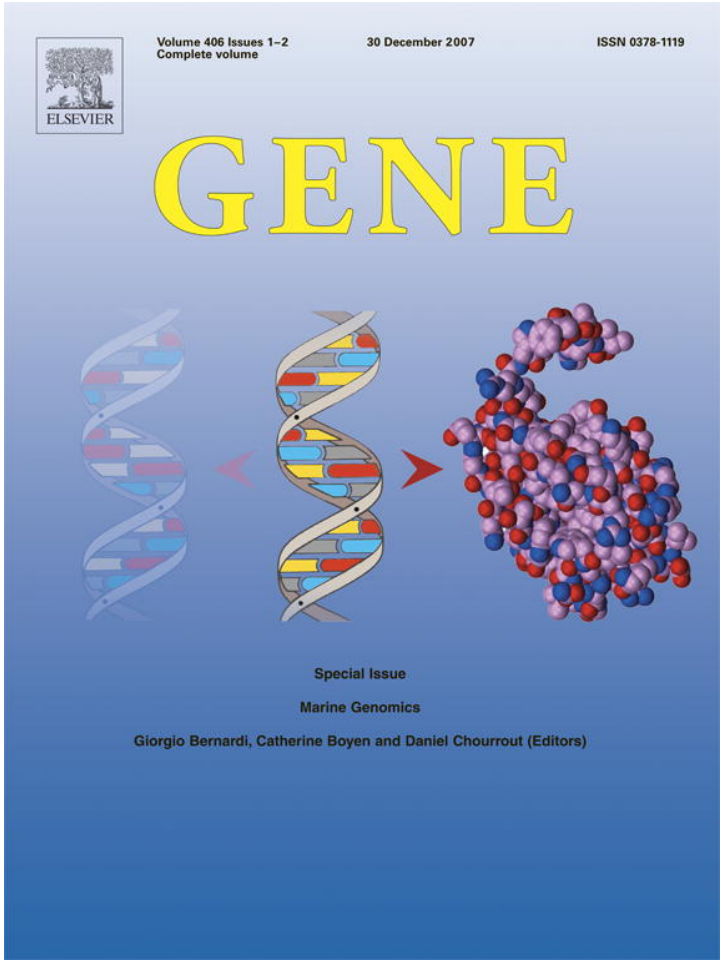

Available online at www.sciencedirect.com
ScienceDirect
Gene 406 (2007) 58–68
GENE
www.elsevier.com/locate/gene

Hemoglobin structure/function and globin-gene evolution in the Arctic fish *Liparis tunicatus* ☆

Daniela Giordano [a], Alessandro Vergara [b], H. Caroline Lee [c], Jack Peisach [c], Marco Balestrieri [a], Lelio Mazzarella [b], Elio Parisi, Guido di Prisco [a], Cinzia Verde [a,*]

[a] *Institute of Protein Biochemistry, CNR, Via Pietro Castellino 111, I-80131 Naples, Italy*
[b] *Dipartimento di Chimica, Università degli Studi di Napoli "Federico II", Complesso Monte S. Angelo, Via Cinthia, I-80126 Naples, Italy*
[c] *Department of Physiology and Biophysics, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Avenue, New York, NY 10461,USA*

Received 19 February 2007; received in revised form 30 May 2007; accepted 4 June 2007
Available online 13 June 2007

Abstract

The importance of the Arctic, in contributing to the knowledge of the overall ensemble of adaptive processes influencing the evolution of marine organisms, calls for investigations on molecular adaptations in Arctic fish.

Unlike the vast majority of Antarctic Notothenioidei, several Arctic species display high hemoglobin multiplicity. The blood of four species, the spotted wolffish of the family Anarhichadidae and three Gadidae, contains three functionally distinct major components. Similar to many Antarctic notothenioids, Arctic *Liparis tunicatus* (suborder Cottoidei, family Liparidae) has one major hemoglobin (Hb 1) accompanied by a minor component (Hb 2). This paper reports the structural and functional characterisation of Hb 1 of *L. tunicatus*. This hemoglobin shows low oxygen affinity, and pronounced Bohr and Root effects. The amino-acid sequence of the β chain displays an unusual substitution in NA2 (β2) at the phosphate-binding site, and the replacement of Val E11 (β67) with Ile.

Similar to some Antarctic fish Hbs, electron paramagnetic resonance spectra reveal the formation of a ferric penta-coordinated species even at physiological pH. The amino-acid sequences have also been used to gain insight into the evolutionary history of globins of polar fish. *L. tunicatus* globins appear close to the notothenioid clades as predicted by teleostean phylogenies. Close phylogenetic relationships between Cottoidei and Notothenioidei, together with their life style, seem to be the main factor driving the globin-sequence evolution.

Keywords: Antarctic; Arctic; Evolution; Hemoglobin; Structure; Unligated ferric state

1. Introduction

The main differences between the Arctic and the Antarctic are the older age and longer isolation of the latter (Brey et al., 1994; Eastman, 1997). The Antarctic remained isolated and cold longer than the Arctic, with ice-sheet development preceding that in the Arctic of at least 10 million years (my). The Antarctic experienced a slow and discontinuous transition from the warm-water system of the early Tertiary (15 °C) to the cold-water system of today (−1.87 °C). The Arctic and Antarctic fish faunas differ in age, endemism and taxonomic diversity. The Arctic fauna has 416 species of 96 families, whereas in the southern ocean the fauna includes 322 species grouped in 50 families (Eastman, 2005). For marine fish, endemism in the Arctic is 25% (Eastman, 1997), but in the Antarctic, it reaches 88% and rises to 97% when only the dominant suborder Notothenioidei is considered.

Unlike Antarctica, where the perciform suborder Notothenioidei is the single dominant group, the Arctic hosts six dominant groups (zoarcoids, gadiforms, cottids, salmonids, pleuronectiforms and chondrichthyans), which account for 58% of the fauna (Eastman, 1997). Only the families Zoarcidae and Liparidae have species at both poles (Eastman, 1997).

Abbreviations: mya, million years ago; ACC, Antarctic Circumpolar Current; Hb, hemoglobin; EPR, Electron Paramagnetic Resonance; DPG, diphosphoglycerate; HbCTn, cathodic hemoglobin of *Trematomus newnesi*.

☆ The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number: P85081 (*L. tunicatus* α^1 chain); P85082 (*L. tunicatus* β^1 chain).

* Corresponding author. Tel./fax: +39 01 6132710.
E-mail address: c.verde@ibp.cnr.it (C. Verde).

0378-1119/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2007.06.002

Polar fish have undergone different regional histories which have engineered physiological diversities. For instance, Antarctic species are more stenothermal than Arctic fish. Geography, oceanography and biology of species inhabiting the polar regions have often been compared (Dayton et al., 1994) to outline the differences between the two polar ecosystems. Understanding the mechanisms of phenotypic response to cold exposure in species living at different latitudes in polar habitats offers fundamental insights into environmental adaptations.

The study of the structure and function of Arctic fish Hbs was initiated only recently, with the molecular characterisation of the oxygen-transport system of the Arctic zoarcoid *Anarhichas minor* and of three species of the family Gadidae (order Gadiformes) (Verde et al., 2002, 2006a).

In an attempt to link polar environmental conditions with the evolution of Hb, this study investigated the structure and function of the oxygen-transport system of the Arctic fish *Liparis tunicatus* (order Scorpaeniforms, suborder Cottoidei, family Liparidae). *L. tunicatus* is a sedentary fish, caught near the coast of Svalbard. Cottoidei appear phylogenetically close to Notothenioidei (Miya et al., 2003; Dettaï and Lecointre, 2004, 2005).

