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The Hemoglobins of the Antarctic Fishes *Artedidraco orianae* and *Pogonophryne scotti*

AMINO ACID SEQUENCE, LACK OF COOPERATIVITY, AND LIGAND BINDING PROPERTIES*

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The oxygen-transport system of two species of Antarctic fishes belonging to the family Artedidraconidae, *Artedidraco orianae* and *Pogonophryne scotti*, was thoroughly investigated. The complete amino acid sequence of the α and β chains of the single hemoglobins of the two species was established. The oxygen-binding properties were also investigated, and were found not to differ significantly from those shown by blood, intact erythrocytes, and unstripped hemolysates. Both hemoglobins have unusually high oxygen affinity and display a relatively small Bohr effect; the Root effect is elicited only by organophosphates and is also reduced. Remarkably, the Hill coefficient is close to one in the whole pH range, indicating absence of cooperative oxygen binding which, in *A. orianae* hemoglobin, could be ascribed to the subunit heterogeneity shown upon oxygen dissociation. In comparison with the other families of the suborder Notothenioidei, the oxygen-transport system of these two species of Artedidraconidae has unique characteristics, which raise interesting questions on the mode of function of a multisubunit molecule and the relationship with cold adaptation.

During adaptation to low temperatures, Antarctic fishes have acquired special hematological features which clearly differentiate them from fishes of temperate and tropical climates. The hematocrit and hemoglobin (Hb)[1] concentration are highly reduced in the blood of Antarctic fishes (1–3), as well as the number of Hb components. At the extreme of such evolution, the 15 species of the family Channichthyidae are characterized by lack of Hb (4).

The hematological peculiarities of Antarctic teleosts prompted an investigation on the molecular structure and ligand binding properties of hemoglobins (Hbs) from these organisms, in order to characterize the adaptation of the oxygen-transport mechanism at the molecular level. We focused our interest on the suborder Notothenioidei, largely endemic and confined south of the Antarctic Polar Front. The suborder includes six families with 120 species, 95 of which are Antarctic (5, 6): Bovichtidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae (in fact, the families might be seven, since recent evidence (7) suggests that Bovichtidae should be grouped into Pseudaphritidae and Bovichtidae).

Notothenioids generally have a single major Hb (Hb 1), often accompanied by a minor component (Hb 2), accounting for approximately 95 and 5% of the total, respectively (8–10). A cathodal Hb (Hb C) is present in trace amounts, except in *Trematomus newnesi*, of the family Nototheniidae, in which Hb C is approximately 25–30% of the total (11).

In this study, the oxygen-transport system of species of the family Artedidraconidae, which comprises 24 of the 80 red-blooded Antarctic species of the suborder Notothenioidei so far identified (5, 6), was thoroughly investigated for the first time. Artedidraconids are benthic fish, have a wide depth distribution, and are largely confined in the Antarctic continental shelf and slope (12). This paper reports the complete amino acid sequence of the α and β chains of the single Hbs of the artedidraconids *Artedidraco orianae* and *Pogonophryne scotti*, along with kinetic and thermodynamic characterization of ligand binding. The functional features of Hbs were very similar to those measured in whole blood, intact erythrocytes, and unstripped hemolysates.

EXPERIMENTAL PROCEDURES

DEAE-cellulose (DE52) was from Whatman; trypsin (EC 3.4.21.4), treated with L-1-tosylamide-2-phenylethylchloromethyl ketone, from Cooper Biomedical; Tris and bisTris from Sigma; dithiothreitol from Fluka; all sequanal-grade reagents from Applied Biosystems; HPLC-grade acetonitrile from Lab-Scan Analytical. All other reagents were of the highest purity commercially available.

A. orianae was caught by means of Agassiz Trawl in the northeastern Weddell Sea, Antarctica, and *P. scotti* by gill nets in the vicinity of Terra Nova Bay Station, Ross Sea, Antarctica. Immediately after catch, fish were transferred to aquariums supplied with running, aerated seawater at approximately −1.0 °C.

Blood samples were drawn from the caudal vein of living animals by means of heparinized syringes; the red blood cells were washed three times in isotonic saline solution (1.7% NaCl, in 1 mM Tris-HCl, pH 8.1). Hemolysates were prepared as described (13).

Hb concentration and purification from minor contaminants was carried out by ion-exchange chromatography on a DE52 column (1 × 3 cm), equilibrated with 10 mM Tris-HCl, pH 8.1, and eluted with 100 mM Tris-HCl, pH 7.1. Gel filtration was performed by fast protein liquid chromatography (Pharmacia) on a ProteinPak 300 SW column (Waters), equilibrated with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, at a flow rate of 0.2 ml/min.

Globins were precipitated with 10 volumes of acetone containing 5 mM HCl at −20 °C; α and β chains were separated by reverse-phase HPLC on a μBondapak C_{18} column (Waters, 0.39 × 30 cm). In *P. scotti*,

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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.

the eluents were (A) 45% acetonitrile containing 0.3% trifluoroacetic acid and (B) 55% acetonitrile; a linear gradient from 0 to 100% of eluent B in 15 min was used. In *A. orianae*, the eluents were (A) 0.1% trifluoroacetic acid and (B) acetonitrile containing 0.08% trifluoroacetic acid; the elution was carried out with a multistep gradient of eluent B. Flow rate was 1 ml/min. Cellulose acetate electrophoresis of hemolysates and purified Hbs and SDS-polyacrylamide gel electrophoresis of the purified globins were carried out as described (14, 15). Globin denaturation, alkylation of the sulfhydryl groups with 4-vinylpyridine, tryptic digestion, and cleavage of Asp-Pro bonds were carried out according to described procedures (16, 17).

Tryptic peptides were purified by reverse-phase HPLC on a μBondapak C_{18} column (Waters, 0.39 × 30 cm), equilibrated with eluent A (0.1% trifluoroacetic acid); peptides were 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 420 A, equipped with the hydrolysis option and with on-line detection of phenylthiocarbamyl amino acids. Alternatively, gas-phase manual hydrolysis was carried out for 1 h at 155 °C in 6 N HCl containing 1% 2-mercaptoethanol and 1% phenol.

