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The hemoglobins of the sub-Antarctic fish *Cottoperca gobio*, a phyletically basal species – oxygen-binding equilibria, kinetics and molecular dynamics

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Database
The protein sequence reported in this paper has been submitted to the UniProt Knowledgebase under the accession numbers P84652 (β chain), P84653 (α^1 chain) and P85083 (α^2 chain). The coordinates of the protein model are available at http://mi.caspur.it/PMDB/ under ID PM0075620.

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The dominant perciform suborder Notothenioidei is an excellent study group for assessing the evolution and functional importance of biochemical adaptations to temperature. The availability of notothenioid taxa in a wide range of latitudes (Antarctic and non-Antarctic) provides a tool to enable identification of physiological and biochemical characteristics gained and lost during evolutionary history. Non-Antarctic notothenioids belonging to the most basal families are a crucial source for understanding the evolution of hemoglobin in high-Antarctic cold-adapted fish. This paper focuses on the structure, function and evolution of the oxygen-transport system of *Cottoperca gobio*, a sub-Antarctic notothenioid fish of the family Bovichtidae, probably derived from ancestral species that evolved in the Antarctic region and later migrated to lower latitudes. Unlike most high-Antarctic notothenioids, but similar to many other acanthomorph teleosts, *C. gobio* has two major hemoglobins having the β chain in common. The oxygen-binding equilibria and kinetics of the two hemoglobins have been measured. Hb1 and Hb2 show strong modulation of oxygen-binding equilibria and kinetics by heterotropic effectors, with marked Bohr and Root effects. In Hb1 and Hb2, oxygen affinity and subunit cooperativity are slightly higher than in most high-Antarctic notothenioid hemoglobins. Hb1 and Hb2 show similar rebinding rates, but also show significant dynamic differences that are likely to have functional consequences. Molecular dynamic simulations of *C. gobio* Hb1 were performed on the dimeric protein in order to obtain a better understanding of the molecular basis of structure/function relationships.

The dominant perciform suborder Notothenioidei is an excellent study group for assessing the evolution and functional importance of biochemical adaptations to temperature in the Antarctic. Unlike temperate fishes, high-Antarctic Notothenioidei (living in the waters south of the Antarctic Polar Front, a roughly circular

Abbreviation
Hb, hemoglobin.

oceanic system running between 50°S and 60°S and extending to 2000 m in depth) express their genomes under an extremely cold thermal regime. To compensate for the rate-depressing effect of low temperature on metabolic processes and the high oxygen concentration in cold waters, many of the biomolecular systems of these fishes have been restructured through mutation and selection [1].

In cold Antarctic waters, which have a much higher concentration of dissolved oxygen than elsewhere, and with the selective evolutionary pressure relaxing oxygen transport, red-blooded high-Antarctic notothenioids have evolved a suite of physiological and molecular adaptations, accompanied by a decrease in hemoglobin (Hb) oxygen affinity and concentration [2]. High-Antarctic notothenioids possess physiological tools to enable survival despite an oxygen-carrier phenotype that facilitates oxygen unloading. The oxygen affinity of Hbs (regulating oxygen binding at the exchange surface and subsequent release to tissues) of many high-Antarctic species is quite low [3], as indicated by the p_{50} values (the oxygen partial pressure required to achieve half-saturation). This feature is probably linked to the high oxygen concentration in the cold Antarctic waters.

Some non-Antarctic notothenioid species belonging to the families Bovichtidae, Pseudaphritidae and Eleginopidae are phylogenetically basal to a clade that is almost exclusively distributed in the Antarctic, providing evidence that vicariance associated with the break-up of Gondwana has been an important factor in the diversification of notothenioids [4]. Non-Antarctic notothenioids are found north of the Antarctic polar front and comprise 22% of all notothenioids (28 of 129 species) [5]. The evolutionary origin of these species is not fully established, but their biochemistry and physiology support an Antarctic ancestry.

The phyletically basal bovichtids, pseudaphritids and eleginopids do not possess antifreeze glycoprotein genes in their genomes, indicating that they diverged and migrated before the tectonic isolation and associated cooling of Antarctica, and the appearance of antifreeze glycoproteins [6]. The split between eleginopids and the Antarctic clade of the five families that have antifreeze glycoproteins and inhabit the cold shelf waters is variously estimated to have occurred 5–14 [7], 27 [4] or 40 million years ago [8]. The 40 million year estimate is based on a fossil calibration [8]. Near [8] discusses the reasons for the discrepancies among these dates.

Bovichtids are notothenioids with a largely non-Antarctic distribution that includes southern South America, south-eastern Australia, New Zealand, and a few isolated islands north of the subtropical convergence. Bovichtids are regarded as the most basal family [5,9,10], with species showing many morphological characters that are considered plesiomorphic for the suborder.

In view of the peculiar thermal history of notothenioids and in an attempt to link polar environmental conditions with Hb evolution and molecular adaptation, this paper describes the structure and function of the oxygen-transport system of the sub-Antarctic notothenioid *C. gobio*.

Oxygen equilibria and CO-rebinding kinetics reveal that the two major Hbs of *C. gobio* (Hb1 and Hb2) show strong, effector-enhanced, Bohr and Root effects. In Hb1 and Hb2, the oxygen affinity and subunit cooperativity are slightly higher than most high-Antarctic notothenioid Hbs. *C. gobio* Hb1 and Hb2 have similar rebinding rates, but also show significant dynamic differences that are likely to have functional consequences. In order to gain a better understanding of the molecular basis for the structure/function relationship of *C. gobio* Hb1, molecular dynamic simulations of the dimeric protein were performed based on a homology model of the available crystal structure of *Trematomus bernacchii* (high-Antarctic notothenioid) [11]. Such simulations have been extensively used for study of the structure/function relationship in Hbs, as they allow analysis of the conformational dynamics of the protein and the characteristics of the protein–ligand interactions. Interestingly, our results show that the β subunit has increased flexibility of the CD loop, a fact that may be related to its oxygen affinity.