Similar to many notothenioids, *L. tunicatus* has one major Hb (Hb 1) and one minor component (Hb 2). Hb 1 shows low oxygen affinity, similar to Antarctic and Arctic Hbs. The Bohr and Root effects are pronounced in the presence of physiological effectors. The sequences of the globins were elucidated for structural purposes. Similar to cathodic Hb (Hb C) of the Antarctic fish *Trematomus newnesi*, Val E11, usually present in the α and β chains at the distal side of the heme, is replaced by Ile. Interestingly, electron paramagnetic resonance (EPR) spectra of both the Antarctic Hb C (Vergara et al., in press) and the Arctic Hb 1 reveal the presence of ferric penta-coordinated species. The amino-acid sequences have been used to gain insight into the evolutionary history of globins of polar fish. *L. tunicatus* globins appear close to the notothenioid clades as predicted by teleostean phylogenies. The close phylogenetic relationships between Cottoidei and Notothenioidei, together with their life style, seem to be the main factor driving the globin-sequence evolution.

2. Materials and methods

2.1. Collection of specimens

Adult *L. tunicatus* was collected by bottom trawling near the coast of the Svalbard islands. Blood samples were drawn from the caudal vein of anesthetised specimens by means of heparinised syringes. Hemolysates were prepared as described (D'Avino and di Prisco, 1988).

2.2. Materials

Mono P HR 5/20 was from Pharmacia, trypsin (EC 3.4.21.4) treated with L-1-tosylamide-2-phenylethylchloromethylketone from Cooper Biomedical, endoproteinase Asp-N and Glu-C (sequencing grade) from Roche, μBondapak-C_{18} from Waters, 4-vinyl pyridine from Sigma, dithiothreitol from Fluka, sequanal-grade reagents from Applied Biosystems, HPLC-grade acetonitrile from Lab-Scan Analytical, oligonucleotides from MWG, *Taq* DNA polymerase from EuroClone. All other reagents were of the highest purity commercially available.

2.3. Purification of Hb 1

All steps were carried out at 0–5 °C. Purification of Hb 1 was achieved by FPLC anion-exchange chromatography on a Mono P HR 5/20 column (Pharmacia, 0.5 × 20 cm). The Hb-containing pooled fractions were dialysed against 10 mM HEPES pH 7.6, and stored in small aliquots at −80 °C until use. For oxygen-binding studies, aliquots of a solution of carbomonoxide Hb 1 were stored at −80 °C prior to use within a maximum of 7 days. For each experiment, one aliquot was thawed, converted to the oxy form by exposure to light and oxygen, and immediately used; no oxidation was spectrophotometrically detectable, indicating that final Met-Hb formation was negligible (<2%).

2.4. Amino-acid sequencing

Alkylation of sulfhydryl groups with 4-vinylpyridine, and tryptic, Asp-N, Glu-C digestions were carried out as described (D'Avino and di Prisco, 1989; Tamburrini et al., 1992, 1996). Globins and peptides were purified by reverse-phase HPLC on a μBondapak-C_{18} column (0.39 × 30 cm) as described (D'Avino and di Prisco, 1989). Cleavage of Asp-Pro bonds was performed on polybrene-coated glass-fibre filters in 70% (v/v) formic acid, for 24 h at 42 °C (Landon, 1977). Asp-Pro-cleaved α-globins were treated with *o*-phthalaldehyde before sequencing (Brauer et al., 1984) in order to block the non-Pro N terminus and reduce the background. Sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids.

2.5. Cloning and sequence analysis of globin cDNAs

Total RNA was isolated from the spleen of *L. tunicatus* spleen using TRI-Reagent® (Sigma Aldrich) as described (Chomczynski and Sacchi, 1987). The first-strand cDNA synthesis was performed according to manufacturer's instructions (Promega) using an oligo(dT)-adaptor primer. The α-globin cDNAs were amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers, and the adaptor primer as reverse primer. Amplifications of cDNA were performed with 2.5 units *Taq* DNA polymerase, 5 pmol each of the above primers and 0.2 mM dNTPs buffered with 160 mM ammonium sulfate, 670 mM TRIS–HCl pH 8.8, 0.1% Tween-20, 1.5 mM $MgCl_2$. The PCR program consisted of 30 cycles of 1 min at 94 °C, 1 min at temperature between 42 and 54 °C and 1 min at 72 °C, and ending with a single cycle of 10 min at 72 °C. Amplified cDNA was purified and ligated in the pDrive vector (Qiagen). *Escherichia coli* cells (strain DH5α) were transformed with the ligation mixtures. Standard molecular biology techniques (Sambrook et al., 1989) were used in the isolation, restriction, and sequence analysis of plasmid DNA. Both strands of the cloned cDNA fragments underwent automated sequencing.