Amino acid sequencing was carried out with an Applied Biosystems automatic sequencer model 477A, equipped with on-line detection of phenylthiohydantoin-derivatives. Sequencing of Asp-Pro-cleaved globins was performed after treatment with *o*-phthalaldehyde (18), to reduce the background due to sequencing from the N terminus. Molecular mass measurements were carried out with a Hewlett-Packard mass spectrometer model 5989B, equipped with the electrospray source model 59987A.

Oxygen saturation experiments were carried out at 2 °C, as described previously (15). Oxygen-equilibrium curves were obtained by the tonometric method (19) at 2, 10, and 20 °C, in the pH range 6.5–8.0. The heat of oxygenation was calculated from the integrated van't Hoff equation,

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

and the values obtained were corrected for the heat of oxygen solubilization (−3 kcal/mol; 1 kcal = 4.184 kJ).

Kinetic measurements were carried out with a Gibson-Durrum stopped-flow system equipped with a 2-cm pathlength observation cell and interfaced to a computer for data acquisition (On Line System, Jefferson, GA). Oxygen dissociation measurements were undertaken mixing oxy-Hb of *A. orianae* (5 μM heme before mixing, in low-ionic strength HEPES buffer, pH 7.0) with sodium dithionite (20 mg/ml after mixing) in buffer at the desired pH at higher ionic strength. For carbon monoxide binding kinetics, deoxy-Hb of *A. orianae* or of *P. scotti* in sodium dithionite at low ionic strength and pH 7.0 (3 μM heme before mixing, in HEPES buffer) were mixed with a higher ionic strength buffer at the desired pH containing a given concentration of dissolved carbon monoxide. Both measurements were performed at 20 °C and monitored at 430 or 419 nm, following the appearance or disappearance of unliganded Hb.

RESULTS

Electrophoretic analysis on cellulose acetate showed that the hemolysates of *A. orianae* and *P. scotti* have a single Hb. The α and β chains of each Hb were purified by HPLC (Fig. 1).

Amino Acid Sequence of the α Chains—The N terminus of the two α chains was unavailable to automated Edman degradation, indicating the presence of a blocking group.

An internal region became accessible in both chains after cleavage of an Asp-Pro bond. After treatment with *o*-phthalaldehyde, sequencing proceeded from Pro^{96} to Thr^{119} in *A. orianae*, and from Pro^{96} to Ala^{122} in *P. scotti*.

Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the patterns of the α chains of *A. orianae* (*panel A*) and *P. scotti* (*panel B*). All peptides were identified and sequenced. The sequence of the blocked N-terminal peptides (residues 1–7) of the two chains was established after incubation in 30% trifluoroacetic acid at 55 °C for 2.5 h. The molecular mass of these peptides, obtained by electrospray mass spectrometry, was 834.6 Da, a value compatible with the presence of an acetyl group at the N terminus. In the α chain of *A. orianae*, mass spectrometry analysis also revealed a minor component having a molecular mass of 592.5 Da, corresponding to the sequence

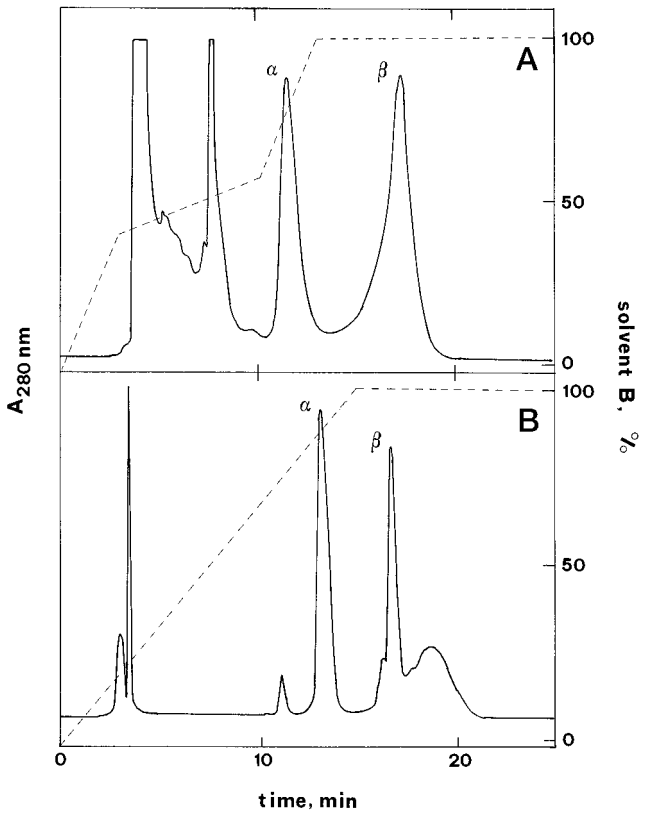

FIG. 1. **Reverse-phase HPLC of the globins of *A. orianae* (*panel A*) and *P. scotti* (*panel B*) Hbs.** Details are given under "Experimental Procedures."

from acetyl-Ser^1 to Lys^5.

In the α chain of *A. orianae* Hb, trypsin partially failed to cleave at Lys^5 and Lys^{62}. Thus, it was possible to recover two forms of peptide T10 (T10A and T10B), having Lys^{62} and Val^{63} at the N terminus, respectively. The sequence obtained after Asp-Pro cleavage provided overlap from T14 to T16.

In the α chain of *P. scotti* Hb, trypsin failed to cleave at Lys^5 and at Arg^{93}; the alignment of T12 and T13 was thus obtained. The sequence obtained after Asp-Pro cleavage provided overlap from T13 to T15.