Results and Discussion

Purification of Hbs and separation of globins

Unlike most high-Antarctic notothenioids [2,3], cellulose–acetate electrophoresis and ion-exchange chromatography showed the presence of two major Hbs in the hemolysate (Figs S1and S2). The less anodal component Hb1 accounts for approximately 40% of the total. The elution profile of the hemolysate, obtained by reverse-phase HPLC, indicated the presence of three globins (Fig. S3). The results for separation of the globins of Hb1 and Hb2, their elution times and their N-terminal sequences indicated that Hb1 and Hb2 differ with respect to the α chains (indicated as α^1 and α^2, respectively), a feature shared with most high-Antarctic notothenioids, and have the β chain in common. Thus, in *C. gobio*, the chain compositions of Hb1 and Hb2 are $\alpha^1{}_2\beta_2$, and $\alpha^2{}_2\beta_2$, respectively.

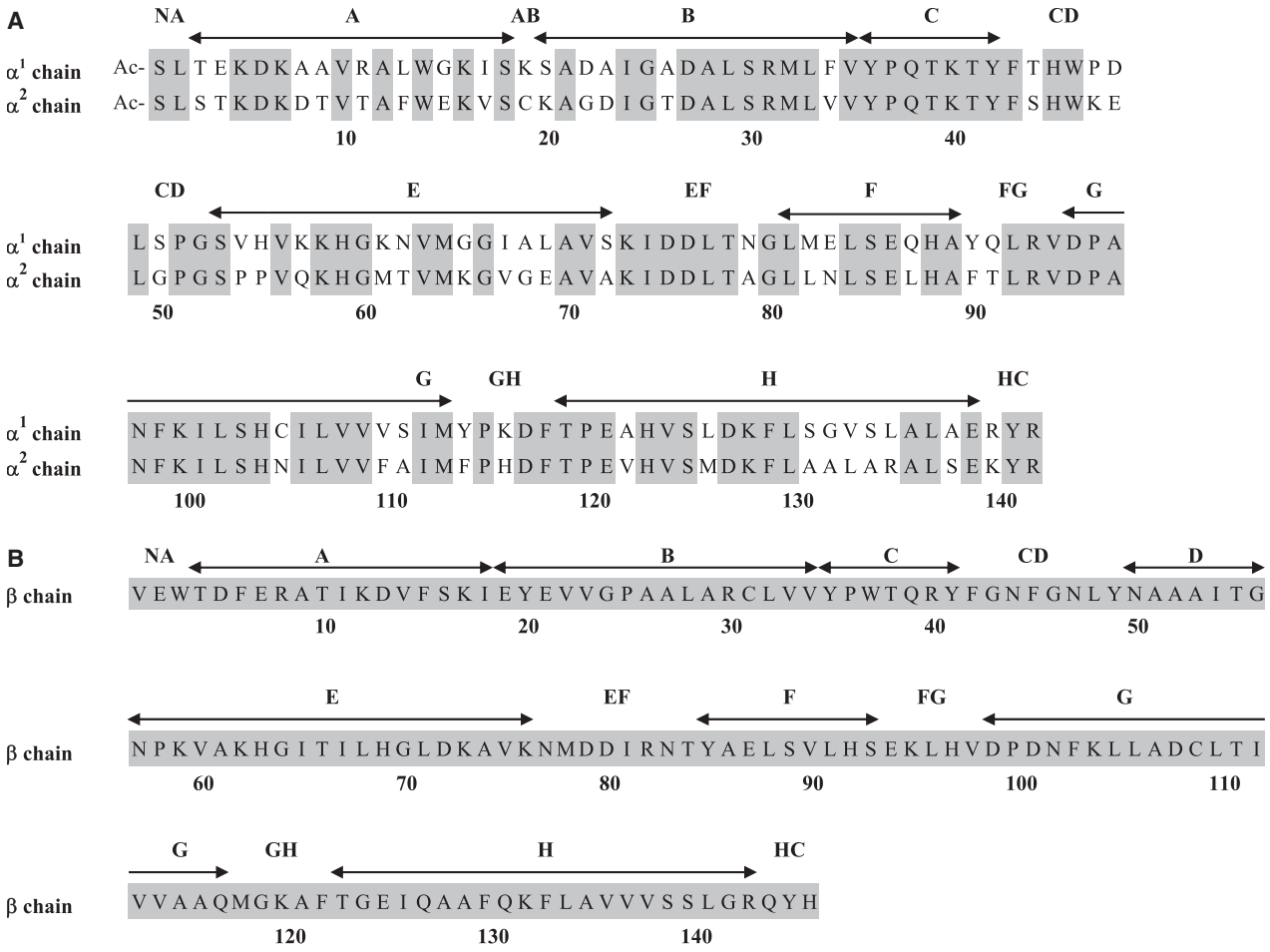

Fig. 1. Amino acid sequences of the α and β chains of *C. gobio* Hb1 and Hb2. Identical residues are shown in grey boxes. The helical regions (A–H) and non-helical regions (NA, A, CD, EF, FG, GH and HC), as established for mammalian Hb, are indicated; helix D is absent in α chains.

Primary structure

The sequences of the α and β chains (142 and 146 residues, respectively) of Hb1 and Hb2 are shown in Fig. 1. The sequences were established by alignment of tryptic and cyanogen bromide-cleaved peptides. The α-chain N-termini were not available for Edman degradation due to N-acetylation. MALDI-TOF mass spectrometry of the N-terminal tryptic peptides confirmed that acetyl was the blocking group, similar to other teleost Hbs sequenced to date. The sequence-deduced molecular masses of *C. gobio* α^1, α^2 and β globins were 15 727, 15 675 and 16 243 Da, respectively. These values are in agreement with those measured by MALDI-TOF. The sequence identity between the α^1 and α^2 chains of *C. gobio* is 66.9%.

The functionally important residues that have been suggested to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs are all conserved [11,12]. Hb1 has two Root-effect motifs typical of *T. bernacchii* Hb (the Asp48α/His55α and Asp69β/His72β pairs), and Hb2 has one (Asp69β/His72β) [11]. In comparison with Hbs of high-Antarctic notothenioids, the primary structure of *C. gobio* Hbs has several non-conservative substitutions. In α^1, Gln replaces Leu in F7 and Asn replaces Lys in E10; in α^2, Leu F7 is conserved, and Thr replaces Lys in E10. Also in α^2, two Pro residues replace Val E2 and His E3; the latter residue is thought to be involved in the Root effect [11].