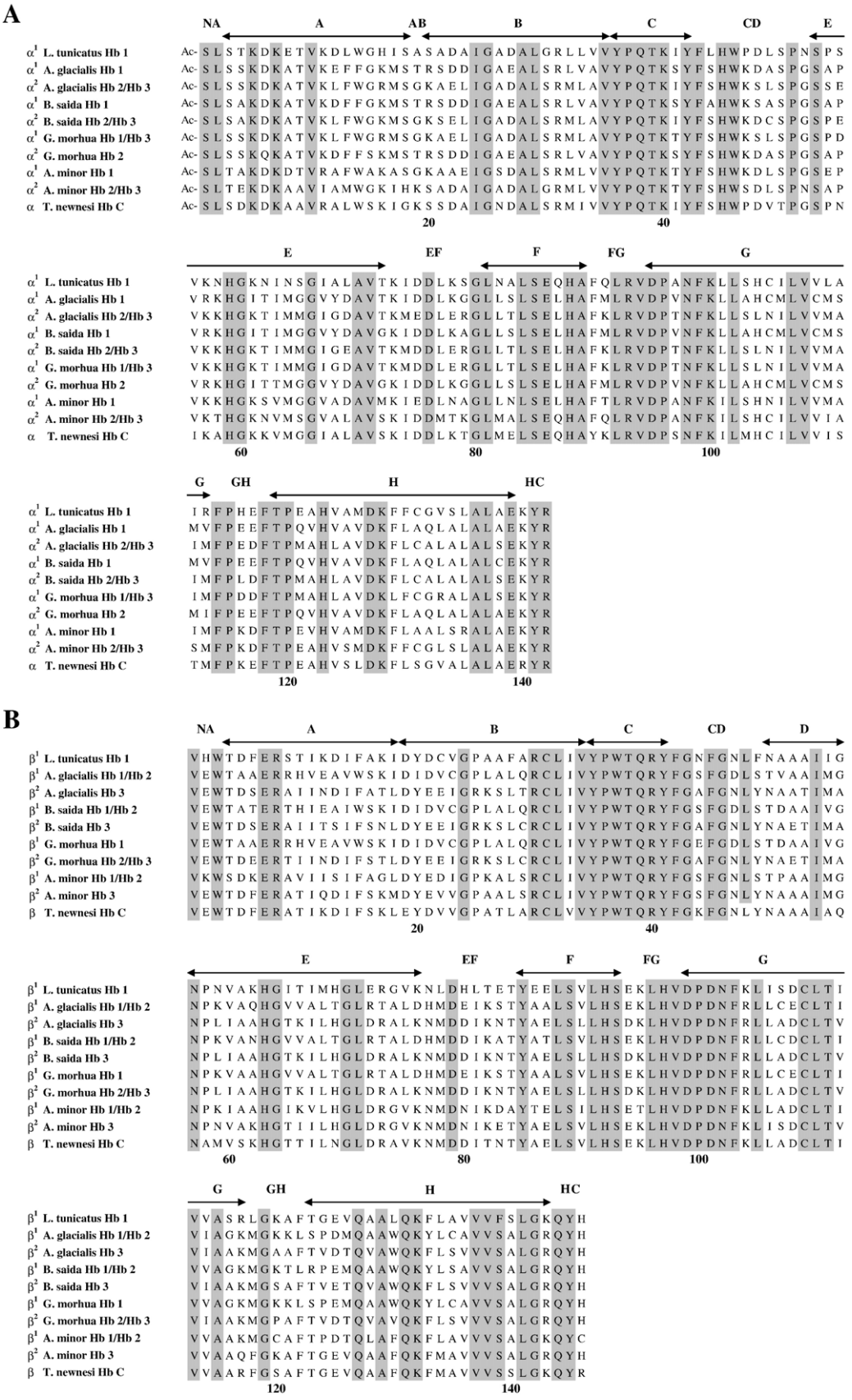

Fig. 1. Alignment of amino-acid sequences of the α (A) and β (B) chains of *L. tunicatus* Hb 1, gadid Hbs, *A. minor* Hbs, and *T. newnesi* Hb C. Identical residues are evidenced in grey boxes, helical and non-helical regions are indicated as in mammalian Hb. Helix D is lacking in the α chain.

Table 1
Species and globin sequences investigated

Order and species	Family	Subunit	Accession number/reference
Coelacanthiformes (outgroup)			
Latimeria chalumnae[a]	Coelacanthidae	α, β	P23740, P23741
Scorpaeniformes			
Chelidonichthys kumu[a]	Trigilidae	α, β	P80270, P80271
Liparis tunicatus	Liparidae	α, β (Hb 1)	This study
Scombroid perciforms			
Thunnus thynnus[a]	Thunnidae	α, β	P11748, P11749
Zoarcoid perciforms			
Anarhichas minor[b]	Anarhichadidae	α (Hb 1), α (Hb 2, Hb 3)	P83270, P83271
		β (Hb 1, Hb 2), β (Hb 3)	P83272, P83273
Sparoid perciforms			
Chrysophrys auratus[a]	Sparidae	α, β (Hb 4)	Stam et al. (1997)
Notothenioid perciforms			
Notothenia coriiceps[c]	Nototheniidae	major α (Hb 1)	P10777
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P16309
Notothenia angustata[d]	Nototheniidae	major α (Hb 1)	P29624
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P29628
Pleuragramma antarcticum[c]	Nototheniidae	α (Hb 1, Hb 2)	Stam et al. (1997)
		β (Hb 1, Hb 3)	Stam et al. (1997)
		minor α (Hb 3), β (Hb 2)	Stam et al. (1997)
Pagothenia borchgrevinki[c]	Nototheniidae	α (Hb 1, Hb 0)	P82344
		major β (Hb 1)	P82346
		minor β (Hb 0)	P83245
Gobionotothen gibberifrons[c]	Nototheniidae	major α, β (Hb 1)	P83611, P83612
		minor α, β (Hb 2)	P83613, P83614s
Aethotaxis mitopteryx[c]	Nototheniidae	α, β	Stam et al. (1997)
Trematomus newnesi[c]	Nototheniidae	major α, β (Hb 1)	P45718, P45720
		minor α (Hb 2), β (Hb C)	P45719, P45721
Trematomus bernacchii[c]	Nototheniidae	major α, β (Hb 1)	P80043, P80044
		minor β (Hb C)	P45722
Cygnodraco mawsoni[c]	Bathydraconidae	α (Hb 1, Hb 2)	P23016
		major β (Hb 1)	P23017
		minor β (Hb 2)	P23018
Gymnodraco acuticeps[c]	Bathydraconidae	α, β	P29623, P29625
Racovitzia glacialis[c]	Bathydraconidae	α, β	Tamburrini et al., unpublished
Bathydraco marri[c]	Bathydraconidae	α, β	Stam et al. (1997)
Pogonophryne scotti[c]	Artedidraconidae	α, β	Stam et al. (1997)
Artedidraco orianae[c]	Artedidraconidae	α, β	Stam et al. (1997)
Salmoniformes			
Salmo salar[a]	Salmonidae	α	P11251
Oncorhynchus mykiss[a]	Salmonidae	α, β (Hb I)	P02019, P02142
		α, β (Hb IV)	P14527, P02141
Gadiformes			
Arctogadus glacialis[b]	Gadidae	α^1 (Hb 1), α^2 (Hb 2, Hb 3)	P84602, P84603
		β^1 (Hb 1, Hb 2), β^2 (Hb 3)	DQ125476, P84604
Boreogadus saida[b]		α^1 (Hb 1), α^2 (Hb 2, Hb 3)	P84605, P84606
		β^1 (Hb 1, Hb 2), β^2 (Hb 3)	P84607, P84608
Gadus morhua[b]		α^1 (Hb 1, Hb 3), α^2 (Hb 2)	P84609, 041425
		β^1 (Hb 1), β^2 (Hb 2, Hb 3)	P84610, P84611
		β (additional chain)	O13077
Anguilliformes			
Anguilla anguilla[a]	Anguillidae	α, β (Hb C)	P80726, P80727
		α, β (Hb A)	P80945, P80946

(continued on next page)

Table 1 (*continued*)

Order and species	Family	Subunit	Accession number/reference
Gymnotiformes			
Electrophorus electricus[a]	Electrophoridae	α, β	P14520, P14521
Siluriformes			
Hoplosternum littorale[a]	Callichthyidae	α, β (Hb C)	P82315, P82316
Cypriniformes			
Cyprinus carpio[a]	Cyprinidae	α, β	P02016, P02139
Carassius auratus[a]	Cyprinidae	α, β	P02018, P02140
Catostomus clarkii[a]	Catostomidae	α	P02017

[a]Temperate freshwater and marine species.
[b]Arctic species.
[c]Antarctic Notothenioidei.
[d]Non-Antarctic Notothenioidei.

2.6. Mass spectrometry

The molecular masses of *S*-pyridylethylated α and β chains and of peptides (less than 10 kDa) were measured by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation. Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration, 5 pmol μL^{-1}) in 4 vol. of matrix, namely 10 mg mL^{-1} sinapinic acid in 30% acetonitrile containing 0.3% trifluoroacetic acid (globins), and 10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% trifluoroacetic acid (peptides).

2.7. Phylogenetic analysis of globin genes

Multiple alignments of the amino-acid sequences of α and β globins were performed with the program CLUSTAL X (Thompson et al., 1997). These alignments are available upon request. Phylogenetic trees of globin sequences were inferred using the Maximum Likelihood (ML) method implemented in the program PHYML (Guindon and Gascuel, 2003) and by Neighbour Joining (NJ) implemented in the software MEGA 3 (available at http://www.megasoftware.net/index.html). The best-fit model was estimated with the software ProtTest (Abascal et al., 2005). Robustness of the ML and NJ trees was assessed by non-parametric bootstrap analysis with 500 and 1000 replicates, respectively. Parametric bootstrap was performed using data sets obtained by simulation on the best estimate of the ML tree inferred with PHYML. Computer simulations were used to produce 500 data sets using the software SEQ-GEN v. 1.3.2 (Rambaut and Grassly, 1997) with parameters estimated by ProtTest. Each of these simulated data sets was used to infer the tree by PHYML. Finally, the resulting trees were used for building a majority-rule consensus tree.