Amino Acid Sequence of the β Chains—Tryptic peptides were purified by reverse-phase HPLC. Fig. 2 shows the patterns of the β chains of *A. orianae* (*panel C*) and *P. scotti* (*panel D*) Hbs. All peptides were identified and sequenced.

Direct sequencing from the N terminus proceeded for 30 (*A. orianae*) and 31 (*P. scotti*) residues, providing overlap from T1 to T3 and from T1 to T4, respectively. After cleavage of the internal Asp-Pro bond and treatment with *o*-phthalaldehyde, sequencing proceeded from Pro^{100} to Val^{114} (*A. orianae*) and from Pro^{100} to Phe^{133} (*P. scotti*), providing overlap from T9 to T10 and from T8 to T11, respectively.

Fig. 3 shows the complete sequence of the α (142 residues) and β (146 residues) chains of the two Hbs. The amino acid compositions are reported in Table I. Alignment of the tryptic peptides was established by the described overlaps and by homology with other known sequences. The calculated molecular masses were: (i) 15,499 and 15,566 (α chains); (ii) 16,203 and 16,129 (β chains), for *A. orianae* and *P. scotti* Hbs, respectively.

Oxygen-binding Properties—At 2 °C (close to physiological conditions) the affinity for oxygen of the two Hbs was high: at pH 8.0, P_{50} was 2.45 and 4.6 mm Hg in the absence, and 3.63 and 5.5 mm Hg in the presence, of chloride and organophosphates, for *A. orianae* and *P. scotti* Hbs, respectively. In com-

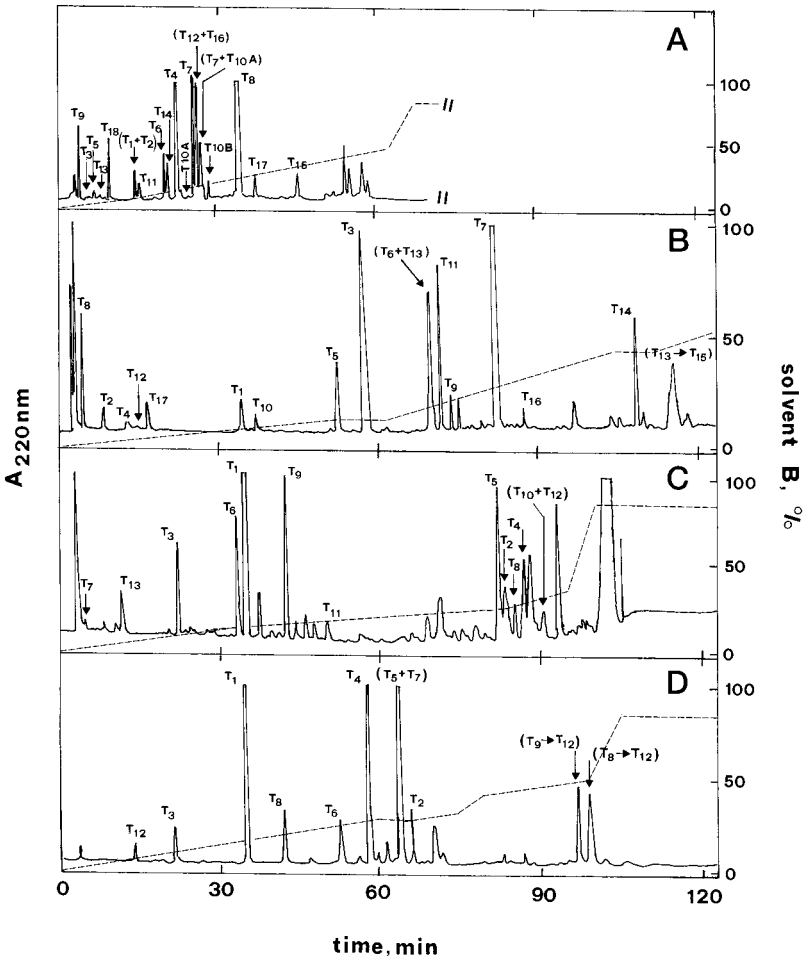

FIG. 2. **Reverse-phase HPLC of the tryptic peptides of the α and β chains of *A. orianae* (*panels A* and *C*, respectively) and *P. scotti* (*panels B* and *D*, respectively) Hbs.** Details are given under "Experimental Procedures."

parison with other notothenioids (10), the Hbs showed a modest, effector-enhanced alkaline Bohr effect (becoming virtually absent at 10 °C in *A. orianae* Hb, not shown) in the pH range 6.5–8.0 (Fig. 4, *panels A* and *B*); the Bohr coefficient $\Delta\log P_{50}/\Delta pH$ was, respectively, −0.35 and −0.31 in the absence, and −0.51 and −0.44 in the presence, of the physiological effectors. Although the overall oxygen affinity of whole blood (as well as erythrocytes and unstripped hemolysate) of *A. orianae* and *P. scotti* (Fig. 4, *panels A* and *B*, respectively) was slightly lower than that measured in Hb in the presence of chloride and phosphates, the slopes of the oxygen-equilibrium curves were very similar in the pH range 7.0–7.5, where the Bohr effect is active.

In the whole pH range, the Hill coefficient of the Hbs of both species was close to one (Fig. 4, *panels C* and *D*), regardless of the presence of chloride and organophosphates, with only slightly higher values observed in *P. scotti*. These results were taken as strong evidence of the absence of cooperative oxygen binding.

In order to ascertain whether the low Hill coefficient values were due to subunit dissociation, *A. orianae* Hb was analyzed by fast protein liquid chromatography gel filtration at pH 8.0 (see "Experimental Procedures" for details). The hemoprotein was eluted from the column at the retention time of tetrameric Hb (not shown), without any apparent dissociation. Therefore, the absence of subunit cooperativity is due to intrinsic properties of the tetrameric molecule; further work is required to establish correlations with molecular structure.