Some of the side chains that form the $\alpha_1\beta_2$ 'dovetailed' switch region in human HbA (Pro αCD2, Thr αC3, Thr αC6, His βFG4) have been replaced. Pro αCD2 is replaced by Thr and Ser in the α^1 and α^2 chains, respectively, and Thr αC3 is replaced by Gln. Thr αC6 and His βFG4 are conserved. The replacements are of special interest because the region that has a primary role in the cooperative, quaternary transition T → R is highly conserved in vertebrate Hbs.

Side-chain packing at this interface is likely to be the major reason for the larger rotation of the two dimers in the R state in *T. bernacchii* Hb compared with human HbA [13].

When compared to major notothenioid β globins, the β chain of *C. gobio* Hb1 and Hb2 shows several substitutions in helix E, i.e. Ala E2 (58β) → Pro, Ala E6 (62β) → Lys, Lys E10 (66β) → Thr, Val E11 (67β) → Ile, and Gly E19 (75β) → Val; in helix G, Gln G19 (117β) is replaced by Lys/Arg. Remarkably, all minor Hbs have these replacements.

Met D6 (β55), which is considered invariant in vertebrates, including most teleosts, is replaced by short-chain Thr. Similar to all notothenioids (high-Antarctic, sub-Antarctic and temperate) and Arctic Gadidae [14], the *C. gobio* β chain contains two cysteyl residues occurring at B13 and G11; these are almost always absent in other teleost β chains.

His A14 (β17), His C7 (β41) and His GH3 (β120) of *T. bernacchii* Hb are replaced by Lys, Tyr and Lys, respectively, in both *C. gobio* Hbs. Evolutionary pressure maintaining a low number of conserved His residues and the likely associated reduction of the specific buffer value of these Hbs, taking into account that there are only few conserved His in teleost Hbs [15], appears to have occurred in non-Antarctic notothenioids, but is relaxed, if not reversed, in some high-Antarctic species.

Oxygen equilibria, subunit cooperativity and root effect

Detailed functional studies were performed on Hb1 and Hb2. The oxygen-binding properties were investigated at 7 and 12 °C in the absence and presence of allosteric effectors, such as protons, chloride ions and organophosphates. Table 1 lists the oxygen-binding properties of Hb1 and Hb2 at pH 8.11 and 8.41.

In comparison with most high-Antarctic notothenioids [2,3], *C. gobio* Hb1 and Hb2 show higher oxygen affinities. In Hb1 and Hb2, in the presence of chloride and ATP at pH 8.11, the p_{50} values are 5.73 and 7.45 mmHg, respectively.

The lower oxygen affinity seen in the species of highest southern latitudes appears typical of the most cold-adapted species. The p_{50} values, traced as a continuous character on the phylogenetic tree showing the phylogeny of high-Antarctic and non-Antarctic notothenioids (C. Verde, unpublished data), indicate that a decrease in Hb affinity occurred along the lineage of the high-Antarctic notothenioids. Taking some notothenioid species as examples, the p_{50} values for *Gymnodraco acuticeps* (high-Antarctic), *Eleginops maclovinus* (sub-Antarctic) (C. Verde, unpublished data) and *Pseudaphritis urvillii* (temperate) Hb1 at pH 8.11 in the presence of effectors are 39.8, 1.26 and 1.99 mmHg, respectively [16,17]. The terminal branches of the high-Antarctic notothenioids *Pleuragramma antarcticum* [18], *T. bernacchii* [19] and *Artedidraco orianae* [20] show higher affinity compared to the other Antarctic notothenioids [3].

Table 1. Oxygen-binding properties of *C. gobio* Hb1 and Hb2 at pH 8.41 and 8.11, and 7 °C. Data shown comprise p_{50} values, subunit cooperativity (n_{Hill}), and the overall oxygenation enthalpy change (ΔH) in 100 mM Hepes, in the absence of effectors (stripped), in the presence of 100 mM NaCl and in the presence of 100 mM NaCl and 3 mM ATP. ΔH values are in kcal·mol^{-1} (1 kcal = 4.184 kJ).

	Hb1		Hb2	
	pH 8.41	pH 8.11	pH 8.41	pH 8.11
p_{50}				
Stripped	3.49	3.39	5.10	5.09
100 mM NaCl	3.05	3.78	5.03	5.61
100 mM NaCl, 3 mM ATP	3.96	5.73	5.50	7.45
n_{Hill}				
Stripped	2.1703	2.1826	1.7602	1.7470
100 mM NaCl	2.0182	2.2979	1.8432	2.0514
100 mM NaCl, 3 mM ATP	2.2621	2.1152	2.2561	2.1070
ΔH				
Stripped	−12.1942	−27.0755	−11.7503	−21.1521
100 mM NaCl	−10.6078	−22.4911	−12.5071	−22.4474
100 mM NaCl, 3 mM ATP	−15.0395	−52.3410	−15.7817	−54.3567

The relationship between the higher oxygen affinity of non-Antarctic notothenioid Hbs and habitat features is not fully understood. Spectroscopic and modelling studies on Hb1 of *P. urvillii* (a temperate notothenioid from coastal waters, estuaries and rivers of southern Australia, e.g. Tasmania, Victoria, New South Wales) have shown that the non-conservative replacements in the primary structure of α and β chains leave the conformation and electrostatic field surrounding the heme pocket essentially unmodified [17] compared with Hb of the high-Antarctic *T. bernacchii* [11,13].

A large Bohr effect was observed under all experimental conditions, and the enhancement by organophosphates was consistently high in Hb1 and Hb2. However, Hb2 had the highest Bohr coefficient ($\varphi = \Delta \log p_{50}/\Delta pH$), which indicates the mean number of protons bound upon heme oxygenation. At pH 7.5, in Hb2, the p_{50} values in the presence of NaCl and ATP correspond to an even lower affinity than that observed at pH 7.0 in the absence of effectors. The exceptional affinity decrease brought about by

ATP at neutral pH in Hb2 species suggests that the ligand is able to lock the proteins in the low-affinity T state at physiological pH values. The transition from the R to the T state appears to start earlier in Hb2, and does not allow measurement of the oxygen affinity (p_{50} value) below pH 7.5. At pH values above neutrality, chloride alone had no strong effect on the oxygen affinity (Table 1).