2.8. Oxygen binding

Hemolysate stripping was carried out as described (Tamburrini et al., 1994). Oxygen equilibria were measured in 100 mM HEPES in the pH range 6.2–8.7, at 5 °C and 10 °C (keeping the pH variation as a function of temperature in due account) at a final Hb concentration of 0.5–1.0 mM on a heme basis. An average standard deviation of ±3% for values of p_{50} was calculated; experiments were performed in duplicate. In order to obtain stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (Weber et al., 1987). pH values were measured with a Radiometer BMS Mk2 thermostatted electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP and 2,3-diphosphoglycerate (DPG) were measured at a final ligand concentration of 3 mM, namely a large excess over tetrameric Hb concentration. Oxygen affinity and cooperativity were calculated from the linearised Hill plot of $\log S/(1-S)$ *vs* $\log pO_2$ at half saturation, where S denotes fractional oxygen saturation.

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

2.9. Electron paramagnetic resonance

Continuous wave EPR spectra were obtained at 12 K using a Varian E112 spectrometer equipped with a Systron–Donner frequency counter and a PC-based data acquisition program. The samples of ferric *L. tunicatus* Hb 1 were examined in 50 mM HEPES pH 7.6 and 50 mM MES pH 6.0. Spectra were recorded at a microwave frequency of 9.29 GHz, a microwave power of 10 mW, a modulation frequency of 100 kHz, and a modulation amplitude of 5 G.

3. Results and discussion

3.1. Purification of Hbs and separation of globins

Ion-exchange chromatography of the hemolysate, using gradient elution with TRIS–HCl pH 7.6 containing NaCl, showed two components (data not shown). The first peak corresponded to Hb 1, the second to Hb 2. The approximate ratio of the two Hbs was 90:10.

The elution profile of the hemolysate, obtained by reverse-phase HPLC, indicated the presence of four globins (data not shown). The separation of the globins of Hb 1 and Hb 2, the

elution times and the N-terminal sequences indicated that Hb 1 and Hb 2 have no chain in common and are made of different α (α^1 and α^2) and β chains (β^1 and β^2). The chain composition of Hb 1 was denoted as $\alpha_2^1\beta_2^1$.

3.2. Primary structure

The amino-acid sequences of the α and β chains of *L. tunicatus* Hb 1 are reported in Fig. 1. The Hb 1 primary structure was established by alignment of tryptic, Asp-N, Glu-C peptides (data not shown). When it was not possible to obtain a peptide in pure form, DNA sequencing was utilised. The N terminus of the α chain was not available to Edman degradation because of the presence of an acetyl blocking group. The sequence-deduced molecular masses of *L. tunicatus* globins were 15,638 Da for α^1 and 16,492 Da for β^1. These values are in agreement with MALDI- TOF mass spectrometry. Hb 1 has 141 residues in the α and 146 in the β chains.

The analysis of the primary structure of Hb 1 revealed several non-conservative substitutions. Remarkably, as in mammals, position NA2 (β2) is occupied by His, in contrast to other teleosts that have Glu or Asp (exceptionally Lys) in this

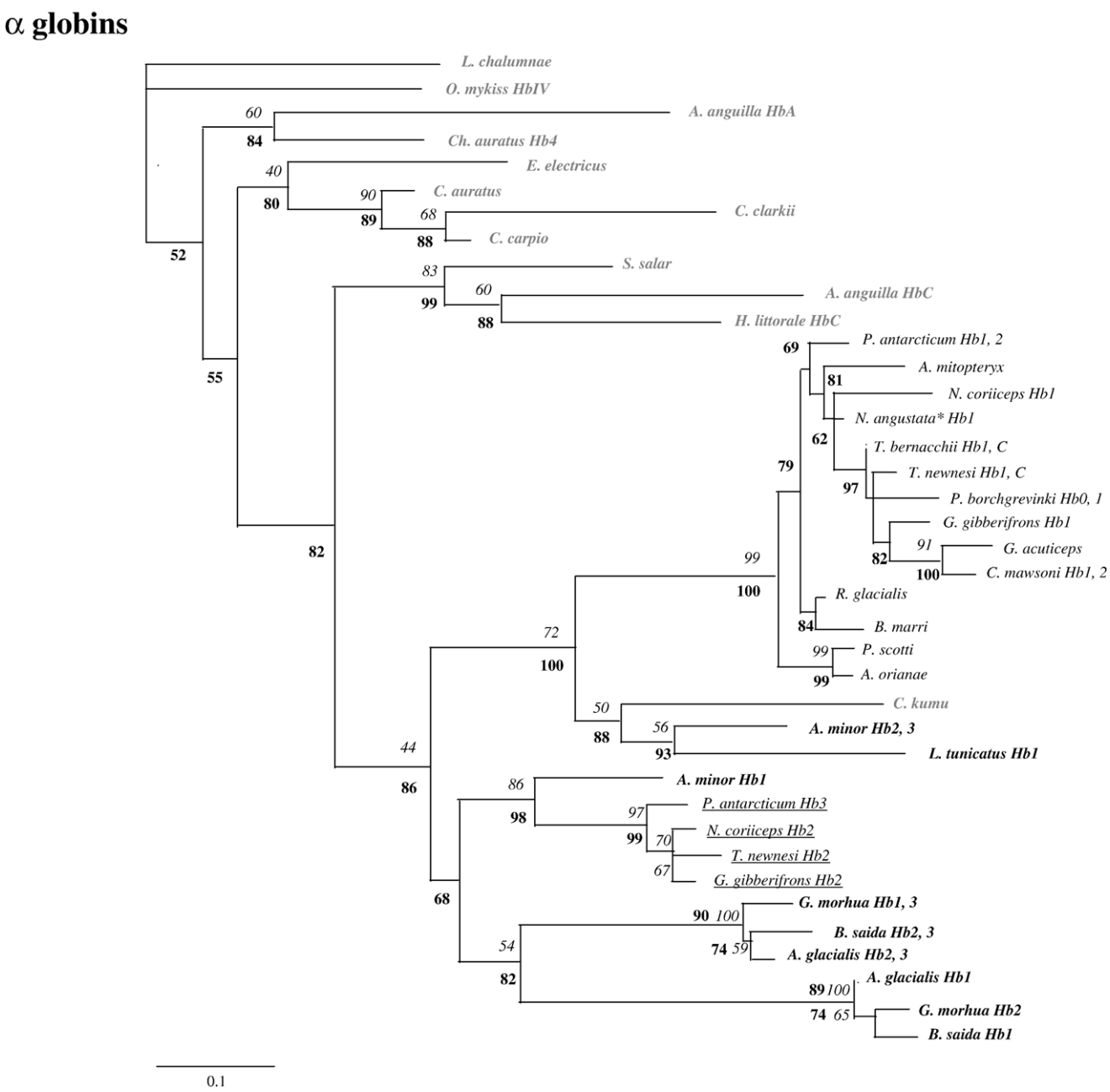

Fig. 2. Maximum likelihood phylogenetic tree of amino-acid sequences of α chains of Arctic, Antarctic and temperate fish Hbs. The Dayhoff substitution model was used with gamma shape parameter set to 1.45 and proportion of invariable sites equal to 0.09. Non-parametric (plain text) and parametric (bold) bootstrap values are given at the nodes. Notothenioid major globins are in black, notothenioid minor globins in black and underscored, non-Antarctic notothenioid globins are indicated by asterisks, Arctic globins in bold black, and temperate globins in bold grey. For full species name, see Table 1.