The Root effect (20, 21) often displayed by fish Hbs, which leads to incomplete saturation of Hb in air, was not found, at 2 °C, in *A. orianae* and *P. scotti* Hbs in the absence of organophosphates, but it was induced to a limited extent by ATP or inositol hexakisphosphate (Fig. 5). The Root effect of *A. orianae* and *P. scotti* blood, erythrocytes, and unstripped hemolysate was very similar to that shown by the isolated Hb (Fig. 5, *panels A* and *B*).

The regulation of the oxygen affinity by temperature was investigated in the range 2–20 °C. A very strong ΔH, larger than that of mammalian Hbs (22), was measured in *A. orianae* Hb at pH 8.0, in the absence and presence of chloride and ATP. However, lowering the pH to 7.0 brought about a dramatic decrease of the oxygen-binding enthalpy, which became almost zero in the presence of effectors (Table II).

Oxygen Dissociation Kinetics—Fig. 6 shows the progress curve for oxygen dissociation of Hb of *A. orianae* at different pH values and at 21 °C, displaying a biphasic process characterized by two exponentials, which can be attributed to a different ligand dissociation behavior for the two subunits α and β of the tetramer, even though the kinetic heterogeneity is not as marked as in the Root effect Hb of the temperate fish *Chelidonichthys kumu* (23). The amplitude of the process decreased as the pH was lowered (Fig. 6), indicating that *A. orianae* Hb was already partially deoxygenated at atmospheric oxygen pressure and at 21 °C, before mixing with sodium dithionite. However, this effect appeared markedly temperature-dependent, since at lower, physiological temperatures, no Root effect, *i.e.* no partial deoxygenation, was observed upon pH lowering at atmospheric pressure (Fig. 5). Both deoxygenation rate constants displayed a pH-dependent behavior in the observed pH range (Fig. 7); the data were analyzed according to,

$$k_{obs} = k_{alk}/(1 + K_a[H^+]) + k_{ac}K_a[H^+]/(1 + K_a[H^+]) \qquad \text{(Eq. 2)}$$

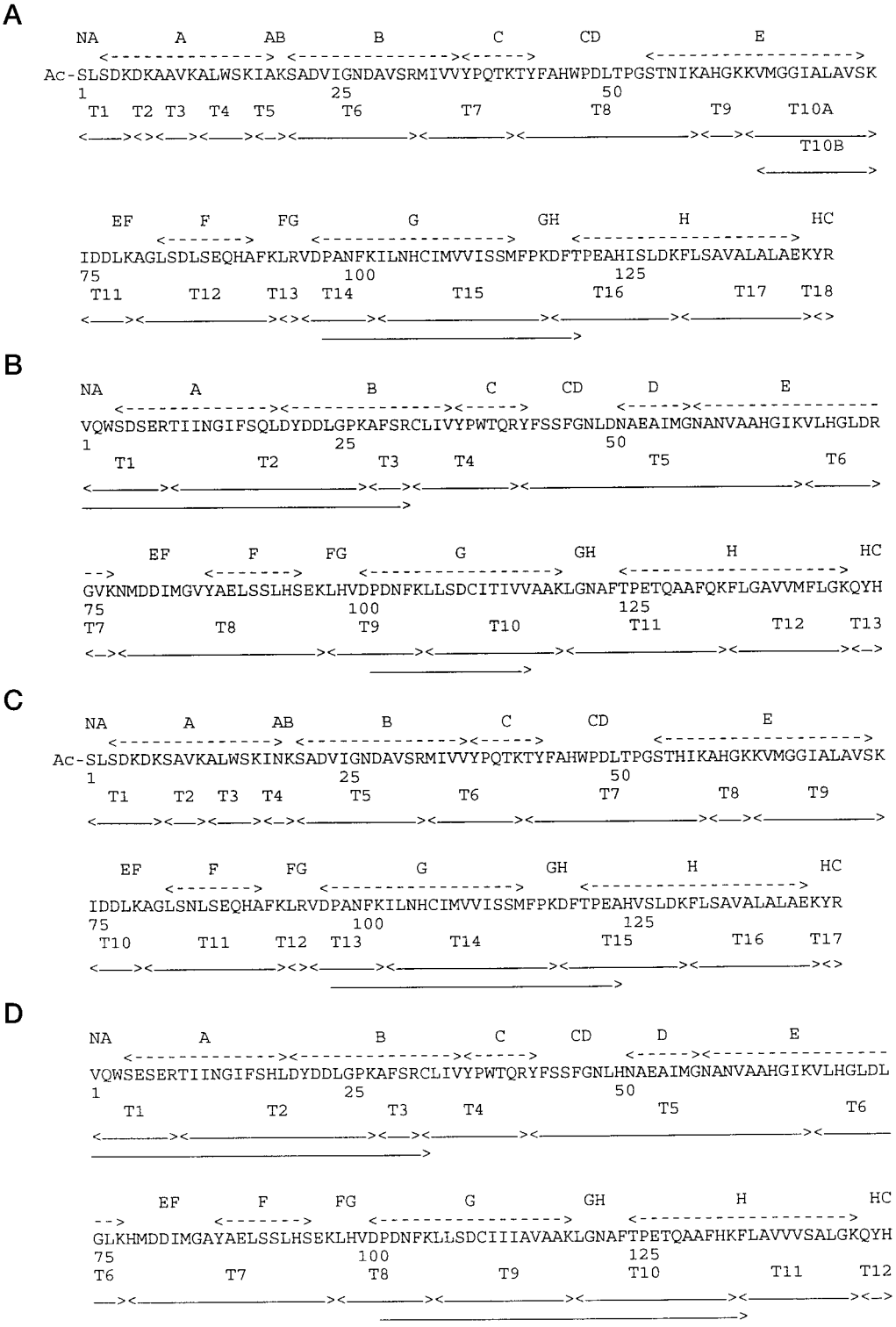

FIG. 3. **Amino acid sequence of the α and β chains of *A. orianae* (*panels A* and *B*, respectively) and *P. scotti* (*panels C* and *D*, respectively) Hbs.** The tryptic peptides (*T*) and the sequence portions elucidated by automated Edman degradation directly from the N terminus or after Asp-Pro cleavage are indicated.

