *T. newnesi* Hb 2 | *P. antarcticum* Hb 3 | *P. urvillii* Hb 1[a] | *N. angustata* Hb 1[a] | *P. antarcticum* Hb 1,2 | *G. acuticeps* | *C. mawsoni* Hb 1,2 | *A. mitopteryx* | *P. bernacchii* Hb 1 | *T. newnesi* Hb 1,C |
| *Notothenia coriiceps* Hb 1 | 59 | 57 | 55 | 63 | 63 | 61 | 69 | 80 | 99 | 95 | 82 | 83 | 83 | 89 | 87 |
| *T. newnesi* Hb 1,C | 58 | 62 | 52 | 66 | 66 | 63 | 68 | 78 | 94 | 89 | 92 | 90 | 90 | 97 | |
| *P. bernacchii* Hb 1 | 64 | 62 | 57 | 70 | 70 | 65 | 69 | 80 | 96 | 92 | 91 | 91 | 90 | | |
| *Aethotaxis mitopteryx* | 62 | 59 | 55 | 65 | 65 | 62 | 65 | 73 | 91 | 88 | 84 | 84 | | | |
| *C. mawsoni* Hb 1,2 | 60 | 62 | 53 | 69 | 69 | 64 | 70 | 78 | 89 | 87 | 93 | | | | |
| *Gymnodraco acuticeps* | 58 | 62 | 53 | 67 | 67 | 65 | 68 | 76 | 89 | 85 | | | | | |
| *P. antarcticum* Hb 1,2 | 66 | 64 | 61 | 70 | 70 | 67 | 72 | 81 | 94 | | | | | | |
| *N. angustata* Hb 1[a] | 65 | 62 | 58 | 69 | 69 | 65 | 70 | 78 | | | | | | | |
| *Pseudaphitis urvillii* Hb 1[a] | 60 | 59 | 60 | 65 | 65 | 62 | 65 | | | | | | | | |
| *P. antarcticum* Hb 3 | 66 | 66 | 65 | 92 | 92 | 90 | | | | | | | | | |
| *T. newnesi* Hb 2 | 61 | 58 | 62 | 93 | 93 | | | | | | | | | | |
| *N. coriiceps* Hb 2 | 63 | 63 | 62 | 100 | | | | | | | | | | | |
| *N. angustata* Hb 2[a] | 63 | 63 | 62 | | | | | | | | | | | | |
| *Oncorhynchus mykiss* Hb I[a] | 66 | 60 | | | | | | | | | | | | | |
| *O. mykiss* Hb IV[a] | 63 | | | | | | | | | | | | | | |

[a] Non-antarctic species.