phosphate-binding region. His NA2 (β2) also occurs in the Hbs of the coelacanth *Latimeria chalumnae* (Bonaventura et al., 1974), the lungfish *Lepidosiren paradoxa* (Rodewald et al., 1984), the sharks and the Amazonian catfish *Hoplosternum littorale* (Weber et al., 2000). The episodic occurence of His NA2 (β2) in elasmobranchs, lungfish and *L. tunicatus* suggests a phylogenetically primitive character, which has been lost in most non-mammalian vertebrates. The functionally important residues involved in the molecular mechanism of the Root effect of fish Hbs are all conserved in *L. tunicatus* Hb 1.

The sequence of *L. tunicatus* Hb 1 exhibits several substitutions in helix E, *i.e.* Ala E6 (62β)→Lys, Lys E10 (66β)→Thr, Val E11 (67β)→Ile when compared to most fish Hbs.

In HbA mutants it has been shown that the bulky side chain of Ile E11 blocks the access of oxygen to the β chain significantly lowering the association (and equilibrium) constant both in the T (Nagai et al., 1987) and R state (Mathews et al., 1989). The replacement of Val E11 with Ile affects the kinetic parameters of oxygen binding, due to the larger *sec*-butyl group that sterically hinders access of ligands to the heme iron (Cupane et al., 1997). It has recently been shown that the Ile E11 reduces the accessibility of oxygen to the iron (Mazzarella et al., 2006).

Interestingly, Val E11 is replaced by Ile in Hb 3 of the Arctic species *A. glacialis*, *B. saida*, *G. morhua* and *A. minor* (Verde et al., 2002, 2006a).

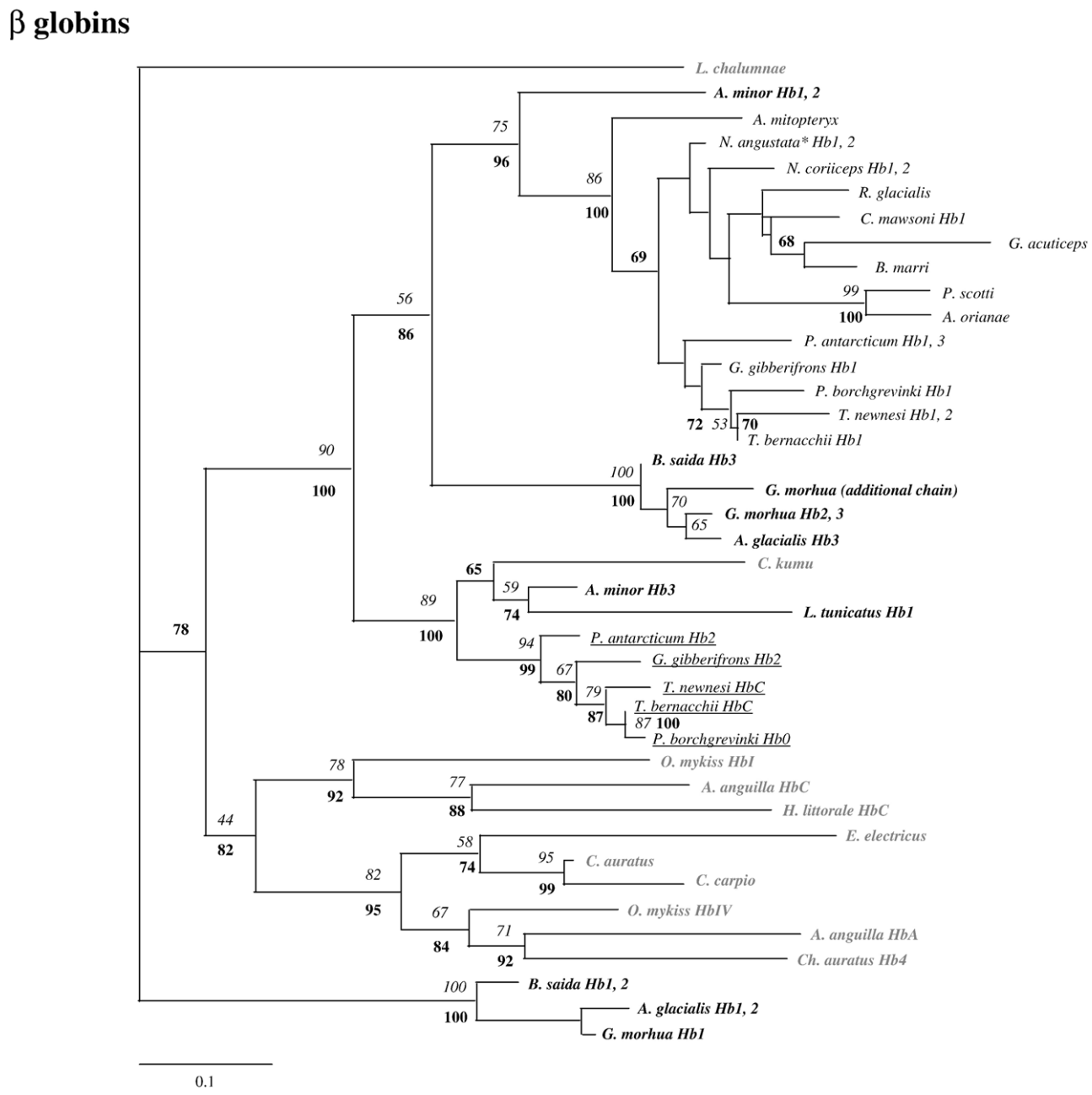

Fig. 3. Phylogenetic tree of amino-acid sequences of β chains of Arctic, Antarctic and temperate fish Hbs. The Whelan And Goldman (WAG) substitution model with gamma shape parameter set to 2.43 and proportion of invariable sites equal to 0.15 was used. For further details, see Fig. 2.

3.3. Phylogenetic analysis of globin genes

Table 1 lists the taxa examined in this study and the accession numbers of α- and β-chain sequences used in the phylogenetic analysis. Figs. 2 and 3 show the trees inferred from the present data set using the ML method. The topologies reported here are congruent with the NJ trees inferred with the software MEGA 3 (data not shown) and with the results of previous analyses (Stam et al., 1997; Verde et al., 2006a). In the tree of α globins (Fig. 2), the Antarctic globins cluster in two paralogous groups suggesting that they originated by gene duplication. The first cluster is formed by the major Antarctic Hbs and by a sister group merging in a basal position that contains the α chains of *C. kumu* Hb, *A. minor* Hb 2 and Hb 3, and *L. tunicatus* Hb 1. A second cluster contains the α chains of minor Antarctic Hbs (Hb 2 and Hb C) and the α chain of *A. minor* Hb 1. Gadid Arctic α chains are organised in two well supported groups of paralogs, one containing the α chains of *A. glacialis* Hb 1, *G. morhua* Hb 2 and *B. saida* Hb 1, the other comprising the α chains of *A. glacialis* Hb 2 and Hb 3, *G. morhua* Hb 1 and Hb 3, and *B. saida* Hb 2 and Hb 3. The presence of these two groups of paralogs argues in favour of a relatively recent duplication event. It must be noted that the branching order of the Arctic globins is not consistent with the generally accepted branching pattern of the relative species.