where k_{obs} is the observed oxygen dissociation rate constant, k_{alk} and k_{ac} are the oxygen dissociation rate constants for the unprotonated and protonated Hb, respectively, and K_a ($=10^{pK_a}$) is the proton binding constant to oxygenated Hb. The pH dependence described by the continuous lines indicated a single protonation event with a $pK_a = 7.1 \pm 0.15$, closely similar for both phases and essentially unaffected by 3 mM ATP (Fig. 7, *panels A* and *B*, and Table III). This behavior suggests that the two subunits of the tetramer, although showing different values of the oxygen dissociation rate constants (as from the biphasic progress curves in Fig. 6), are functionally regulated by the same protonation process, indicating that differences in the observed rates should be related to variations in the conformation of the distal side of the heme pocket.

Carbon Monoxide Binding Kinetics—Fig. 8 shows the pH dependence of the rate constant for the monophasic carbon

TABLE I
Amino acid composition of the α (A and B) and β (C and D) chains of A. orianae and P. scotti, respectively

The number of residues from the sequence are indicated in parentheses.

Amino acid	A	B	C	D
Asp/Asn	12.6 (15)	15.2 (15)	13.8 (19)	16.2 (16)
Glu/Gln	6.4 (5)	5.2 (5)	11.9 (11)	11.7 (10)
Ser	10.1 (13)	14.3 (14)	8.0 (10)	10.1 (11)
Gly	6.7 (6)	6.8 (6)	10.7 (11)	10.7 (10)
His	8.6 (5)	6.1 (6)	7.2 (5)	8.2 (9)
Arg	3.7 (3)	3.0 (3)	4.1 (4)	3.0 (3)
Thr	5.4 (5)	5.3 (5)	5.0 (5)	4.6 (4)
Ala	14.7 (18)	16.9 (16)	12.8 (13)	15.1 (16)
Pro	6.7 (6)	6.4 (6)	4.0 (4)	3.8 (4)
Tyr	3.7 (3)	3.2 (3)	5.6 (5)	5.2 (5)
Val	9.9 (11)	10.7 (12)	10.1 (11)	7.7 (9)
Met	1.6 (4)	4.5 (4)	1.2 (4)	3.6 (3)
Cys	ND[a] (1)	ND (1)	ND (2)	1.6 (2)
Ile	10.1 (10)	7.9 (9)	8.7 (9)	7.8 (10)
Leu	14.2 (13)	13.0 (13)	14.2 (14)	15.0 (16)
Phe	6.7 (6)	6.2 (6)	8.5 (9)	8.1 (8)
Lys	15.2 (16)	15.4 (16)	8.7 (8)	8.1 (8)
Trp	ND (2)	ND (2)	ND (2)	ND (2)
No. of residues	142	142	146	146
Molecular mass (Da)	15,499	15,566	16,203	16,129

[a] ND, not determined.

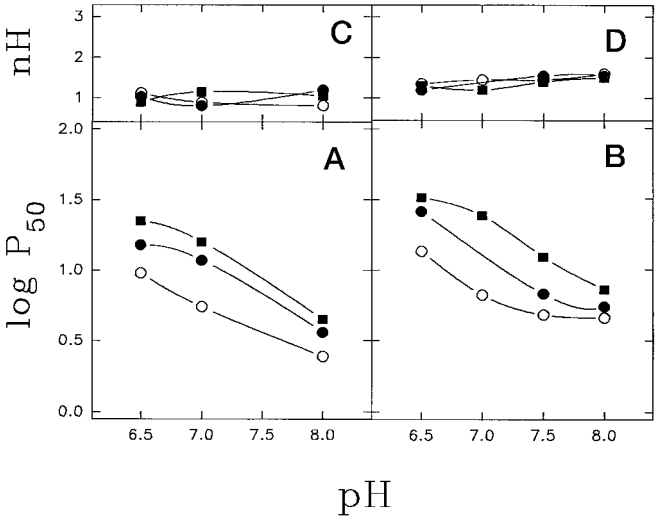

FIG. 4. **Oxygen-equilibrium isotherms (*panels A* and *B*) and cooperativity of ligand binding (*panels C* and *D*) as a function of pH, at 2 °C, of *A. orianae* and *P. scotti* Hbs, respectively.** Experimental conditions: 100 mM Tris-HCl or bisTris-HCl, in the absence (*open circles*) and presence (*filled circles*) of 100 mM NaCl and 3 mM ATP (*A. orianae*) or inositol hexakisphosphate (*P. scotti*). *Filled squares* refer to experiments carried out on whole blood in isotonic buffer.

monoxide binding process to both Hbs. The behavior, substantially unaffected by 3 mM ATP (data not shown), was investigated over a very wide pH range, spanning from pH 2.5 to 9.0. This range has been shown to be suitable in order to follow the effect of the protonation of the proximal and distal histidine on the CO binding process, since over this pH interval both events are taking place (24, 25). The pH dependence was analyzed according to Equation 2 modified only in that k values of Equation 2 are substituted by l' since the rate constants are referring to the bimolecular carbon monoxide binding rate constants. Furthermore, it must be outlined that the K_a value measured in CO binding kinetics refers to the proton binding constant to unliganded Hb. The continuous lines refer to the fitting of the experimental data from the Hbs of *A. orianae* and *P. scotti* according to Equation 2, employing the set of parameters reported in Table IV.