In Hb1, cooperativity is high in the alkaline pH range (n_{Hill} = 2.2); only when the affinity reaches the highest values, namely at the maximal allosteric constraint, is the apparent cooperativity abolished (Table 1). In *C. gobio* Hb2, the dramatic decrease in affinity with ATP already observed at pH 7.5 (to a much higher extent than that observed in Hb1) does not allow measurement of the lack of subunit cooperativity at low pH values. In Hb2, the cooperativity is slightly, but significantly, higher in presence of chloride.

As shown in Fig. 2A,B, in which oxygen saturation at atmospheric pressure is reported as a function of pH, Hb1 (Fig. 2A) and Hb2 (Fig. 2B) show a marked Root effect. The oxygen release induced by protons is enhanced by ATP in Hb1 and Hb2. This is not a novel finding, but once again it highlights the physiological role of anions and in particular of organophosphates, which, in addition to modulating the oxygen affinity, appear to finely control the region of pH in which the Root effect is operative. The magnitude of the Root effect, expressed as percentage of the decrease in oxygen saturation at pH 6.25 in the presence of the physiological effectors NaCl and ATP, is very large (60% in Hb1; 70% in Hb2). While the appearance of the Root effect in Hb1 corresponds to the loss of subunit cooperativity, the magnitude of the Root effect in Hb2 does not fully correspond to the decrease of subunit cooperativity. These results suggest differences in pH regulation of the allosteric transition from R to T in the two Hbs. Such differences may well be correlated with the presence of the two Pro residues in the α^2 chain, one of which replaces His, and produce functional differences in the biochemical properties of the two Hbs.

A weakening in the Bohr and Root effects is generally noted in the high-Antarctic notothenioid lineage in comparison with non-Antarctic species [3].

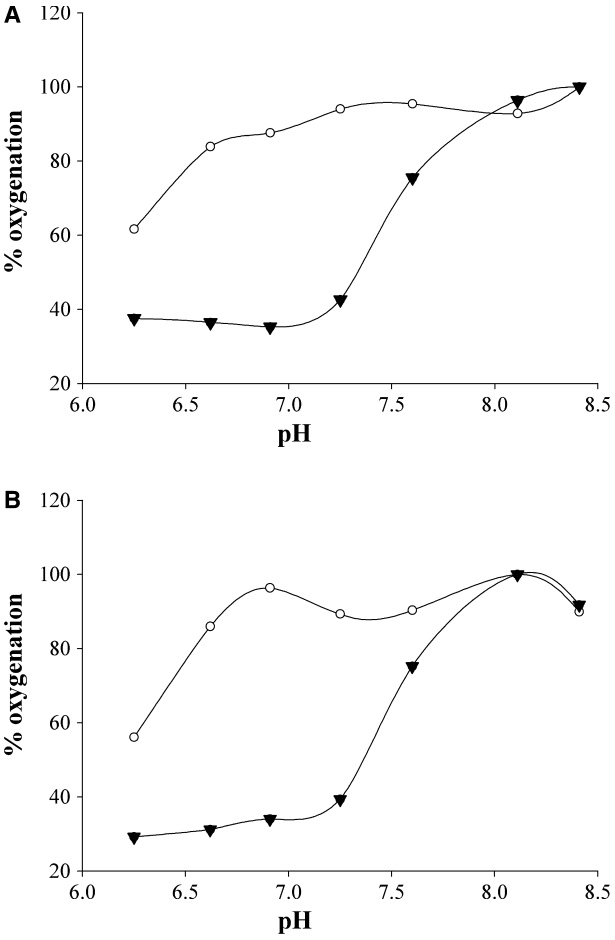

Fig. 2. Root effect as a function of pH, at 7 °C, for *C. gobio* Hb1 (A) and *C. gobio* Hb2 (B). Values were obtained in 100 mM Hepes in the absence of effectors (open circles) or in the presence of 100 mM NaCl and 3 mM ATP (filled triangles).

Thermodynamics of oxygen binding

Regulation of the oxygen affinity of Hb1 and Hb2 by temperature, calculated in terms of the overall oxygenation enthalpy change (ΔH), was investigated in the range 7–12 °C (Table 1). Thermodynamic analysis showed that the oxygenation enthalpy change in both Hbs is high when compared with Antarctic notothenioid fish, and is very similar to the values seen in the Hbs of temperate fish. Relying upon Hbs with reduced ΔH values may thus be a frequent evolutionary strategy of cold-adapted fish. It appears that, in polar fish, evolutionary development has often favored a decrease in the temperature sensitivity of Hb affinity [21].

In Hb1 and Hb2, in the absence and presence either of chloride alone, or of both chloride and organophosphates, the highest values (in absolute terms) were found at pH 8.11, where the molecules may be considered as locked in the high-affinity R state. At lower pH values, the overall enthalpy change progressively decreased as a function of increasing proton concentration, due to the endothermic contribution of the heterotropic effectors released upon oxygen binding and the allosteric transition. Under all conditions, the

difference in the ΔH of oxygenation between the two states was 10–15 kcal·mol^{-1}, but the overall ΔH drastically increased between pH 7.6 and 8.1 in both Hbs, with a difference in the ΔH of oxygenation of 50 kcal·mol^{-1} (in absolute terms).

Rebinding kinetics

The question now arises as to whether distinct equilibrium properties at different pH in Root-effect Hbs correspond to kinetically different behaviors, and we found that such a correlation does indeed exist in *C. gobio* Hbs.

Measurements of the transient absorption, which follows CO rebinding, were measured in CO-liganded Hbs under various conditions. For comparison, we also report the CO-rebinding kinetics for the high-Antarctic Root-effect *T. bernacchii* Hb, and contrast them with those of the Hb of a species that lacks the Root effect, *G. acuticeps* Hb [16].