Fig. 3 shows the topology of the β globins, which appears to be somewhat more complex than that of α chains. The β chains shared by *A. glacialis* Hb 1 and Hb 2, *B. saida* Hb 1 and Hb 2 and the β chain of *G. morhua* Hb 1 form a basal divergence lineage. The monophyletic origin of this clade is supported by high bootstrap (100%). In contrast, the group including the *G. morhua* β chain shared by Hb 2 and Hb 3 plus an additional putative larval form from the same species, together with the β chains of Hb 3 of *A. glacialis* and *B. saida*, form a lineage basal to the cluster of the major Antarctic globins that includes also the globin of *A. minor* Hb 1 and Hb 2. The splitting of the Arctic β globins in two distinct groups suggests that an early duplication event occurred well before the segregation of the major Arctic/Antarctic groups, leading to the formation of the β chains of *A. glacialis* Hb 1 and Hb 2, *B. saida* Hb 1 and Hb 2, and of *G. morhua* Hb 1. Apparently, this event preceded the divergence of the other β-globin lineages during fish evolution (see also Verde et al., 2006a, 2006b).

Another duplication event generated the group of the gadid globins, major Antarctic globins and β chains of *A. minor* Hb 1 and Hb 2, and the cluster containing the minor Antarctic globins and the β chains of *A. minor* Hb 3 and *L. tunicatus* Hb 1. It is worth noting that in the majority of notothenioids, in the adult stage, minor α and β globins are expressed in trace or limited amounts (Verde et al., 2006b).

When evolutionary pressures and rates of change are the same across taxa, similarity is proportional to phylogeny, and in that case the gene (or protein) tree reflects the species tree. Showing low identity with temperate species, the globin sequences of Arctic *L. tunicatus* are consistent with species history that places cottoids (liparids) and zoarcoids close to notothenioids in the teleostean phylogeny (Miya et al., 2003; Dettaï and Lecointre, 2004, 2005). In contrast, as discussed above, the branching order of the globins of the Arctic gadid does not reflect the expected phylogeny.

We speculate that the life style of a benthic species such as *L. tunicatus* is similar to that of Antarctic notothenioids, and that such a similarity is mirrored to some extent by Hb evolution. Possibly, the constant physico-chemical conditions of the Antarctic ocean and the inactive life style of non-migratory Arctic species, such as the zoarcoid *A. minor* and the cottoid *L. tunicatus*, contribute to grouping their globins with the

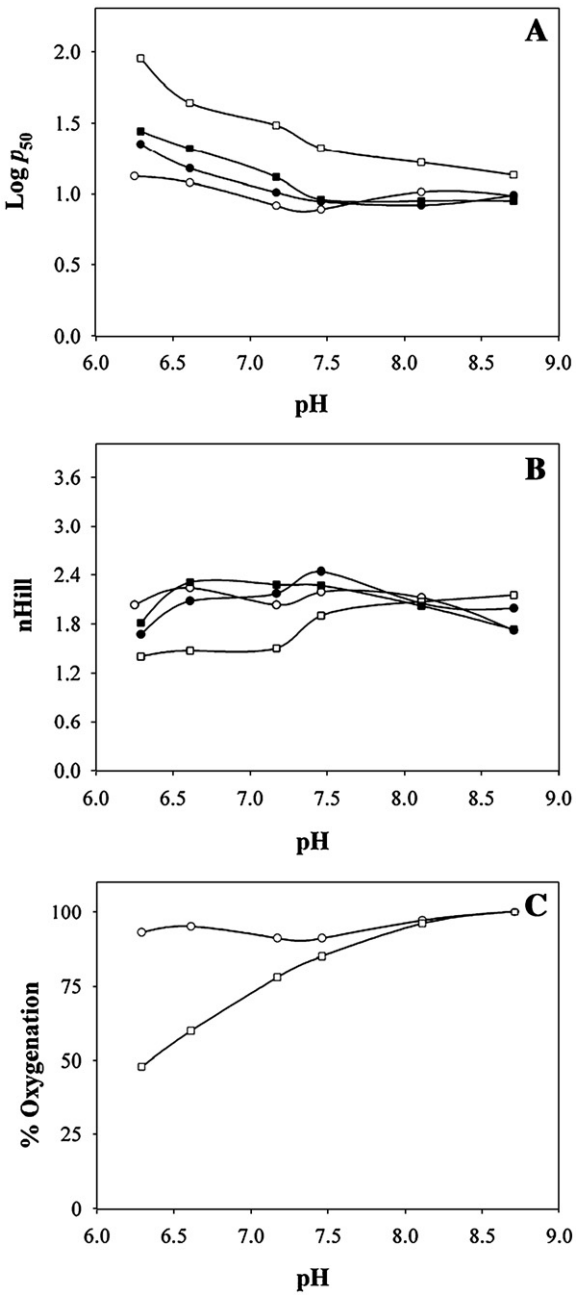

Fig. 4. (A) Oxygen-equilibrium isotherms (Bohr effect); (B) Subunit cooperativity; (C) Root effect, as a function of pH, of *L. tunicatus* Hb 1, at 5 °C. 100 mM HEPES, in the absence of effectors (open circles), in the presence of 100 mM NaCl (filled circles), 100 mM NaCl, 3 mM ATP (open squares), 100 mM NaCl, 3 mM 2,3-DPG (filled squares).

Antarctic globin sequences, whereas the variations typical of the Arctic ocean, in conjunction with the pelagic and migratory life style of gadids, correspond to high divergence of their globin sequences.

3.4. Oxygen binding

Similar to Antarctic notothenioids and most Arctic fish, *L. tunicatus* Hb 1 displayed a relatively low oxygen affinity, as indicated by the oxygen partial pressure required to achieve half saturation p_{50} (p_{50}=8.2 mmHg at pH 8.1, 5 °C, in the presence of NaCl; p_{50}=16.5 mmHg at pH 8.1, 5 °C, in the presence of NaCl and ATP). Low oxygen affinity in the species of the highest northern latitudes seems typical of the most cold-adapted species. This feature is probably linked to the high oxygen concentration in cold waters. Chloride and 2,3-DPG had no effect on the oxygen affinities. "Mammalian DPG-binding" His NA2 (β2) seems to confer no selective advantage for DPG binding because Hb 1 exhibits higher sensitivity to ATP.

The Bohr effect of stripped Hb 1 is not very strong, but is enhanced by the physiological ligand ATP (Fig. 4A). The Bohr coefficient ($\varphi = \Delta\log p_{50}/\Delta\mathrm{pH}$), which indicates a measure of the average number of protons bound upon oxygenation, is −0.56 at 5 °C in the presence of NaCl and ATP. The Hill coefficient (nHill) is about 2.0 in the whole pH range in the absence of effectors (Fig. 4B); but at acidic pH and in the presence of NaCl and ATP, it falls significantly, reflecting lower subunit cooperativity (Fig. 4B). The Root effect is strongly enhanced by ATP (Fig. 4C).