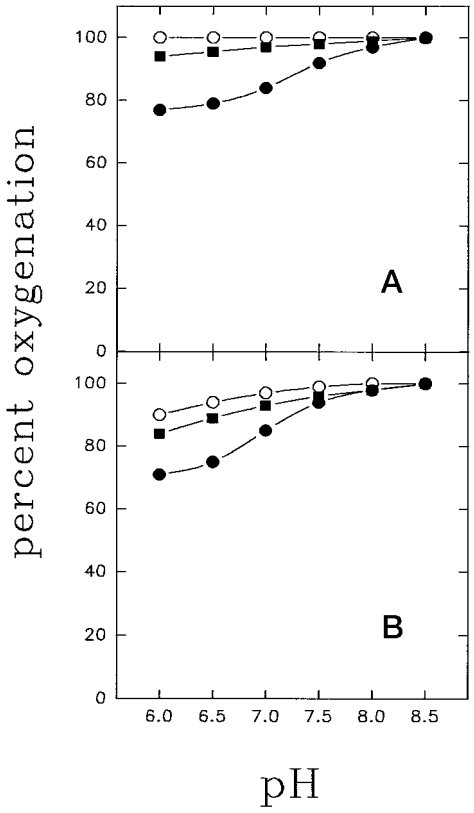

FIG. 5. **Oxygen saturation as a function of pH, at 2 °C, of *A. orianae* (*panel A*) and *P. scotti* (*panel B*) Hbs.** Experimental conditions: 100 mM Tris-HCl or bisTris-HCl, in the absence (*open circles*) and presence (*filled circles*) of 3 mM ATP (*A. orianae*) or inositol hexakisphosphate (*P. scotti*). *Filled squares* refer to experiments carried out on whole blood in isotonic buffer.

TABLE II
Apparent heat of oxygenation of A. orianae and P. scotti Hbs

	ΔH		
	pH 7.0	pH 7.5	pH 8.0
	kcal mol^{-1} *oxygen*		
Without effectors			
A. orianae	−6.75		−19.6
P. scotti		−9.9	
With effectors			
A. orianae	−1.55		−15.1
P. scotti		−12.0	

DISCUSSION

Species of the Antarctic family Artedidraconidae have only one Hb (26–29) in the adult stage. This is the first report of the complete primary structure of the single Hbs of two artedidraconid species, *A. orianae* and *P. scotti*.

A very high degree of sequence identity (96% for the α chains, and 90% for the β) was found between the Hbs of *A. orianae* and *P. scotti* (Table V), higher than the identity with the major Hbs of species belonging to other Antarctic families (82–91 and 77–83%, respectively). As usual (10), the identity with minor Hbs (Hb 2 and Hb C) of Antarctic fish and with Hbs of non-Antarctic species was substantially lower (51–68%).

Although cladograms (6, 30) indicate the family Bathydraconidae to be evolutionarily farther apart from Nototheniidae than Artedidraconidae, the artedidraconid sequences show lesser identity with those of two nototheniid species (*T. newnesi* and *Trematomus bernacchii*) than the identity between the nototheniids and two bathydraconids (*Gymnodraco acuticeps* and *Cygnodraco mawsoni*). Sequences of Hbs of other nototheh-

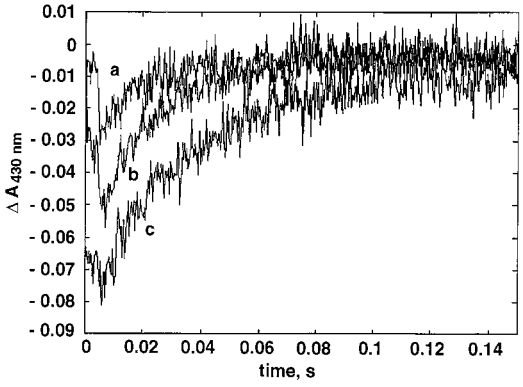

FIG. 6. **Oxygen dissociation progress curves of *A. orianae* Hb at pH 6.0 (*a*), 7.0 (*b*), and 9.0 (*c*) in the absence of ATP.** Temperature, 21 °C. Details are given in the text.

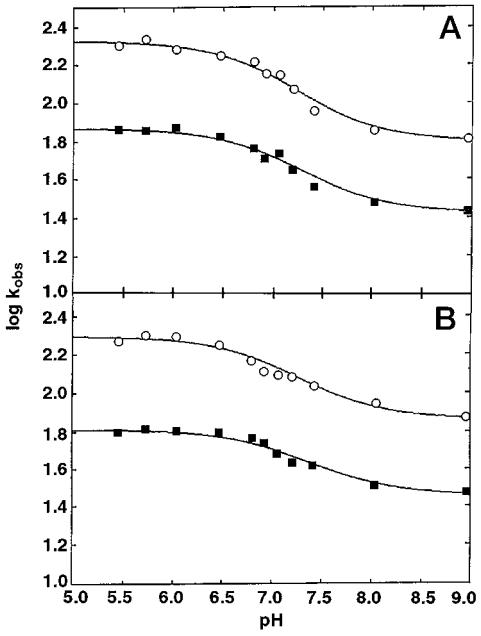

FIG. 7. **pH dependence of oxygen dissociation rate constant (k; s^{-1}) of *A. orianae* Hb, in the absence (*panel A*) and presence (*panel B*) of 3 mM ATP.** Wavelength, 430 nm; temperature, 21 °C. *Continuous lines* are the best fitting according to Equation 2, with parameters reported in Table IV. *Open circles*, fast phase; *filled squares*, slow phase. Details are given in the text.

TABLE III

Parameters obtained from the analysis according to Equation 2 of the pH dependence of oxygen dissociation of *A. orianae* Hb in the absence and presence of 3 mM ATP, at 20 °C

	k_{ac}	k_{alk}	pK_a
	s^{-1}		
Without ATP			
Fast phase	62.6 ± 2.4	214.4 ± 5.7	6.97 ± 0.12
Slow phase	26.2 ± 0.7	74.4 ± 3.3	7.06 ± 0.10
With ATP			
Fast phase	73.0 ± 2.9	198.3 ± 5.9	7.04 ± 0.11
Slow phase	28.6 ± 1.0	65.2 ± 2.8	7.20 ± 0.12

nioids, together with evidence from phylogenetic analysis based on partial sequences of 12 S and 16 S mitochondrial ribosomal RNA (31), will hopefully contribute to understanding the evolutionary history.