Figure 3A,B shows the CO-rebinding kinetics at 3.5 °C and pH 6.0 and 8.4, respectively, in *C. gobio* Hb1 and Hb2 (marked a and b, respectively), and *T. bernacchii* Hb and *G. acuticeps* Hb (marked c and d, respectively) in the absence of ATP. Figure 3C shows the kinetics of CO rebinding in *C. gobio* Hb1 at pH 6.0 in the absence and presence of 3 mM ATP (marked a and b, respectively), whereas Fig. 3D shows the same comparison at pH 7.0 in the absence and presence of 3 mM ATP (marked a and b, respectively). *T. bernacchii* Hb, similar to *C. gobio* Hbs, shows strong pH dependence of the kinetic and equilibrium properties.

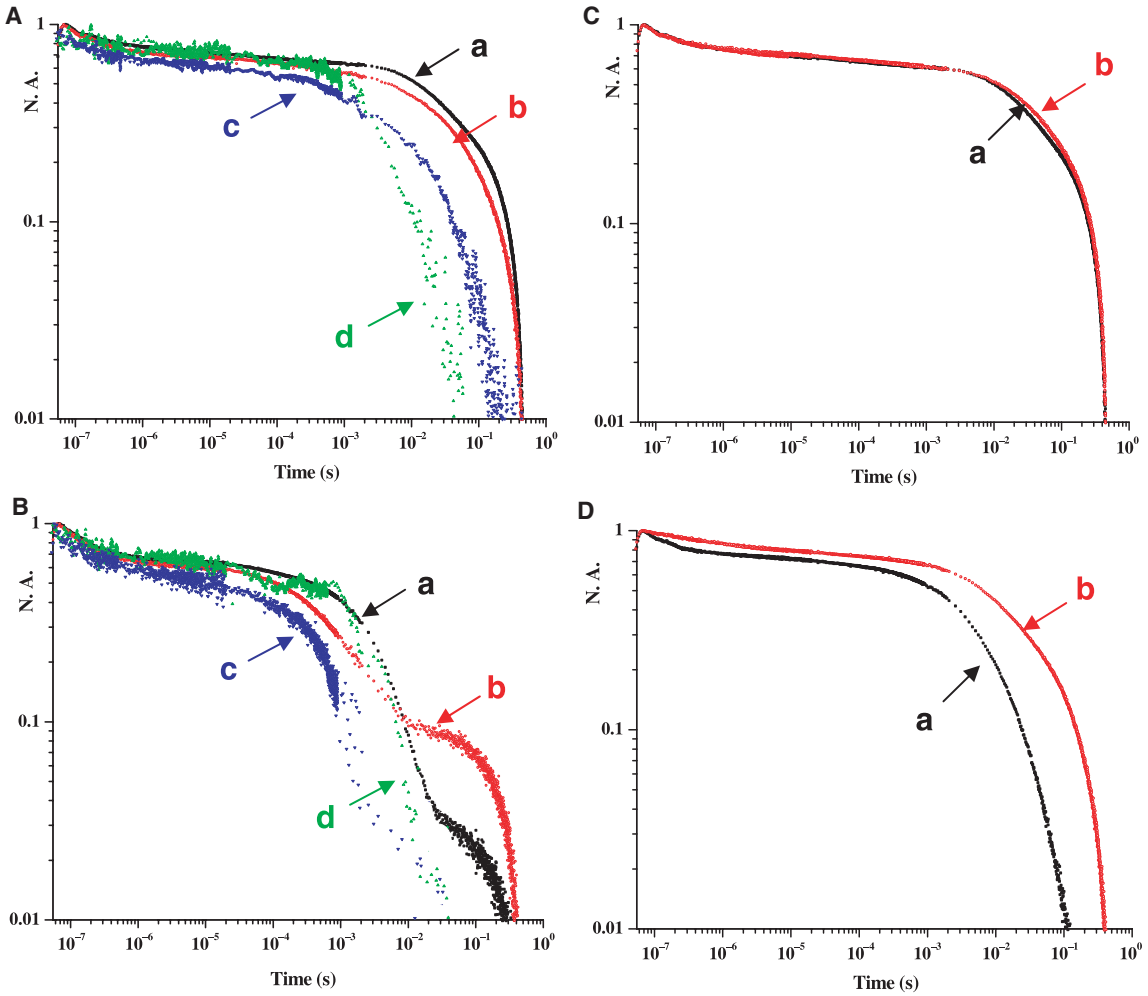

Fig. 3. CO-rebinding kinetics. (A) Hb1 (trace a, black), Hb2 (trace b, red), *T. bernacchii* Hb (trace c, blue) and *G. acuticeps* Hb (trace d, green) at pH 6.0 in Mes buffer. (B) Hb1 (trace a, black), Hb2 (trace b, red), *T. bernacchii* Hb (trace c, blue) and *G. acuticeps* Hb (trace d, green) at pH 8.4 in Tris/HCl or Bicine buffer. (C) Hb1, at pH 6.0, in the absence (trace a, black) or presence (trace b, red) of 3 mM ATP. (D) Hb1, at pH 7.0, in the absence (trace a, black) or presence (trace b, red) of 3 mM ATP.

However, as seen in Fig. 3, rebinding in both *C. gobio* Hbs is always slower than in *T. bernacchii* Hb.

The kinetic traces of *G. acuticeps* Hb are the counterpart of the pH-independent equilibrium properties of this non-Root-effect Hb. The invariant rebinding kinetics of *G. acuticeps* Hb correspond to the rebinding kinetics of *T. bernacchii* Hb at physiological pH.

As in most Hbs studied, the higher the affinity in *C. gobio* Hb1 and Hb2, the higher the CO-rebinding rate. When liganded Hb is photodissociated, there is a period of time (< 1 μs for solution-phase samples in aqueous buffer) during which the photodissociated ligand remains within the protein. This composite species is often referred to as the geminate pair. The geminate pair can decay either by having the ligand escape into the bulk solvent or by recombining with the heme iron to which it was originally bound. Geminate recombination is the name given to this unimolecular rebinding process. If the ligand escapes from the protein into the bulk solvent, the subsequent rebinding, termed solvent-phase recombination, becomes a bimolecular process. In the case of *C. gobio* Hbs, the results of the kinetic measurements show a clear gradual decrease in the geminate yield as well as in the rate of the bimolecular rebinding as pH decreases and/or ATP is added. At alkaline pH, higher affinity and cooperativity correspond to higher rebinding rate (< 1 ms). Conversely, lower affinity and cooperativity at acidic pH correspond to lower rebinding rate (> 0.01 s). The progression in the geminate recombination and bimolecular recombination probably parallels the changes in the quaternary state.