Oxygen-binding equilibria were investigated in the range 5–10 °C. The oxygenation-enthalpy change was low when compared with temperate fish and very similar to the values seen in Antarctic and Arctic fish Hbs (Verde et al., 2006a). Relying upon Hbs with reduced ΔH values may thus be a frequent evolutionary strategy of cold-adapted fish. It seems that in polar fish, evolutionary development has often favoured a decrease in temperature sensitivity of Hb oxygen affinity (di Prisco et al., 1991).

3.5. Electron paramagnetic resonance

The EPR spectra of ferric *L. tunicatus* Hb 1, at pH 7.6 and 6.0, at 12 K, contain both axial (g=5.88, 2.01) and rhombic high-spin ferric signals (Fig. 5) as well as some residual rhombic low-spin ferric signals (g≈3.2, 3.0, 2.2; Fig. 5A, inset). The high-spin signals are similar to those observed in cathodic Hb (HbCTn) of *T. newnesi* (Vergara et al., in press); however, whereas the EPR of HbCTn shows a low-spin ferric signal (g=2.9, 2.2; arising from a cytochrome *b*-like hemichrome [Blumberg and Peisach, 1972]) indicating comparable high-spin and low-spin populations, rhombic low-spin forms are barely resolved in ferric *L. tunicatus* Hb 1, indicating the ferric hemes are predominantly high spin in this protein.

The prominent high-spin signal corresponds to that of an aquo-met form. Similar to HbCTn, but unlike four other tetrameric Hbs from the Antarctic fish *T. bernacchii*, *Gymnodraco acuticeps*, and *T. newnesi* Hb 1, the signal from a low-spin ferric hydroxide complex (g=2.6, 2.2. 1.8; Blumberg and Peisach, 1972) was not resolved, indicating that the p*K*a's of the bound water in HbCTn and *L. tunicatus* Hb 1 are both higher than that of the other four Antarctic fish Hbs.

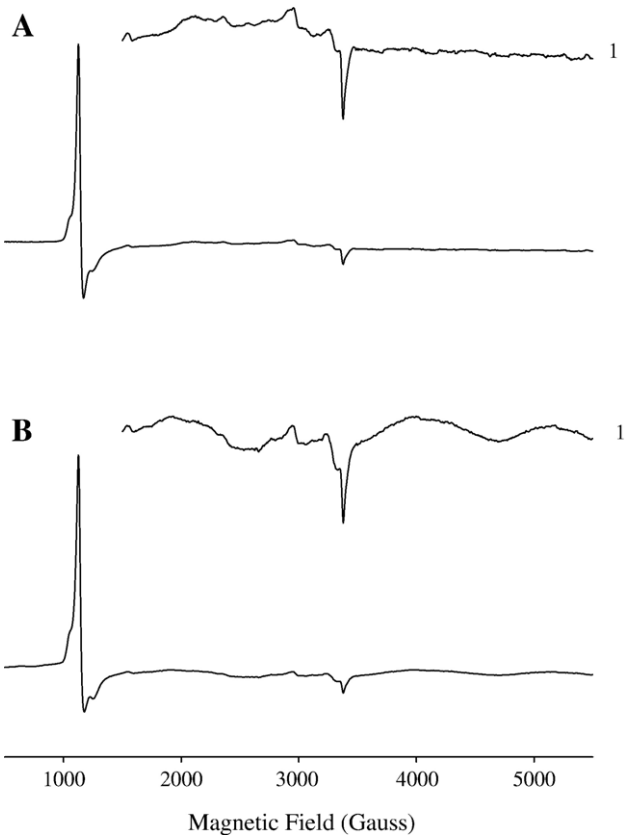

Fig. 5. Continuous wave EPR spectrum (band X) of 2 mM *L. tunicatus* Hb 1. A, 50 mM HEPES pH 7.6; B, 50 mM MES pH 6.0. Spectra were recorded at 12 K at a microwave frequency of 9.29 GHz, a microwave power of 10 mW, a modulation frequency of 100 kHz, and a modulation amplitude of 5 G. Curves labelled 1 show the low-spin region replotted on a ×5.7 intensity scale.

The rhombic high-spin signals suggest the formation of unligated penta-coordinated Fe(III). Such forms have been previously observed in distal-His mutants of myoglobin (Ikeda-Saito et al., 1992; Quillin et al., 1993), peroxidases (Smulevich et al., 2005), a flavo Hb (Ilari et al., 2002), a giant Hb at acidic pH (Marmo Moreira et al., 2006), *Scapharca inequivalvis* Hb (Boffi et al., 1994) and HbCTn (Vergara et al., in press). The current results provide the spectroscopic indication of an unligated ferric form also in this tetrameric Arctic fish Hb. Probably the replacement of Val E11 (67β) with bulkier Ile in *T. newnesi* Hb C and *L. tunicatus* Hb 1 generates unfavourable interactions in the heme pocket, thus negatively affecting the bis-His adduct formation in the β chains (Vergara et al., in press).

4. Concluding remarks

Antarctic notothenioids fill a varied range of ecological niches normally occupied by taxonomically diverse fish communities in

temperate waters. However, as they are absent from the Arctic, comparisons needed to study adaptations to the cold can be made only with taxonomically distant groups (like gadids, for instance). However some "minor" fish families, represented in both polar oceans, are extremely useful for comparing Arctic and Antarctic evolution. Fishes of the family Liparidae and those of the suborder Zoarcoidei are the second and third components of the Antarctic ichthyofauna after the Notothenioidei. More interestingly, they exhibit anti-tropical major geographic distributions. The comparison of the molecular phylogenies of each of the two groups, based on genes from multigene families (like globins) involved in the adaptation to the cold would reveal evolutionary constraints on the latter and allow tests of adaptive scenarios. Moreover, comparing a trait in phyletically unrelated taxa permits to study convergent and parallel evolutionary trends. The different phylogenetic histories of Arctic and Antarctic fish depend on the differences in the respective habitats. In the absence of competition with other fish groups, Notothenioids have filled most niches on the shelf and upper slope and may constitute a species flock (Eastman and McCune, 2000).

Antarctic waters are dominated by a single taxonomic group, whereas Arctic waters are characterised by high diversity, reflected in the phylogeny of a given trait. The life style of benthic species such as *L. tunicatus* and *A. minor*, unlikely to disperse across wide latitude and temperature gradients, corresponds to Hb evolution which is stable enough to recover the close phylogenetic relationships among zoarcoids, cottoids and notothenioids (Dettaï and Lecointre, 2005).

Acknowledgements

This study is financially supported by the Italian National Programme for Antarctic Research (PNRA). It is in the framework of the programme Evolution and Biodiversity in the Antarctic (EBA) endorsed by the Scientific Committee on Antarctic Research (SCAR), and of the cruises TUNU I and TUNU II (Greenland) in 2003 and 2005. Amino-acid sequencing by Mr. V. Carratore is gratefully acknowledged. A. Vergara acknowledges the University of Naples and the Albert Einstein College of Medicine (AECOM) for travel grants. Work carried out at AECOM was supported by National Institutes of Health grants GM040168 and HL071064-03004 (awarded to J. Peisach).

References

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21, 2104–2105.

Blumberg, W.E., Peisach, J., 1972. Low-spin ferric forms of hemoglobin and other heme proteins. Wenner-Gren Cent. Int. Symp. Ser. 18, 219–225.

Bonaventura, J., Gillen, R.G., Riggs, A., 1974. The hemoglobin of the Crosspterygian fish, *Latimeria chalumnae* (Smith). Arch. Biochem. Biophys. 163, 728–734.