Among the functionally important amino acid residues (32), in the β chain of both Hbs, Ser F9, Glu FG1, Gln HC1, and His HC3 are conserved, Arg H21 is conservatively replaced by Lys,

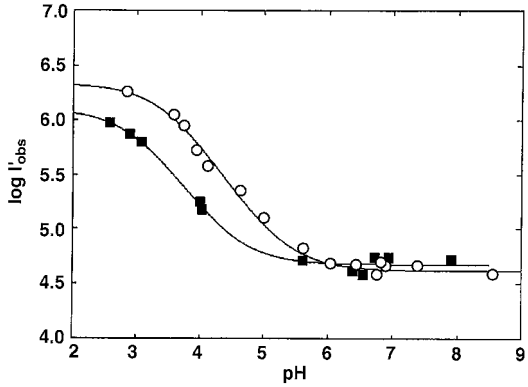

FIG. 8. **pH dependence of carbon monoxide binding rate constant (l' $M^{-1}s^{-1}$) to *A. orianae* (*open circles*) and *P. scotti* (filled squares) Hbs in the absence of ATP.** Wavelength, 419 nm; temperature, 21 °C. *Continuous lines* are the best fitting according to Equation 2, with parameters reported in Table V. Details are given in the text.

TABLE IV

Kinetic parameters of carbon monoxide binding

Parameters obtained from the analysis according to Equation 2 of the pH dependence of carbon monoxide binding to *A. orianae* and *P. scotti* Hbs, at 20 °C. l', second order carbon monoxide association rate constant.

	l'_{ac}	l'_{alk}	pK_a
	$M^{-1}\,s^{-1}$		
A. orianae	2.2 (±0.3) × 10^6	4.1 (±0.4) × 10^4	3.52 ± 0.27
P. scotti	1.3 (±0.2) × 10^6	4.7 (±0.4) × 10^4	3.04 ± 0.17

whereas Asp NA2 and Lys EF6 are replaced by Gln and Met, respectively. At the $\alpha_1\beta_2$ interface, the residues forming the flexible joint between the α_1 FG corner and the β_2 C helix (Arg βC6, Trp βC3, Arg αFG4, Asp αG1, and Pro αG2) are conserved; among the residues forming the switch region between the α_1 C helix and the β_2 FG corner, His βFG4 and Thr αC6 are conserved, whereas Thr αC3 and Pro αCD2 are replaced by Gln (as in all fish hemoglobins) and Ala (as in *Cyprinus carpio* and *Catostomus clarkii*), respectively.

The Hbs of *A. orianae* and *P. scotti* (as well as those of other species of Artedidraconidae, such as *Artedidraco shackletoni*, *D. longedorsalis*, *Pogonophryne* sp. 1, sp. 2 and sp. 3)[2,3] are characterized by a modest Bohr effect, very weak or no Root effect, and very low cooperativity of oxygen binding. Similar results were obtained with blood, intact erythrocytes, or unstripped hemolysates. ATP slightly enhances the Bohr effect, and induces the Root effect to a limited extent. The Root effect is further induced upon addition of ATP to blood, intact erythrocytes, or unstripped hemolysates, even in specimens that had recovered from the stress of capture, suggesting that the organic phosphate is never in the erythrocytes at saturating concentrations. This similarity in oxygen-binding behavior confirms previous findings on other Antarctic fish Hbs (33–35).

The study of the regulation of the oxygen affinity by temperature in *A. orianae* Hb clearly indicates a marked temperature dependence of the Bohr effect, which is enhanced when the temperature is lowered, underlying an efficient coupling of oxygen and proton transport only at very low temperatures. In particular, very high values of oxygenation enthalpy are measured at alkaline pH values.

These observations deserve some comments, at both functional and structural level. The α and β chains of the two Hbs show a substitution at position B10. This residue (Leu in all the

[2] G. di Prisco, unpublished data.
[3] M. Tamburrini and G. di Prisco, unpublished data.

TABLE V
Sequence identity (%) in α and β chains of some fish hemoglobins

Species	*C. carpio*[a]	*S. irid*[a] Hb IV	*S. irid*[a] Hb I	*T. newnesi* Hb 2	*N. coriiceps* Hb 2	*A. orianae*	*P. scotti*	*G. acuticeps*	*C. mawsoni* Hb 1, Hb 2	*T. bernacchii* Hb 1	*T. newnesi* Hb 1, Hb C
α Chains											
N. coriiceps Hb 1	59	57	55	61	63	91	91	82	83	89	87
T. newnesi Hb 1, Hb C	58	62	52	63	66	86	85	92	90	97	
T. bernacchii Hb 1	64	62	57	65	70	87	88	91	91		
C. mawsoni Hb 1, Hb 2	60	62	53	64	69	84	85	93			
G. acuticeps	58	62	53	65	67	82	82				
P. scotti	66	60	60	65	68	96					
A. orianae	67	61	60	64	68						
N. coriiceps Hb 2	63	63	62	93							
T. newnesi Hb 2	61	58	62								
Salmo irideus[a] Hb I	66	60									
S. irideus[a] Hb IV	63										

	C. carpio[a]	*S. irid*[a] Hb IV	*S. irid*[a] Hb I	*C. mawsoni* Hb 2	*T. newnesi* Hb C	*A. orianae*	*P. scotti*	*G. acuticeps*	*C. mawsoni* Hb 1	*T. bernacchii* Hb 1	*T. newnesi* Hb 1, Hb 2
β Chains											
N. coriiceps Hb 1, Hb 2	57	63	53	65	70	83	82	80	88	90	86
T. newnesi Hb 1, Hb 2	57	62	53	64	68	79	77	80	84	93	
T. bernacchii Hb 1	61	66	58	66	70	80	79	83	87		
C. mawsoni Hb 1	56	62	53	67	70	81	83	85			
G. acuticeps	56	59	55	65	67	77	78				
P. scotti	56	60	51	62	65	90					
A. orianae	59	62	54	64	68						
T. newnesi Hb C	57	62	57	89							
C. mawsoni Hb 2	55	60	54								
S. irideus[a] Hb I	64	59									
S. irideus[a] Hb IV	73										