TABLE II
*Sequence identity (%) in β chains of fish hemoglobins*

| Species | Non-Notothenioidei | | | Notothenioidei | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C. carpio*[a] | *O. mykiss* Hb IV[a] | *O. mykiss* Hb 1[a] | *P. urvillii* Hb 2[a] | *C. mawsoni* Hb 2 | *P. antarcticum* Hb 2 | *P. bernacchii* Hb C | *T. newnesi* Hb C | *P. urvillii* Hb 1[a] | *N. angustata* Hb 1[a] | *P. antarcticum* Hb 1,3 | *G. acuticeps* | *C. mawsoni* Hb 1 | *A. mitopteryx* | *P. bernacchii* Hb 1 | *T. newnesi* Hb 1,2 |
| *N. coriiceps* Hb 1,2 | 57 | 63 | 53 | 70 | 65 | 72 | 70 | 70 | 77 | 93 | 86 | 80 | 88 | 82 | 90 | 86 |
| *T. newnesi* Hb 1,2 | 57 | 62 | 53 | 67 | 64 | 69 | 68 | 68 | 77 | 85 | 84 | 80 | 84 | 83 | 93 | |
| *P. bernacchii* Hb 1 | 61 | 66 | 58 | 70 | 66 | 72 | 70 | 70 | 82 | 91 | 90 | 83 | 87 | 86 | | |
| *A. mitopteryx* | 58 | 60 | 54 | 66 | 62 | 69 | 66 | 67 | 77 | 84 | 84 | 77 | 80 | | | |
| *C. mawsoni* Hb 1 | 56 | 62 | 53 | 66 | 67 | 68 | 68 | 70 | 75 | 87 | 82 | 85 | | | | |
| *G. acuticeps* | 56 | 59 | 55 | 65 | 65 | 69 | 66 | 67 | 75 | 81 | 80 | | | | | |
| *P. antarcticum* Hb 1,3 | 61 | 63 | 57 | 70 | 67 | 72 | 69 | 69 | 77 | 88 | | | | | | |
| *N. angustata* Hb 1[a] | 61 | 64 | 57 | 71 | 66 | 73 | 71 | 71 | 81 | | | | | | | |
| *P. urvillii* Hb 1[a] | 63 | 66 | 61 | 69 | 66 | 70 | 69 | 69 | | | | | | | | |
| *T. newnesi* Hb C | 57 | 62 | 57 | 86 | 89 | 91 | 95 | | | | | | | | | |
| *P. bernacchii* Hb C | 60 | 63 | 55 | 88 | 90 | 92 | | | | | | | | | | |
| *P. antarcticum* Hb 2 | 63 | 66 | 58 | 84 | 86 | | | | | | | | | | | |
| *C. mawsoni* Hb 2 | 55 | 60 | 54 | 85 | | | | | | | | | | | | |
| *P. urvillii* Hb 2[a] | 58 | 64 | 53 | | | | | | | | | | | | | |
| *O. mykiss* Hb I[a] | 64 | 59 | | | | | | | | | | | | | | |
| *O. mykiss* Hb IV[a] | 73 | | | | | | | | | | | | | | | |

[a] Non-antarctic species.

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 *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, $\alpha^a{}_2\beta^a{}_2$, $\alpha^a{}_2\beta^b{}_2$ and $\alpha^b{}_2\beta^a{}_2$. 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 $\alpha^b$ 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 *Cygnodraco mawsoni* Hb 2 (Caruso *et al.*, 1991) and *Trematomus newnesi* Hb C (D'Avino *et al.*, 1994), respectively.

*Oxygen Binding Properties*—Hb 1, Hb 2, and Hb 3 showed a very strong, effector-enhanced dependence of oxygen affinity on pH (alkaline Bohr effect). At 2 °C the Bohr coefficient ($\phi = \Delta\log P_{50}/\Delta pH$, where $P_{50}$ is the partial pressure of oxygen required to saturate 50% of the hemes) ranged from −0.9 to −1.0 in the absence and from −1.0 to −1.23 in the presence, of chloride and organophosphates (Fig. 4). The oxygen affinity of the three Hbs was very high, $P_{50}$ at pH 8.0 ranging from 1.97 to 2.99 mm of Hg in the absence and from 2.14 to 3.8 mm of Hg in the presence of the physiological effectors. In comparison with Hb 1 and Hb 2, at 2 °C Hb 3 showed a lower cooperativity (enhanced by organophosphates) of oxygen binding, as indicated by the Hill coefficient $n_H$. At pH 6.5, the values of $n_H$ were close to one in all components.

The Root effect (Root, 1931; Brittain, 1987) was also displayed by the three components, and its amplitude was enhanced by the effectors (Fig. 5), indicating a strong pH dependence of Hb oxygenation in air.

The effect of temperature on the oxygen affinity showed that Hb 1, Hb 2, and Hb 3 had different values of heat of oxygenation both in the absence and the presence of the effectors (Table III). Moreover, in their absence, Hb 3 showed the largest $\Delta H$ variation between pH 7.0 and 8.0 (16.4 kcal/mol; 2.5 and 2.8 kcal/mol were shown by Hb 1 and Hb 2, respectively), whereas in the presence of the effectors, it showed the smallest variation (3.5 kcal/mol; Hb 1 and Hb 2 showed 8.8 and 6.3 kcal/mol, respectively).