When comparing the CO-rebinding kinetics of Hb1 and Hb2 at pH 8.4 (Fig. 3B), there is a large difference in the R-state bimolecular-rebinding rate, suggesting a difference in the R-state affinity. There is also a small difference in the onset time of the slower T-state component, which may indicate a difference in the R → T barrier. However, CO-geminate rebinding is similar for Hb1 and Hb2, indicating that the liganded R structures of Hb1 and Hb2 are similar, whereas differences in the bimolecular-rebinding kinetics demonstrate the existence of differences in the dynamics, possibly indicating that the Hb2 deoxy R intermediates have a higher ligand affinity compared with the Hb1 deoxy R intermediates.

In contrast, both *C. gobio* Hbs show similar CO-rebinding kinetics at pH 6.0 (Fig. 3A) and pH 7.0, both in the presence and absence of ATP, leading to the conclusion that their low-affinity T states are similar. Interestingly, in both Hbs, addition of ATP does not affect the rebinding rate at pH 6.0 (Fig. 3C, Hb1), but it does at pH 7.0 (Fig. 3D, Hb1), leading to a significant decrease in the rebinding rate. Similar results were obtained with Hb2. These results suggest that, even in the absence of effectors, *C. gobio* Hbs are already fully in the T state at pH 6.0, such that addition of effectors will have no further or limited effect on the rebinding rates.

Simulations

As the two Hbs share the functionally relevant β chain and the sequence identity (66.9%) between the α^1 and α^2 chains of *C. gobio* covers all the functionally important domains, we decided to perform the simulation on Hb1 only. The structure of the model of *C. gobio* Hb1 was constructed as described in Experimental procedures (PMDB ID PM0075620). The dimer structure is stable during the whole 10 ns period of the simulation, suggesting that the model properly describes the subunit interactions. The model also correctly predicts the presence of the proximal His F8 as the heme ligand and of His E7 in the distal cavity.

For this dimer simulation, the root mean square fluctuation was plotted against residue for each subunit separately in the oxy and deoxy forms (Fig. 4). The root mean square fluctuation measures the mobility of each residue by calculating the standard deviation from the mean structure. In both the oxy and deoxy structures, the fluctuations in the CD loop region (residues 10–40) are higher in the β subunit than in the α^1 subunit. The fluctuations in the remaining part of the protein are small and similar in the α^1 and β subunits. In addition, the simulations indicate that the presence of coordinated oxygen increases the magnitude of the fluctuations in the β subunits.

The simulations of the deoxy proteins also showed that His E7 acts as a gate to possibly regulate ligand entry in *C. gobio* Hb1, as is known to occur in most globins [22]. In all deoxy simulations (α^1, β and the dimer $\alpha^1\beta$), starting with His E7 buried inside the distal pocket of the protein, in < 2 ns of simulation time, His rotates around the C–C bond and opens the pocket to the solvent, as shown in Fig. 5. His E7 remains totally exposed to the solvent during the remainder of the simulation. This opening event allows one or two water molecules to enter the distal site.

It is common knowledge that the main control of oxygen affinity in globins involves the ligands of the distal pocket, particularly His E7 [22]. The stronger the hydrogen bonds between bound oxygen and the distal residues, the higher the oxygen affinity, due to slower dissociation rates. The simulations of both oxygenated α^1 and β of *C. gobio* Hb1 show that the only residue capable of interacting with bound oxygen is

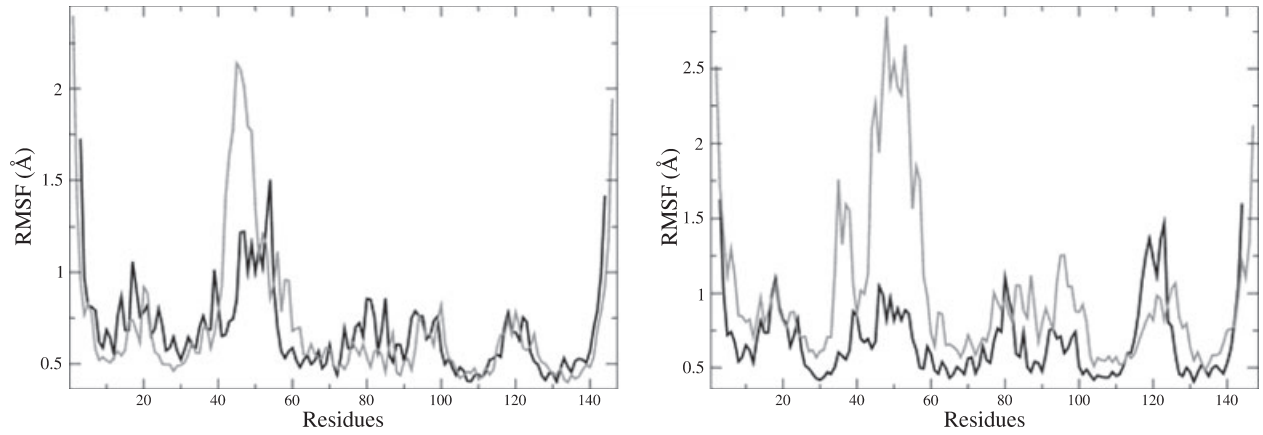

Fig. 4. Structure and flexibility comparison between states. Root mean square fluctuation (RMSF) along the sequence for the backbone atoms of the deoxy (left panel) and oxy (right panel) proteins during the molecular dynamic run. The results for the α^1 and β chains are shown in grey and black, respectively.