[a] Non-Antarctic species.

other Antarctic fish Hb sequences) is changed to Val in the α chains and to Phe in the β chains. Since position B10 has been suggested to have an important functional role (36, 37), we may argue that the substitution in this position produces small, or lack of, Bohr and Root effects in *A. orianae* and *P. scotti* Hbs. Furthermore, this substitution, which occurs on the distal side of the heme pocket, might be at least partially responsible for the different kinetic behavior displayed by the two subunits, and thus for their functional dynamic heterogeneity.

In CO binding kinetics the rate constant values of *A. orianae* and *P. scotti* Hbs are significantly lower than in human HbA (24). Moreover, the rate enhancement observed as pH is lowered (Fig. 8) can be attributed to the protonation of proximal His, associated to the cleavage (or severe weakening) of the proximal bond (24, 25). This event has been shown to bring about a dramatic enhancement of the CO binding rate constant (24, 25), and the resulting pK_a is reflecting the strength of the proximal bond and the stereochemical impairing represented by the ligand-linked movement of proximal His. Therefore, the close similarity for the pK_a value of the cleavage of the proximal bond in *A. orianae* and *P. scotti* Hbs with respect to that observed in most other hemoproteins, including human HbA (24, 25), indeed suggests that in both fish Hbs the conformation of the proximal side is similar to that of other mammalian hemoproteins, and that, probably, the lower binding rate constant is mostly attributable to an alteration of the barrier for ligand entry through the distal portion of the heme pocket, possibly related to the presence of a bulky residue in βB10. Further investigations on the tridimensional structures are needed and, in view of the high sequence identity, protein engineering may be a suitable additional approach to ascertain these hypotheses.

The subunit heterogeneity upon oxygen dissociation in *A. orianae* Hb, although limited, could be enough to account for the very low levels, or lack, of subunit cooperativity. The lack of cooperativity of *A. orianae* Hb implies that the Hb-oxygen dissociation curve is hyperbolic (Fig. 9) rather than sigmoidal,

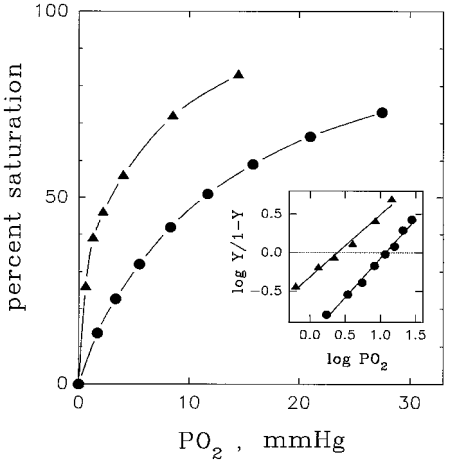

FIG. 9. **Oxygen dissociation curve of *A. orianae* Hb at 10 °C (*circles*) and at 2 °C (*triangles*).** Conditions: 100 mM Tris-HCl, pH 8.0. The *inset* shows the Hill plots obtained for the two experiments.

thereby not allowing large volumes of oxygen to be bound or released in response to small changes in the blood oxygen partial pressure. The data reported in Fig. 9 are well described by the Hill plot (see *inset*). The Hill coefficient, at pH 8.0, is 1.01 at 10 °C and 0.81 at 2 °C, suggesting that the Hb of *A. orianae*, in temperature conditions close to the physiological ones, is actually characterized by negative cooperativity of ligand binding. These observations, together with the high values of oxygenation enthalpy measured under alkaline conditions, raise the question whether or not this Hb undergoes the T → R conformational transition upon going from the deoxy to the oxy state. This question on such a crucial point of the structure-function relationship of *A. orianae* Hb remains open.

The oxygen-transport system of these fish has strong functional similarities with that of a Triassic reptilian relict, *Sphenodon punctatus* (38, 39); the exceptionally low resting meta-

bolic rate and low temperature of a primitive vertebrate is considered to be consistent with reduced Bohr effect, absence of cooperative oxygen binding, and high oxygen affinity (decreased by ATP with no effect on cooperativity) displayed by the hemolysate and Hbs of this organism. These remarkable characteristics are shared by the Hbs of *A. orianae* and *P. scotti*. Are these hematological features a result of life-style adaptation to extreme conditions, or did "deleterious" Hb mutations force these fish to migrate to a low-temperature habitat? This question remains open. In fact it is difficult to establish consensus on objective criteria to identify a phenotypic trait as an adaptation, which remains a "slippery concept" (40, 41). However, it is astonishing that the Hbs of Artedidraconidae (which are not primitive vertebrates) display functional properties typical of primitive organisms. Bearing in mind that Antarctic fishes have very low metabolic rates and decreased dependence on Hb-mediated oxygen transport (10, 26) and that in fact the blood of Channichthyidae, the most phyletically derived notothenioid family, is devoid of Hb (4), this feature may be correlated to the less critical role of Hb in sluggish Artedidraconidae. In these fishes, the Hb physiological role might merely be that of an "oxygen store" when the animal encounters anoxic conditions.

The high sequence identity of major Hbs of other notothenioids (which show cooperative interactions) with the Hbs of Artedidraconidae induces to consider the latter Hbs as "modern." As a consequence of the above arguments, interesting questions arise on the evolution and mode of function of multisubunit molecules.

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The Hemoglobins of the Antarctic Fishes *Artedidraco orianae* and *Pogonophryne scotti* : AMINO ACID SEQUENCE, LACK OF COOPERATIVITY, AND LIGAND BINDING PROPERTIES

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