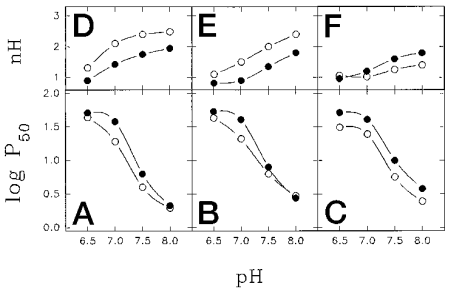


FIG. 4. **Oxygen equilibrium isotherms (*panels A, B,* and *C*) and subunit cooperativity (*panels D, E, F*) as a function of pH, of Hb 1 (*A* and *D*), Hb 2 (*B* and *E*) and Hb 3 (*C* and *F*).** Experiments were carried out in 100 mM Tris-HCl or bisTris-HCl, at 2 °C, in the absence (○) and the presence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

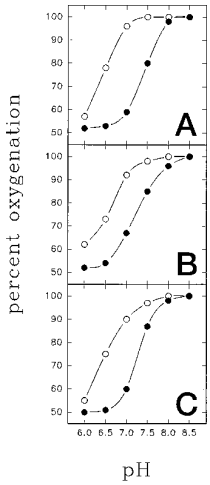


FIG. 5. **Oxygen saturation at atmospheric pressure as a function of pH of Hb 1, Hb 2, and Hb 3 (*panels A, B,* and *C*, respectively).** Experiments were carried out at 2 °C in 100 mM Tris-HCl or bisTris-HCl in the absence (○) and presence (●) of 3 mM ATP.

## DISCUSSION

Most Notothenioidei have a single Hb (Hb 1), often accompanied by a minor component (Hb 2). Hb 1 and Hb 2 have identical $\beta$ 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 $\alpha$ chain in common (Caruso *et al.*, 1991). A cathodal Hb (Hb C), with the $\alpha$ 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 high sequence identity between the chains of *P. antarcticum* Hb 1 and those of major Hbs from antarctic 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 Val$\beta$E11 → 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 $\alpha$ chain is identical). Furthermore, the substitution GluFG1 → Gln in the $\beta^a$ 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 Glu$\beta$FG1 and His$\beta$HC3, 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* Asp$\alpha$G1, Asp$\beta$G3, and Asp$\beta$G1 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 Mylvaganam *et al.* (1996) to be responsible for the other half of the Root effect in Spot HbCO, Val$\beta$NA1, Lys$\beta$H21, and His$\beta$HC3 are conserved, but Lys$\beta$EF6 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 Gln$\beta$HC1 and Glu$\beta$FG1 in Hb 1 and Hb 3, because in FG1 Glu is replaced by Gln.

TABLE III
*Heat of oxygenation of P. antarcticum Hbs*

| | 100 mM NaCl, 3 mM ATP | $\Delta H$ (kcal/mol $O_2$) | |
|---|---|---|---|
| | | 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 |

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, Lys$\alpha$C5, Ser$\beta$F9, Gln$\beta$HC1, and His$\beta$HC3 are conserved in the three Hbs. In the phosphate binding site, Asp$\beta$NA2 is conservatively replaced by Glu; in the $\beta^a$ chain, Arg$\beta$H21 is conservatively replaced by Lys, similar to all other Hbs of antarctic fish (di Prisco *et al.*, 1991), and Lys$\beta$EF6 is replaced by Ala and Thr in the $\beta^a$ and $\beta^b$ chain, respectively. The substitution of Lys$\beta$EF6 with nonpolar 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 ($\Delta H$ 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

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 $\alpha$-globin-related sequences has been demonstrated (Cocca *et al.*, 1995).

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# The Unique Hemoglobin System of *Pleuragramma antarcticum*, an Antarctic Migratory Teleost: STRUCTURE AND FUNCTION OF THE THREE COMPONENTS

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