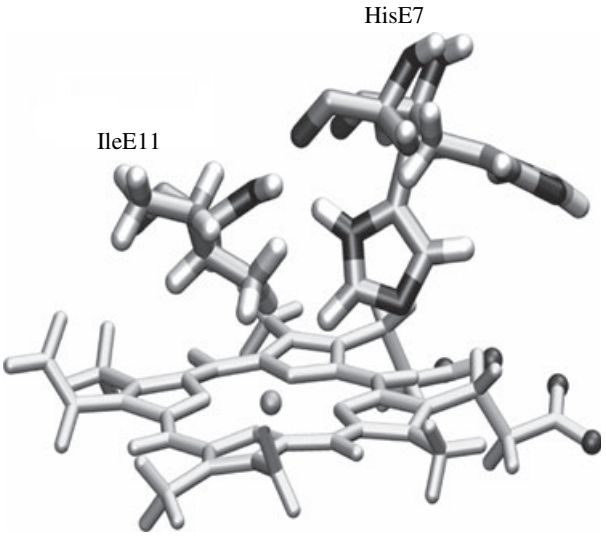

Fig. 5. His E7 in superimposed open and closed conformations in the β chain of the dimer $\alpha^1\beta$ of Hb1. The heme and residue Ile E11 are shown. This gate-opening movement of His E7 has also been observed in the α^1 chain.

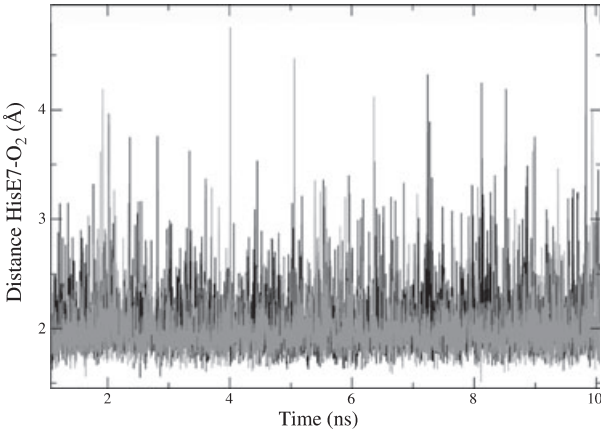

Fig. 6. Distance between His E7 and oxygen in oxy Hb1. Plot of distance among hydrogen bound to nitrogen epsilon of His E7 (HNE2) of His E7 and the more distal oxygen atom of the oxygen molecule coordinated to Fe. Results for the α^1 chain are shown in black; those for the β chain are shown in grey.

His E7. To analyze this interaction in both subunits, the hydrogen-bond distance between the Hδ of His E7 and the coordinated oxygen was calculated (Fig. 6). It can be seen from this simulation that a strong hydrogen bond is present in both subunits over the entire duration of the simulation.

Conclusions

Fish living in the polar regions are exposed to strong environmental constraints. Understanding how they have evolved to cope with these challenges requires elucidation of the molecular mechanisms of physiological/biochemical adaptations. The study of gains, losses and changes in the Hb function over evolutionary time may offer an explanation to how Hbs of sub-Antarctic species have evolved to become high-Antarctic Hbs.

As a result of the isolation of Antarctica, the genotype of Notothenioidei diverged with respect to other fish groups in a way that is interpreted as typical of a species flock [23]. Unlike most high-Antarctic notothenioids, but similar to many other acanthomorph teleosts, adult *C. gobio*, which thrives in sub-Antarctic waters just north of the Antarctic polar front, has two major Hbs having the β chain in common. In the two Hbs, oxygen binding is strongly modulated by

heterotropic effectors, with marked Bohr and Root effects. Higher multiplicity was also observed in *Bovichtus diacanthus*, one of the most northern notothenioids (C. Verde, unpublished data), which belongs to the same family. Although the presence of multiple Hbs in the blood of non-Antarctic notothenioids may be considered a plesiomorphic condition for many perciform fishes, the more complex oxygen-transport system in *C. gobio* and *B. diacanthus* may have been maintained by positive selection to deal with large temperature changes in waters north of the Antarctic polar front. Rather than relying on a single respiratory protein, many organisms, including teleosts, express multiple oxygen carriers with various oxygen-binding properties, in order to meet oxygen demand under changing environmental conditions or metabolic challenges.

In the phylogenetic trees, the basal position of *C. gobio* and *B. diacanthus* Hbs appears to agree with the postulated divergence before the appearance of antifreeze glycoproteins [3]. The α chain of *C. gobio* Hb1 branches off the clade of the major Antarctic Hbs, whereas the β chain shared by Hb1 and Hb2 is surprisingly included in the clade of the minor Antarctic Hbs [24]. In the majority of notothenioids, 'embryonic' α and β globins are expressed in trace or limited amounts in the adult stage, although in at least three species, namely *Trematomus newnesi* [25], *Pagothenia borchgrevinki* [26] and *P. antarcticum* [18], 'embryonic' globins are expressed at significant levels. In these three species, however, the majority of the β chains are included in the 'notothenioid major adult Hb group'. Thus, a complete 'switch' to exclusive expression of the embryonic β globin gene was found in adult *C. gobio*. Constitutive expression of the embryonic globins in adult fish may have survived exclusively along the lineage leading to *C. gobio* [24].

The kinetic results show that, over the whole pH range, the high-Antarctic *T. bernacchii* Hb CO-rebinding kinetics are faster than those of the *C. gobio* Hbs. This suggests that both the R-state and T-state conformations of *T. bernacchii* Hb have higher rebinding rates compared to the respective states of *C. gobio* Hbs, e.g. at pH 8.4, the respective R-state rebinding rates are 10^{-3} versus approximately 10^{-2} s. An interesting dynamic property with functional implications that was observed in the ligand rebinding kinetics studies, but not in equilibrium studies, is relaxation of the protein conformation from the higher-affinity R state to the lower-affinity T state following ligand photolysis. This conformational relaxation, causing the switch from the R to the T state, appears as an additional slower kinetic phase in the ligand-rebinding kinetic trace, and is due to a slowdown in the rebinding kinetics due to the new lower-affinity conformation of the protein (T state) compared to its initial higher-affinity conformation (R state) at the time of photolysis. In *T. bernacchii* Hb with its faster rebinding kinetics, this crossover point is harder to see but seems to occur at around approximately 10^{-3} s, at an earlier time than the R → T switch point of *C. gobio* Hbs.