Boffi, A., Takahashi, S., Spagnolo, C., Rousseau, D.L., Chiancone, E., 1994. Structural characterization of oxidized dimeric *Scapharca inaequivalvis* hemoglobin by resonance Raman spectroscopy. J. Biol. Chem. 269, 20437–20440.

Brauer, A.W., Oman, C.L., Margolies, M.N., 1984. Use of *o*-phthalaldehyde to reduce background during automated Edman degradation. Anal. Biochem. 137, 134–142.

Brey, T., et al., 1994. Antarctic benthic diversity. Nature 368, 297.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Cupane, A., et al., 1997. Modification of α-chain or β-chain heme pocket polarity by Val (E11)→Thr substitution has different effects on the steric, dynamic, and functional properties of human recombinant hemoglobin. Deoxy derivatives. J. Biol. Chem. 272, 26271–26278.

Dayton, P.K., Mordida, B.J., Bacon, F., 1994. Polar marine communities. Am. Zool. 34, 90–99.

D'Avino, R., di Prisco, G., 1988. Antarctic fish haemoglobin: an outline of the molecular structure and oxygen binding properties. I. Molecular structure. Comp. Biochem. Physiol. 90B, 579–584.

D'Avino, R., di Prisco, G., 1989. Haemoglobin from the Antarctic fish *Notothenia coriiceps neglecta*. 1. Purification and characterisation. Eur. J. Biochem. 179, 699–705.

Dettaï, A., Lecointre, G., 2004. In search for notothenioid (Teleostei) relatives. Antarct. Sci. 16, 71–85.

Dettaï, A., Lecointre, G., 2005. Further support for the clades obtained by multiple molecular phylogenies in the acanthomorph bush. C.R. Biol. 328, 674–689.

di Prisco, G., Condò, S.G., Tamburrini, M., Giardina, B., 1991. Oxygen transport in extreme environments. Trends Biochem. Sci. 16, 471–474.

Eastman, J.T., 1997. Comparison of the Antarctic and Arctic fish faunas. Cybium 12, 276–287.

Eastman, J.T., 2005. The nature of the diversity of Antarctic fishes. Polar Biol. 28, 93–107.

Eastman, J.T., McCune, A.R., 2000. Fishes on the Antarctic continental shelf: evolution of a marine species flock? J. Fish Biol. 57 (Suppl. A), 84–102.

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Ikeda-Saito, M., et al., 1992. Coordination structure of the ferric heme iron in engineered distal histidine myoglobin mutants. J. Biol. Chem. 267, 22843–22852.

Ilari, A., Bonamore, A., Farina, A., Johnson, K., Boffi, A., 2002. The X-ray structure of ferric *Escherichia coli* flavohemoglobin reveals an unexpected geometry of the distal heme pocket. J. Biol. Chem. 26, 23725–23732.

Landon, M., 1977. Cleavage at aspartyl–prolyl bonds. Methods Enzymol. 47, 145–149.

Marmo Moreira, L., Lima Poli, A., Costa-Filho, A.J., Imasato, H., 2006. Pentacoordinate and hexacoordinate ferric hemes in acid medium: EPR, UV–Vis and CD studies of the giant extracellular hemoglobin of *Glossoscolex paulistus*. Biophys. Chem. 124, 62–72.

Mathews, H.J., Rohlfs, R.J., Olson, J.S., Tame, J., Renaud, J.P., Nagai, K., 1989. The effects of E7 and E11 mutations on the kinetics of ligand binding to R state human hemoglobin. J. Biol. Chem. 264, 16573–16583.

Mazzarella, L., et al., 2006. Minimal structural requirements for Root effect: crystal structure of the cathodic hemoglobin isolated from the Antarctic fish *Trematomus newnesi*. Proteins Struct. Funct. Bioinf. 62, 316–321.

Miya, M., et al., 2003. Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. Mol. Phylogenet. Evol. 26, 121–138.

Nagai, K., et al., 1987. Distal residues in the oxygen binding site of haemoglobin studied by protein engineering. Nature 329, 858–860.

Quillin, M., Arduini, R., Olson, J., Phillips, G.J., 1993. High-resolution crystal structures of distal histidine mutants of sperm whale myoglobin. J. Mol. Biol. 234, 140–155.

Rambaut, A., Grassly, N.C., 1997. Seq-gen: an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic tree. Comput. Appl. Biosci. 13, 235–238.

Rodewald, K., Stangl, A., Braunitzer, G., 1984. Primary structure, biochemical and physiological aspects of hemoglobin from South American lungfish (*Lepidosiren paradoxus*, Dipnoi). Hoppe Seylers Z. Physiol. Chem. 365, 639–649.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Smulevich, G., Feis, A., Howes, B.D., 2005. Fifteen years of Raman spectroscopy of engineered heme containing peroxidases: what have we learned? Acc. Chem. Res. 38, 433–440.

Stam, W.T., Beintema, J.J., D'Avino, R., Tamburrini, M., di Prisco, G., 1997. Molecular evolution of hemoglobins of Antarctic fishes (Notothenioidei). J. Mol. Evol. 45, 437–445.

Tamburrini, M., Brancaccio, A., Ippoliti, R., di Prisco, G., 1992. The amino acid sequence and oxygen-binding properties of the single haemoglobin of the cold-adapted Antarctic teleost *Gymnodraco acuticeps*. Arch. Biochem. Biophys. 292, 295–302.

Tamburrini, M., Condò, S.G., di Prisco, G., Giardina, B., 1994. Adaptation to estreme environments: structure–function relationships in Emperor penguin haemoglobin. J. Mol. Biol. 237, 615–621.

Tamburrini, M., D'Avino, R., Fago, A., Carratore, V., Kunzmann, A., di Prisco, G., 1996. The unique haemoglobin system of *Pleuragramma antarcticum*, an Antarctic migratory teleost: structure and function of the three components. J. Biol. Chem. 271, 23780–23785.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Verde, C., Carratore, V., Riccio, A., Tamburrini, M., Parisi, E., di Prisco, G., 2002. The functionally distinct hemoglobins of the Arctic spotted wolffish *Anarhichas minor*. J. Biol. Chem. 277, 36312–36320.

Verde, C., Balestrieri, M., de Pascale, D., Pagnozzi, D., Lecointre, G., di Prisco, G., 2006a. The oxygen-transport system in three species of the boreal fish family Gadidae. Molecular phylogeny of haemoglobin. J. Biol. Chem. 281, 22073–22084.

Verde, C., Parisi, E., di Prisco, G., 2006b. The evolution of hemoglobin of thermal adaptation in polar fish. Gene 385, 137–145.

Vergara, A., Franzese, M., Merlino, A., Vitagliano, L., Verde, C., di Prisco, G., Lee, H.C., Peisach, J., Mazzarella, L., in press. Structural characterization of ferric hemoglobins from three Antarctic fish species of the suborder Notothenioidei. Biophys. J. doi:10.1529/biophysj.107.105700.

Weber, R.E., Jensen, F.B., Cox, R.P., 1987. Analysis of teleost haemoglobin by Adair and Monod–Wyman–Changeux models: effects of nucleoside triphosphates and pH on oxygenation of tench haemoglobin. J. Comp. Physiol., B 157, 145–152.

Weber, R.E., et al., 2000. Isohemoglobin differentiation in the bimodal-breathing Amazon catfish *Hoplosternum littorale*. J. Biol. Chem. 275, 17297–17305.