When comparing *C. gobio* Hb1 and Hb2, the kinetic results show many similarities. At low pH, their kinetic traces are very similar, suggesting that the low-affinity T states are similar. Furthermore, the geminate rebinding at pH 8.4 is very similar in Hb1 and Hb2, suggesting that the liganded R structures of the two proteins are similar. However, at pH 8.4, there is large difference in the R-state bimolecular rebinding rate, suggesting a difference in the R-state affinities of the two species, possibly indicating that the deoxy R intermediates of *C. gobio* Hb2 have a higher ligand affinity compared with the Hb1 deoxy R intermediates. Interestingly, there is also a small difference in the onset time of the slower T-state component, which may indicate a difference in the R → T barrier. These results may explain why *C. gobio* expresses two Hbs. Thus, although the liganded R-state and T-state structures of *C. gobio* Hb1 and Hb2 have similar rebinding rates, they also show significant dynamic differences that are likely to have functional consequences.

Experimental procedures

Collection of specimens

Specimens of adult *C. gobio* were collected near the Falkland Islands by bottom trawling from the research vessel Nathaniel B. Palmer during the ICEFISH 2004 cruise. Blood was taken using heparinized syringes from the caudal vein. Saline-washed erythrocytes were frozen at −80 °C until use.

Purification and amino acid sequencing

Hemolysates were prepared as described previously [27]. Separation of Hbs was achieved using AKTA-FPLC (Pharmacia, Uppsala, Sweden) anion-exchange chromatography on a DE 52 column. The Hb-containing pooled fractions were dialyzed against 10 mM Hepes, pH 7.7. All steps were performed at 0–5 °C. No oxidation was spectrophotometrically detectable. Hb solutions were stored in small aliquots at −80 °C until use.

Purification of globins by reverse-phase HPLC, fractionation of tryptic and cyanogen bromide-cleaved peptides, and subsequent amino acid sequencing were carried out as previously described [28].

Mass spectrometry

The molecular masses of the S-pyridylethylated α and β chains and of the peptides (less than 10 kDa) were measured by MALDI-TOF mass spectrometry using a PerSeptive Biosystems (Applied Biosystems, Framingham, MA, USA). Voyager-DE biospectrometry workstation, as described previously [28].

Oxygen binding and Root effect

Hemolysate stripping was performed as described previously [29]. Oxygen equilibria were measured in 100 mM Hepes/Mes in the pH range 6.5–8.4 at 7 and 12 °C (bearing in mind the pH variation as a function of temperature) at a final Hb concentration of 0.5–1.0 mM on a heme basis. Experiments were performed in duplicate, a standard deviation of ± 3% for p_{50} values was calculated. In order to achieve stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air [30]. pH values were measured using a Radiometer (Copenhagen, Denmark) BMS Mk2 thermostatted electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP were measured at a final ligand concentration of 3 mM, a large excess over the tetrameric Hb concentration. Oxygen affinity (measured as p_{50}) and cooperativity (n_{Hill}) were calculated from the linearized Hill plot of log $S/(1 - S)$ against log pO_2 at half saturation, where S is the fractional oxygen saturation. The amount of metHb formed after each measurement was < 2% .

The Root effect was determined in 100 mM Hepes/Mes in the pH range 6.2–8.4 at 10 °C by calculating the mean absorbance difference at three wavelengths (540, 560 and 575 nm) between the spectra at pH 8.4 (fully oxygenated Hb) and pH 6.2 and the spectra after deoxygenation using sodium dithionite.

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

Rebinding kinetics

All chemicals were obtained at the highest purity commercially available and used without further purification. Nitrogen and oxygen were purchased from Tech Air (White Plains, NY, USA), carbon monoxide (CO) from Matheson (East Rutherford, NJ, USA), and ATP from Sigma (St Louis, MO, USA). The previously described [31] flash-photolysis equipment used for the CO-rebinding rate experiments of the total, was based on a pump–probe optical set-up in which the frequency-doubled output of an Nd:YAG laser (7 ns, Surelite and Minilite lasers, Continuum, Santa Clara, CA, USA) at 532 nm and 2 Hz repetition rate was used to photolyze the CO derivatives. Recombination of CO was monitored by measuring the change in sample absorption at 441.6 nm (continuous wave, HeCd laser, Liconix, Santa Clara, CA, USA). The HeCd probe laser beam was passed through the sample nearly collinear with but in the opposite direction to the Nd:YAG laser while using a dichroic mirror used for rebinding kinetics. Samples containing 0.2–0.5 mM Hb on a heme basis were assayed in 1 mm cuvettes (NSG Precision Cells Inc., Farmingdale, NY, USA) at either 3.5 or 7 °C. They were flushed with CO and monitored using UV/Vis absorption spectra. The buffers were 50 mM Mes at pH 6.0 and 50 mM Bicine and Tris/HCl at pH 8.4. All buffers were purchased from Sigma.

Simulations

A model of *C. gobio* Hb1 was built using the structure of *T. bernacchii* Hb at pH 8.4 as the template (Protein Data Bank code 2H8D). The proteins share 71% sequence identity [11].

We used the MODELLER program [32] to obtain ten possible model structures. Based on visual inspection and the stereochemistry of the model residues, the best model was selected. We modelled one α^1 and one β subunit, and the dimeric structure $\alpha^1\beta$. In addition, we built models of oxygenated proteins by adding an oxygen molecule to the heme.

For each of the systems α^1deoxy, α^1oxy, βdeoxy, βoxy and deoxy $\alpha^1\beta$, a 10 ns molecular dynamic simulation was performed using an AMBER9 force field in explicit water environment (8746 molecules in the dimeric structure, 6499 molecules in the monomeric structure) [33]. The simulation parameters are the same as successfully used in a previous study on neuroglobin by our group, and further details of the simulation have been given previously [34].

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Supporting information

The following supplementary material is available:
Fig. S1. Cellulose–acetate electrophoresis of *C. gobio* hemolysate, Hb1 and Hb2.
Fig. S2. Ion-exchange chromatography of *C. gobio* hemolysate and the mixture of *C. gobio* Hb1 and Hb2.
Fig. S3. Reverse-phase HPLC of *C. gobio* hemolysate, Hb1 and Hb2.

This supplementary material can be found in the online version of this article.

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